

edison – a novel model of otitis media

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A thesis presented for the degree of Doctor of Philosophy (D.Phil)

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A thesis presented for D.Phil, Trinity term, 2014

Abstract

Otitis media (OM) is characterised by inflammation of the middle ear and is a common cause of conductive hearing impairment that places a substantial social, medical and economic burden on healthcare systems globally. Despite the importance of the disease, the aetiology of chronic middle ear inflammatory disease remains poorly understood. The development and persistence of chronic OM is multifactorial with a significant genetic component. A new mouse model of chronic OM, *edison*, was generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis and discovered in a recessive screen at MRC Harwell.

Homozygous *edison* mice have craniofacial abnormalities, an emphysema-like lung phenotype and spontaneously develop a conductive hearing loss at 28 days as measured by ABR. Histological analysis shows the hearing loss is associated with the development of chronic OM in the middle ear, characterised by mucosal inflammation and highly cellular ear exudates. Similar to the *Jeff* and *Junbo* mutants, *edison* shows raised levels of *Vegfa*, *Tnfa* and *Il-1 β* in middle ear fluids. A putative functional mutation was identified, resulting in a missense Leu972Pro change in a relatively unknown gene, *Nischarin* (*Nisch*). The identification of additional ENU-induced *Nisch* alleles, and subsequent characterisation, validated *Nisch* as the causative gene in *edison*.

NISCH selectively binds ITGA5, which is thought to have a role in modulating VEGF signalling through SRC and FAK kinases. A significant genetic interaction between *Nisch* and *Itga5* exists and impacts upon development of chronic OM. Mice heterozygous for *Itga5*-null and homozygous for *edison* alleles show a significantly increased penetrance and severity of chronic OM. Analysis of downstream pathways suggests that the *edison* allele is impacting upon both RAC1 and TGF- β /SMAD signalling.

I also explored the potential use of the *edison* mouse as a model for bacterial challenge with the human otopathogen, *NTHi*. Similar to the *Junbo* infection model at MRC Harwell, the *edison* mouse was identified as a robust OM model for bacterial *NTHi* infection.

The *edison* mouse highlights a new candidate gene for susceptibility to chronic OM and will provide further insight into the genetic pathways and pathogenic processes involved in chronic OM.

Acknowledgements

Thank you goes first and foremost to my supervisors Steve Brown and Martin Burton for providing me with the opportunity to study for a D.Phil and for all the support, scientific discussion and encouragement that they have provided throughout.

I am extremely grateful to all the members of the Deafness laboratory who have made the lab an enjoyable place to work, contributed towards this project through discussion at group meetings and given advice in the lab. Particular thanks go to Hilda Tateossian, Tom Purnell, Hayley Tyrer and Andy Parker for being so generous with their time.

A very special thank you is extended to all the core services at MRC Harwell. In particular I would like to thank Sara Wells of the Mary Lyon Centre and all the staff of ward 4, especially Andy Hinton, Lisa Ireson and Lucie Vizor. Thank you to all the other core facilities at MRC Harwell who have processed many samples on my behalf: Jim Humphries, Dave Shipston, Kate Vowell, Naomi Busk and Lynne Beresford of the necropsy team; Jenny Corrigan, Liz Darley, Adele Austin and Caroline Barker of the histology team. Thank you also goes to GEMS core who were a big help with screening the ENU-DNA archive, particularly Adele Traynor and Deen Quwailid. Thank you also to Debbie Williams of GEMS core, for teaching me how to work with RNA and analyse RT-qPCR data.

Importantly, I would like to thank my family for always supporting me and the biggest thank you goes to my wonderful partner, Anna Jarvis. I honestly do not know what I would do without you.

Thank you also to the Nuffield Department of Surgical Sciences for funding this project and to the Medical Research Council Mammalian Genetics Unit for providing the laboratory space and facilities to conduct the work outlined in this thesis.

Statement of the Contribution of Others

This thesis is original, unpublished work done primarily by the author, M. Crompton. However, this thesis could not have been completed without the contributions of others. The Mary Lyon Centre provided all animal care, and basic husbandry was completed by Ward 4 animal technicians. All histological processes for fixing, wax embedding and sectioning of tissues were performed by the MRC Harwell core histology facility. Additionally, staining of sections for F4/80, H&E and PAS were also prepared by the MRC Harwell core histology facility.

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Abbreviations

aa	amino acid
ABR	auditory brainstem response
ALI	acute lung injury
AOM	acute otitis media
ASOM	acute suppurative otitis media
BHI-LEV	brain heart infusion-levinthal's
Cas	CRISPR-associated protein
CBY1	chibby homolog 1
cDNA	complementary DNA
cfu	colony forming units
COM	chronic otitis media
COME	chronic otitis media with effusion
Cre	cyclisation recombinase enzyme
CRISPR	clustered regularly interspaced short palindromic repeat
CSOM	chronic suppurative otitis media
DAB	days after birth
dB	Decibel
dB SPL	Sound Pressure Level
DNA	deoxyribonucleic acid
DNAHC	dynein, axonemal, heavy chain
ECM	extracellular matrix
ENU	<i>N</i> -ethyl- <i>N</i> -nitrosourea
ET	Eustachian tube
EVI1	ecotropic virus integration site 1
EYA	eyes absent homolog
FAK	focal adhesion kinase
FBXO11	F-box protein 11
FESA	Frozen Embryo and Sperm Archive group
GAG	glucose amino glycan
GEMS	Genotyping and Mutation detection Screens group
H&E	hematoxylin and eosin
HDR	homology-directed repair
HIF	hypoxia inducible factor
HR	homologous recombination
HRM	high resolution melting
HSP90	heat shock protein 90
IDS	Iduronatesulfatase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IKK	I κ B kinase
IKMC	International Knockout Mouse Consortium
IL	Interleukin
IP	Intraperitoneal

ITGA5	integrin alpha-5
IVF	<i>in vitro</i> fertilisation
I κ B	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JNK	c-Jun N-terminal kinase
KO	Knockout
LAP	latency associated peptide
LIMK1	LIM domain kinase 1
LKB1	liver kinase B1
loxP	locus of crossing-over of bacteriophage P1
LRR	leucine-rich repeat
LTBP	latent TGF- β binding protein
MAP	mitogen activated protein
MCh	Methacholine
ME	middle ear
MEC	middle ear cavity
MPS	Mucopolysaccharidoses
MRC	Medical Research Council
MyD88	myeloid differentiation primary response gene 88
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next-generation sequencing
NHEJ	non-homologous end-joining
NIK	NF- κ B inducing kinase
NISCH	Nischarin
NP	nasopharynx
NTHi	non-typeable <i>Haemophilus influenza</i>
OM	otitis media
OME	otitis media with effusion
OMV	outer membrane vesicle
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PCD	primary ciliary dyskinesia
PCR	polymerase chain reaction
Penh	enhanced pause
PI(3)P	phosphatidylinositol 3-phosphate
PMN	polymorphonuclear leukocytes
PROVEAN	Protein Variation Effect Analyzer
PRR	proline-rich region
PTK	protein tyrosine kinase
PX	phox-homology domain
RAC1	Ras-related C3 botulinum toxin substrate 1
RAOM	recurrent acute otitis media
RNA	ribonucleic acid
RT-qPCR	real time-quantitative PCR
SEM	scanning electron microscopy

SEM (statistics)	standard error of mean
SHIRPA	SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment
SIFT	Sorting Intolerant From Tolerant
siRNA	small interfering RNA
SNAP	Screening for Non-Acceptable Polymorphisms
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SPF	specific pathogen free
TALEN	transcription activator-like effector nuclease
TGF- β	transforming growth factor beta
TGIF	TGFB-induced factor homeobox
TLR	toll-like receptor
TNF	tumour necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon- β
URTI	upper respiratory tract infection
VEGF	vascular endothelial growth factor
WBC	white blood cell
WHO	World Health Organisation
wk	weeks
ZFN	zinc finger nuclease

CHAPTER 1: Introduction

1.1 Overview

Hearing impairment is a leading cause of disability worldwide (WHO 2008). Childhood hearing impairment, despite being less common than adult-onset hearing impairment, can result in more serious implications due to its potential for interfering with language acquisition leading to inability to interpret speech sounds, economic and educational disadvantage and social isolation. Otitis media (OM) is the most common cause of hearing impairment in children and is primarily characterised by inflammation of the middle ear mucosa (Davidson, Hyde et al. 1989; Kubba, Pearson et al. 2000). The pathogenesis of OM is complex with many key genes and signalling pathways still being identified, through utilising human and mouse genetics, and some of these will be discussed below. One recently identified mouse model of chronic OM is *edison*. The research described in this thesis attempts to characterise the cellular and molecular basis of the phenotypic defects in the *edison* mouse mutant and identify the downstream components of the pathway that are involved in OM. Before discussing *edison*, however, I will describe important recent developments in our understanding of OM, with an emphasis on chronic OM.

1.2 Anatomy of the human ear

The ear is the organ by which we can detect sounds and also aids in balance. The structure of the human ear can be divided broadly into three sections; the outer ear, the middle ear and the inner ear (Figure 1.1). Sound waves from our external environment are funnelled into the outer ear, transferred through the middle ear to the inner ear, and are transmitted to the brain to be registered as sound.

The outer ear includes the pinna, the external auditory canal and the tympanic membrane (commonly known as the ear drum). The role of the outer ear is to funnel sounds from the environment down the external auditory canal and create an

efficient transfer of energy to the tympanic membrane, from sound waves to vibrations. The location of the tympanic membrane means that it is protected from trauma and maintained at a constant temperature and humidity for optimal function. The tympanic membrane of the human ear is an extremely efficient vibrating surface. The majority of this surface area is taut, known as the pars tensa, and consists of three layers: skin, fibrous tissue and mucosa. The centre of the entire membrane is pulled inwards towards the centre of the head, due to the embedding of the malleus (the first bone of the ossicular chain) into the fibrous layer of the pars tensa.

The middle ear (ME) is an air-filled cavity behind the tympanic membrane, enclosed by the temporal bone. The ossicular chain, enclosed within the ME, consists of three small bones (malleus, incus and stapes) and acts to reduce the amplitude of vibrations received from the tympanic membrane. Overall it increases the energy transmitted to the inner ear as vibrations are transferred from the tympanic membrane and down through the malleus, incus and stapes. This energy is ultimately transferred to the inner ear when the stapes footplate presses onto the oval window and causes movement of fluid within the cochlea (a portion of the inner ear). The combined effects of the primary increased pressure and leverage action at the malleus results in a pressure increase at the stapes 40-45 times greater than it would be were the sound to impinge directly on its surface (Emanuel, Maroonroge et al. 2009). Unlike the external auditory canal, the air of the ME is not in direct contact with the atmosphere outside the body. The Eustachian tube (ET) connects from the ME cavity to the nasopharynx (NP). The ET plays an important role in pressure regulation (through equilibrating gas pressure in the ME with atmospheric pressure); protection from potentially damaging sound and secretions; and removal of dead cells and secretions produced in the ME (Bluestone 1996). Optimal sound conduction through maximal mobility of the tympanic membrane is achieved when the ME air pressure is equivalent to that in the external auditory canal (Felding, Rasmussen et

al. 1987). Ventilation of the ME is provided mainly by means of gas exchange through the ciliated ME mucosa, with some exchange occurring through the ET (Felding, Rasmussen et al. 1987). Normally the ET is collapsed (closed), but opening of the tube is enabled with swallowing, through muscle contraction. During swallowing, small volumes of air enter the ME and this helps replace some of the gas lost through diffusion driven gradients across the ME mucosa (Sadé and Ar 1997; Ar, Herman et al. 2007). A functioning ET opens once every minute while awake and every five minutes during sleep. The narrow central section of the ET tube (the isthmus) in healthy ears prevents nasopharyngeal secretions containing bacteria from passing into the ME from the NP and causing infection.

The inner ear is composed of the bony labyrinth and the membranous labyrinth (which runs inside the bony labyrinth separated by a layer of perilymph). The bony labyrinth itself contains the cochlea, involved in hearing, and the vestibular system (semicircular canals and vestibule), associated with balance (Torres and Giráldez 1998). The connection between the middle and inner ear is provided through the oval window, where the pressure from the stapes footplate of the ossicular chain results in displacement of the perilymph within the cochlea. The movement of the perilymph results in displacement of the auditory hair cells, located within the organ of Corti on a thin basilar membrane in the cochlea. Hair cells are the sensory receptors of the auditory system and form bundles made up of three outer rows and one inner row of hair cells which contain stereocilia (Bryant, Goodyear et al. 2002). Each hair cell is connected to both afferent and efferent neurons. Deflection of the hair cell stereocilia results in mechanotransduction and release of a neurotransmitter chemical that communicates with the auditory nerve, transmitting nervous impulses to the brain stem. The human cochlea contains around 30,000 afferent neurons and 1,800 efferent neurons (Martin and Clark 2009). This complex series of neural connections acts in a co-ordinated manner to receive, process and transmit these nervous impulses along the auditory pathway so that we can recognise sound.

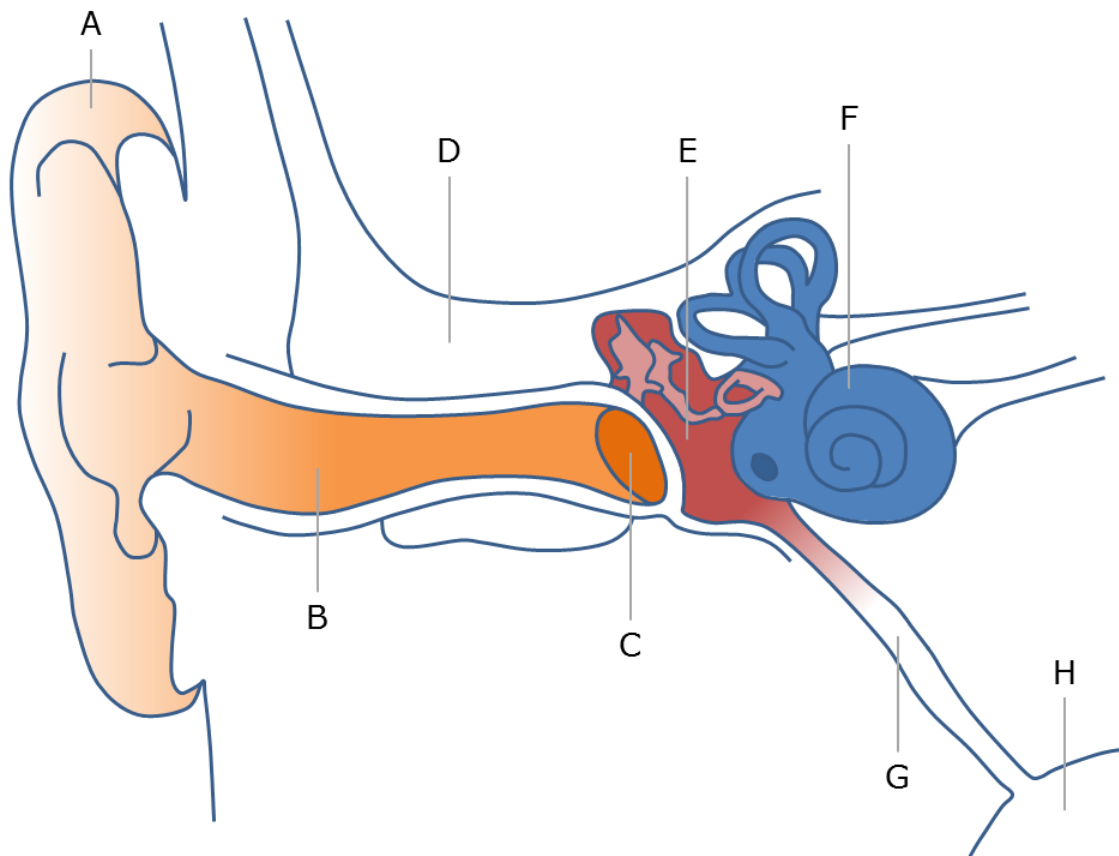


Figure 1.1| The anatomy of the human ear.

The human ear can be divided into three sections; the outer, middle and inner ear (orange, red and blue respectively). **(A)** The outer ear consists of the pinna, **(B)** the external auditory canal and **(C)** the tympanic membrane (also known as the ear drum). **(D)** The movement of the tympanic membrane is aided in humans by the presence of the mastoid cavity although it should be noted that this structure is absent in rodents. **(E)** The middle ear is an air filled cavity containing the malleus, incus and stapes (collectively known as the ossicles). The ossicles transfer mechanical sound waves from the outer ear to the inner ear when the stapes footplate presses onto the oval window. **(F)** The inner ear contains the fluid filled cochlea which converts mechanical sound waves to nervous impulses transmitted to the brain stem via the auditory nerve. The semi-circular canals of the inner ear are involved in balance. **(G)** The Eustachian tube links **(H)** the nasopharynx to the middle ear. Image adapted from Chittka and Brockmann 2005.

1.3 Prevalence of hearing loss

Hearing impairment is a leading cause of disease burden worldwide (WHO 2008). In developed countries alone, hearing impairment is known to be the third most

prevalent chronic condition in older adults (48–92 years), after hypertension and arthritis, with considerable implications for physical and mental health (Cruickshanks, Wiley et al. 1998; Yueh, Shapiro et al. 2003). Stevens *et al.*, recently estimated that the global prevalence of hearing impairment was 1.4% for children aged 5–14 years, 9.8% for females >15 years of age and 12.2% for males >15 years of age (Stevens, Flaxman et al. 2013). In 2012, the World Health Organisation (WHO) estimated that there were 360 million persons in the world with disabling hearing loss (5.3% of the world's population), where 328 million (91%) were adults and 32 million (9%) were children (Table 1.1).

Table 1.1| Prevalence of hearing loss: WHO global estimates.

Figures sourced from the World Health Organisation (WHO).

Population type	Number of people affected worldwide
Children	32 million
Adults	328 million
(Male	183 million)
(Female	145 million)
TOTAL	360 million

While the number of children with a disabling hearing loss is significantly lower than that of adults, studies have suggested that children prone to recurrent ear infections, characterised by prolonged periods of ME effusion, may suffer delays in language acquisition and social development (Teele, Klein et al. 1984). The ability to hear properly as a child is a vital means by which to learn speech and language. As adults, this ability enables us both to communicate effectively with others and remain acutely aware of our surroundings. Not being able to hear properly, as a consequence of childhood ear infections, noise-induced hearing loss or age-related hearing loss, can be distressing and results in large medical and social costs.

Hearing impairment can be sensorineural or conductive. Sensorineural hearing loss is the total or partial inability to hear sound in one or both ears resulting from a dysfunction of the inner ear. It usually results from damage to the hair cells within the cochlea or the auditory nerve (or both). Conductive hearing loss is the total or partial inability to hear sound in one or both ears because of some mechanical problem causing a reduction in sound transmission from the outer to the inner ear. The ossicular chain may fail to conduct sound to the cochlea, or the tympanic membrane may fail to vibrate in response to sound as a result of fluid being present in the ME. ME inflammation, known as otitis media can result in conductive hearing loss and is the most common cause of hearing impairment in children.

1.4 Otitis media

Otitis media (OM) is commonly defined as inflammation of the ME and is a common disease of childhood in both the developed and developing world. OM is an often neglected condition, despite having associated morbidity levels in developing countries that are comparable with conditions such as polio and trachoma (WHO 1993). It is perceived by many to be a transient affliction that in reality places a substantial social, medical and economic burden on healthcare systems globally (Monasta, Ronfani et al. 2012).

OM is a condition that comprises a continuum of related ME diseases of which there are three main types (AOM, OME and CSOM) and no standardised definitions (Cripps, Otczyk et al. 2005) (Figure 1.2). Generally, acute OM (AOM) in children is usually a short-term inflammation of the ME, characterised by the rapid onset of several signs or symptoms of inflammation such as otalgia (earache), otorrhoea (discharge), fever or irritability in the presence of a ME effusion (Bluestone 2003). AOM is often preceded by upper respiratory symptoms, including a cough and rhinorrhoea (runny

nose) (Gates, Klein et al. 2002; SIGN 2003; Rovers, Schilder et al. 2004). This can be self-limiting but the condition can progress to chronic OM. OM with effusion (OME or “glue ear”), is defined as inflammation of the ME, accompanied by the accumulation of fluid, with no signs or symptoms of acute inflammation, and may occur independently of any preceding disease (Bluestone 2003). OME has often been attributed to poor ET function, and will be discussed in more detail in section 1.4.2.1 (Rosenfeld, Culpepper et al. 2004). Acute suppurative OM (ASOM) is an acute inflammation of the ME by pyogenic organisms, resulting in the presence of purulent fluid in the ME (Rowe 1975). It is often accompanied by a bulging, inflamed tympanic membrane that is usually associated with pain and fever. Recurrent acute OM (RAOM) is the term used for individuals that have frequent episodes of AOM, with the clinical definition of RAOM as the presentation of four or more episodes of AOM in the preceding twelve months (Rosenfeld and Kay 2003). Chronic suppurative OM (CSOM) is a persistent inflammation of the ME, which presents with a suppurative ME fluid, rich in neutrophils and leukocytes, and perforation of the tympanic membrane can occur. Here, untreated chronic disease and associated complications can result in irreversible hearing loss (Murphy 1981) and the potential onset of life-threatening secondary conditions such as meningitis (WHO 1993). It is recognised that all OM phenotypes are linked (Bhutta 2014), whereby the presence of one form of OM in an individual is associated with a coincident or subsequent risk of presentation of another form of OM (Rosito, da Costa et al. 2007; Selaimen da Costa, Rosito et al. 2008). This associated risk has been termed ‘otitis prone’ (Howie, Ploussard et al. 1975).

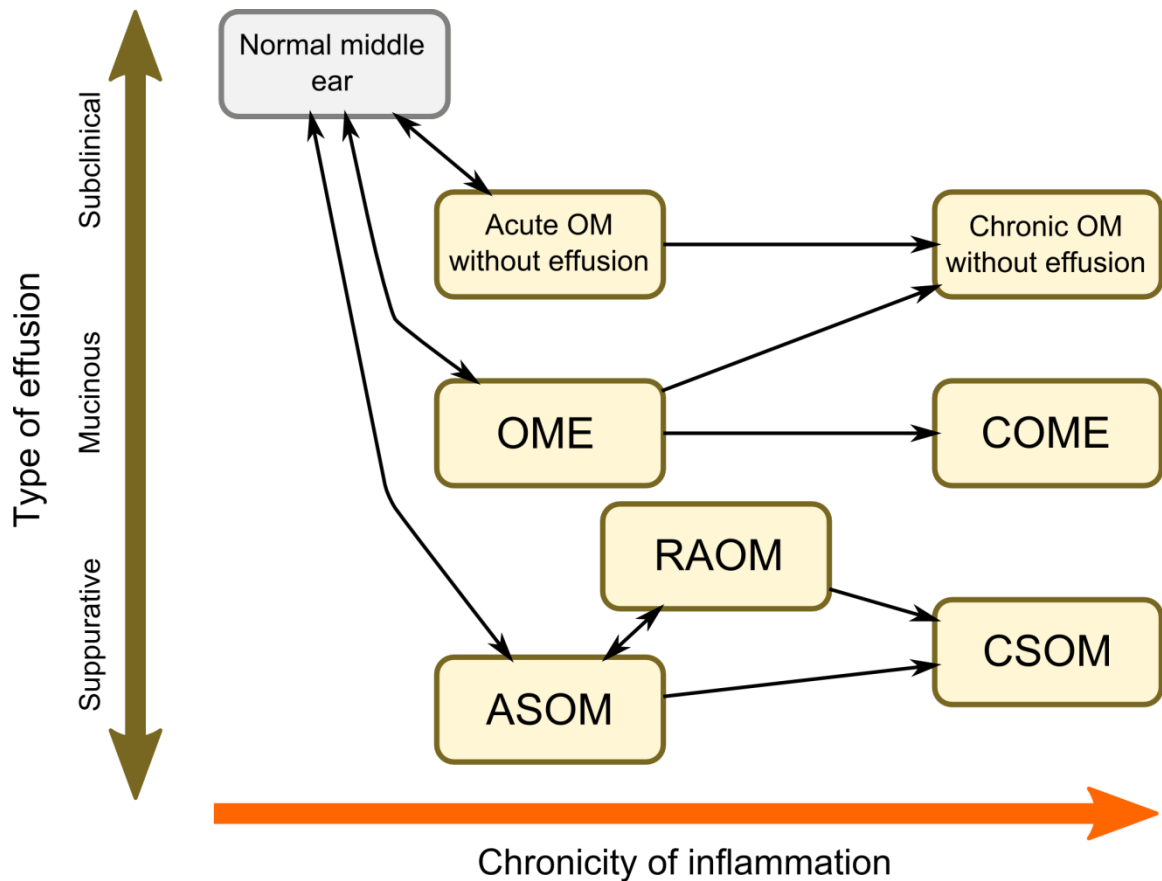


Figure 1.2| Otitis media (OM) is a continuum of related middle ear conditions.

OM can be split into two main types, acute and chronic. There are still no standardised definitions for OM. Taken from Rye *et al.*, 2011. OME, OM with effusion; COME, chronic OM with effusion; RAOM, recurrent acute OM; ASOM, acute suppurative OM; CSOM, chronic suppurative OM.

1.4.1 Burden of disease caused by OM

The incidence of most of the related forms of OM varies between developed and developing countries, however AOM is extremely common in young children in all countries (Davidson, Hyde *et al.* 1989). Studies from developed countries show that around 80% of children experience an episode of AOM before the age of three (Teele, Klein *et al.* 1989; Vergison, Dagan *et al.* 2010), with almost 40% of children experiencing more than six episodes by the age of seven (Casselbrant, Mandel *et al.* 1999). In a recent review of 114 papers on OM epidemiology, the global estimate for the incidence rate for AOM was predicted to be 10.85% (709 million cases each

year), with the highest incidence rate seen between one and four years of age at 60.99% (Monasta, Ronfani et al. 2012). In the UK, and other developed countries, OME is cited as the most common cause of hearing loss in children under the age of twelve (Davidson, Hyde et al. 1989). A prospective study of the duration and recurrence of OME in children from birth to 3 years found that nearly one fifth of children in the study had either unilateral or bilateral OME for more than half of their first three years of life (Hogan, Stratford et al. 1997). Interestingly, the mean effusion free period (the time spent between episodes of effusion) in the children with a low incidence of OME (31 weeks) was three times longer than the children who experienced a high incidence of OME (10 weeks) (Hogan, Stratford et al. 1997). With the high prevalence and the progression to chronic disease in a large number of children, OME results in 30,000 operations for the insertion of tympanostomy tubes (also known as grommets or ventilation tubes) per annum in the UK (Sood and Waddell 2007). Along with grommets, treatment of OME requires consultations, referrals and antibiotic prescriptions, which are responsible for the considerable costs to the NHS of up to £300 million annually (Williamson, Bengt et al. 2006). In the USA this significant burden is increased, where the recorded 2.2 million annual episodes of OME have a cost of approximately \$4 billion (Lous, Burton et al. 2005; Trune and Zheng 2009). Serious secondary complications as a result of chronic ME disease, such as meningitis, have declined in developed countries as a consequence of antibiotic use (Davidson, Hyde et al. 1989). However, this is not observed in the developing world where CSOM is a greater burden, estimated to affect 65-330 million individuals worldwide (WHO 2004). CSOM accounts for 60-80% of ME disease in Africa, Tanzania and South East Asia and suppurative intracranial complications contributing to high mortality rates occur in almost 10% of cases (Davidson, Hyde et al. 1989).

1.4.2 Risk factors for OM

The main risk factors that influence the development of OM can be host-related factors (such as craniofacial and ET abnormalities) or environmental factors (such as passive or parental smoking). A summary of the proposed risk factors can be found in Table 1.2. This can primarily include young age, gender, race, social class, smoking/passive smoking, day-care and respiratory infections, no/short duration breast feeding, exposure to other children/siblings and pollutants (Daly, Hoffman et al. 2010). The number of known risk factors demonstrates the multi-factorial nature of this condition.

Table 1.2| Summary of the proposed risk factors in the development of OM.

Adapted from Kong and Coates 2009.

	Risk Factors	Comment	References
Host-related	<i>Age</i>	Highest incidence between 1 and 4 years of age	Monasta <i>et al.</i> , 2012
	<i>Sex</i>	Higher incidence among boys, reasons unknown	Teele <i>et al.</i> , 1989
	<i>Ethnicity</i>	Particularly prevalent in indigenous groups, e.g. the Aboriginal Australian	Boswell <i>et al.</i> , 1996
	<i>Premature birth</i>	Increased risk	Engel <i>et al.</i> , 1999
	<i>Allergy</i>	Association is recorded, but pathways unclear	Tomonaga <i>et al.</i> , 1988; Pukander <i>et al.</i> , 1985
	<i>Immune system dysfunction</i>	Subtle immune deficiencies often noted in RAOM	Prellner <i>et al.</i> , 1989
	<i>Genetic predisposition</i>	Estimated heritability for time spent with ME effusion is 72%	Casselbrant <i>et al.</i> , 2004
	<i>Craniofacial / Eustachian tube abnormalities</i>	Increased incidence in children with cleft palate, Down syndrome and craniofacial anomalies	Engel <i>et al.</i> , 1999
Environmental	<i>Seasonality</i>	Higher incidence in winter and lower incidence in summer	Castagno <i>et al.</i> , 2002
	<i>Lack of breastfeeding</i>	Being breastfed has a protective effect	Uhari <i>et al.</i> , 1996
	<i>Child daycare / Overcrowding</i>	Higher incidence with daycare attendance and overcrowded family home	Wald <i>et al.</i> , 1988; Pukander <i>et al.</i> , 1985
	<i>Youngest sibling</i>	Increased risk with older siblings	Pukander <i>et al.</i> , 1985
	<i>Use of dummy (pacifier)</i>	Increased risk after 11 months of age	Uhari <i>et al.</i> , 1996
	<i>Low socioeconomic status</i>	Variable but generally increased risk with lower status	Smith <i>et al.</i> , 2010
	<i>Upper respiratory tract infection</i>	Viruses predispose to otitis media	Ruuskanen <i>et al.</i> , 1994
	<i>Parental / Passive smoking</i>	Increased risk	Uhari <i>et al.</i> , 1996; Jacoby <i>et al.</i> , 2008

1.4.2.1 Eustachian tube

The precise sequences of events that lead to OM development have been hotly contested. According to the “hydrops ex vacuo” theory (Politzer, Ballin et al. 1902), a primary obstruction of the ET causes negative pressure to develop in the ME which leads to exudate formation through transudation (fluid entry from mucosal blood vessels) or exudation (as a consequence of nasopharyngeal secretions being aspirated into the ME via the ET). Young children and children with craniofacial abnormalities, with ETs deemed to be shorter, more horizontal in orientation and “floppy” were proposed to have a primary dysfunction of the ET, be at a higher risk for bacterial ascension to the ME and consequently at an increased risk of developing OM. Using different ET function tests (automatic Toynbee test, tympanometry and Valsalva’s manoeuvre), ET dysfunction was observed more frequently in patients with CSOM compared to control patients (Yüçetürk, Unlü et al. 1997). Down syndrome and craniofacial abnormalities, such as cleft palate, affect both the anatomy and function of the ET and also predispose to CSOM (Nelson 1988). However, it is possible that the development of ME disease in these syndromes is achieved through unique pathobiological mechanisms that would not translate to non-syndromic OM (Bhutta and Burton 2013). In contrast, others disputed that the obstruction of the ET was a secondary event caused by the formation of a mucus plug generated after an initial inflammatory process (Sadé and Ar 1997).

Currently it is thought that new episodes of OM are usually a complication of an upper respiratory tract infection (URTI), which causes ET dysfunction through reducing ME ventilation, ciliary clearance and drainage (Darrow, Dash et al. 2003). Various studies have found that URTI can cause damage to the ME mucosa, resulting in easier bacterial ascension and persistence in the ME (Bakaletz 2010). However, a clear *in vivo* association has not been established between a particular viral species,

causing the concomitant URTI, and a specific OM bacterial pathogen adhering to and colonising mucosal surfaces (Kleemola, Nokso-Koivisto et al. 2006). Damage to the ME mucosa has been observed through *in vitro* studies as a reduction in ciliary beat frequency: firstly, as a consequence of leukocyte produced elastase (Smallman, Hill et al. 1984); secondly, from toxins produced by *Haemophilus* bacterial species (Wilson, Roberts et al. 1985); and finally, from acidic pH (Luk and Dulfano 1983). Passive smoking has also been observed to reduce ciliary function in CSOM patients (Agius, Wake et al. 1995). Accumulation of ME secretions, which reduce ciliary function further, has been proposed as a method to facilitate the progression from AOM/OME to CSOM (Agius, Wake et al. 1995; Atef and Ayad 2004). The importance of correctly functioning cilia in the ME mucosa is further illustrated by the autosomal recessive disorder, primary cilia dyskinesia (PCD). PCD is characterised by impaired mucociliary clearance through abnormal or reduced cilia beating and common symptoms include chronic rhinitis, sinusitis and OME (Campbell, Birman et al. 2009). Onset of OM appears to reflect a complex interplay between the nasopharyngeal microbiota and host mucosal immunology.

1.4.2.2 Microbiology of OM

In humans, the NP is colonized with an assortment of non-pathogenic bacteria, including viridans streptococcus, non-hemolytic streptococcus and diphtheroids (Faden, Duffy et al. 1997). Varieties of other bacterial species are also carried asymptotically in the NP, yet can contribute to disease if allowed to dominate. Three bacterial species that are commonly cultured from children with AOM are non-typeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Hong, Peng et al. 2010). Nasopharyngeal colonisation with these three species begins shortly after birth, with most NTHi colonisation occurring before one year of age (Faden, Duffy et al. 1996). Children have been shown to carry a single

predominant strain of *NTHi*, although the NP of a child can become colonised with up to seven different strains over the first two years of life (Faden, Duffy et al. 1996). The use of pneumococcal vaccines in recent years has meant that *NTHi* has become the most prevalent isolated pathogen in AOM (Block, Hedrick et al. 2004; Casey and Pichichero 2004).

Several studies have indicated that OM is more likely to occur in children that have either an earlier or increased nasopharyngeal colonisation of the three bacterial species commonly associated with ME disease (Faden, Duffy et al. 1996; Faden, Duffy et al. 1997). Colonisation of pathogens stimulates the production of mucosal as well as serum antibodies. Specific Immunoglobulin-A (IgA) mucosal antibody limits the duration and frequency of colonisation, whereas serum IgG antibody protects children against the development of OM but does not affect colonisation (Faden 2001). Infection with *S. pneumoniae* or *NTHi* in the first year of life has been shown to increase the risk of becoming 'otitis-prone' (four or more episodes of OM in twelve months) (Faden 2001). It has been proposed that otitis-prone children may generate poor mucosal immune responses, whereby they have lower levels of the antibodies IgA and IgG. A persistent and recurrent colonisation by *NTHi* is observed in children with lower IgA levels, whereas children with increased immune responses are usually only colonised briefly (Harabuchi, Faden et al. 1994). More recently, it has been suggested that reduced IgG responses to pneumococcal antigens in otitis-prone children may be caused by poor generation of memory B-cells (Sharma, Casey et al. 2012). This all supports evidence suggesting that breastfeeding has a protective effect on the development of ME disease related to the immunological properties of breast milk (Pettigrew, Khodaei et al. 2003; Chantry, Howard et al. 2006).

1.4.2.3 Genetic contributions of OM

Heritability refers to the proportion of risk of disease in populations that can be attributed to inherited, genetic factors. Evidence suggests that chronic OM (COM) clusters in families and exhibits substantial heritability, with estimates of around 40-70% (Kvaerner, Tambs et al. 1997; Daly, Brown et al. 2004; Rye, Bhutta et al. 2011). By controlling for common environmental factors shared between siblings, prospective studies found that children who have siblings with a history of COME/RAOM have a higher risk of developing OM themselves (Sipilä, Karma et al. 1988; Teele, Klein et al. 1989; Rasmussen 1993). In a three year twin and triplet study, Casselbrant *et al.* concluded that there was a strong genetic component to the amount of time spent with ME effusion and the number of episodes of AOM in children, estimating the heritability at 72% (Casselbrant, Mandel et al. 1999; Casselbrant, Mandel et al. 2004). Similarly, a retrospective questionnaire study of heritability for RAOM in twins has been estimated at 49% (Rovers, Haggard et al. 2002). There is a lack of basic epidemiological data on the development of CSOM due to its low prevalence, but reports of familial clustering suggest a genetic component is also likely for CSOM (Jensen, Homøe et al. 2011). Unfortunately, many of the genes involved in OM susceptibility have still yet to be identified.

1.4.2.3.1 Genome wide linkage studies

Genome wide linkage studies (GWLS) enable the identification of susceptibility loci within the genome of affected individuals by typing microsatellite markers or single nucleotide polymorphisms (SNPs) between sets of related individuals. Only two GWLS to identify OM susceptibility loci have been carried out and published to date. The first was performed using the Minnesota COME/ROM Family Study (Daly, Rich et al. 1996), and linkage analysis identified regions of linkage at chromosome 10q26.3 and 19q13.4 (Daly, Brown et al. 2004). Fine mapping narrowed the linkage region at

19q13.4, however failed to identify the underlying disease gene(s) (Chen, Allen et al. 2011). The second GWLS was conducted by University of Pittsburgh using a different population containing 403 Caucasian and 26 African American families (Casselbrant, Mandel et al. 2009). Linkage analysis in the Caucasian population revealed regions of linkage at 4p15.2, 6q25.1, 7q33, 10q22.3 and 17q12. Combined analysis of Caucasian and African American pedigrees only showed increased significance at 10q22.3. Again, further analysis failed to identify the causative gene(s).

Differences in the findings of the two GWLS can be attributed to a potential lack of statistical power in these types of study and also disparate definitions of OM phenotypes upon patient recruitment. While the susceptibility genes within these linkage regions have not yet been identified, some potential susceptibility loci have been tentatively identified. *ADAM8*, a gene associated with allergy, has been proposed as a potential candidate underlying the 10q26.3 linkage (Daly, Brown et al. 2004). A variety of genes encoding proteins forming part of the inflammasome complex have been postulated as being responsible for the linkage at 19q13.4 (Chen, Allen et al. 2011). Additionally, a cluster of chemokine genes and surfactant protein genes have been identified as possible candidate genes at 17q12 and 10q22.3 respectively (Casselbrant, Mandel et al. 2009).

1.4.2.3.2 Candidate gene studies

Candidate gene studies are used to investigate the potential contribution of a gene towards disease susceptibility. These types of studies have been carried out worldwide for OM, with positive associations for many genes involved in immune and inflammation pathways; including *IGH* (G2m(23) allotype) (Prellner, Hallberg et al. 1985), *HLA-A* (Kalm, Johnson et al. 1991), *SP-A1* (Rämet, Löfgren et al. 2001; Pettigrew, Gent et al. 2006), *IL1 α* (Joki-Erkkilä, Puhakka et al. 2002), *IFN γ* (Gentile,

Doyle et al. 2003), *MBL2* (Nuytinck, De Meester et al. 2006; Wiertsema, Herpers et al. 2006), *TGF- β 1* (Patel, Nair et al. 2006), CD14 (Wiertsema, Khoo et al. 2006), *TNFa* (Patel, Nair et al. 2006; Emonts, Veenhoven et al. 2007), *IL6* (Patel, Nair et al. 2006; Emonts, Veenhoven et al. 2007; Alper, Winther et al. 2009; Revai, Patel et al. 2009), *FBXO11* (Segade, Daly et al. 2006; Rye, Wiertsema et al. 2011), *PAI-1* (Emonts, Wiertsema et al. 2007), *TLR4* (Emonts, Veenhoven et al. 2007; Alpay, Etem et al. 2010; Sale, Chen et al. 2011; MacArthur, Wilmot et al. 2014), *IL10* (Emonts, Veenhoven et al. 2007; Alper, Winther et al. 2009; Sale, Chen et al. 2011), *MUC5* (Sale, Chen et al. 2011), *MUC5AC* (Ubell, Khampang et al. 2010), *SMAD2* and *SMAD4* (Rye, Wiertsema et al. 2011; MacArthur, Wilmot et al. 2014), *SLC11A1* (Rye, Wiertsema et al. 2013), and finally *MUC5B* (MacArthur, Wilmot et al. 2014). Equally, many genes have also been identified as not being significantly associated with OM in these studies; including but not limited to *NOS2A*, *PARP* and *C1INH* (Emonts, Veenhoven et al. 2007), *TNF* and *IL-6* (Alper, Winther et al. 2009), *EVI1* (Rye, Wiertsema et al. 2011; Sale, Chen et al. 2011), *MUC2* (Ubell, Khampang et al. 2010), *SMAD3* and *SMAD7* (Rye, Wiertsema et al. 2011). It should be noted however that these different studies all phenotyped OM slightly differently and this may contribute towards the results obtained.

1.4.2.3.3 Genome wide association studies

Genome wide association studies (GWAS) involving direct testing of genetic polymorphisms in large series of cases versus controls, provide a powerful approach to identify lower penetrance alleles that cannot be detected by GWLS (Easton and Eeles 2008). Two GWAS of OM have been published to date. The first GWAS of OM was conducted in Western Australia using the Western Australian Pregnancy (Raine) cohort and identified two chromosomal areas of interest for OM (Rye, Warrington et al. 2012). *CAPN14* and *GALNT14* on chromosome 2p23.1 and the *BPIFA* cluster on

chromosome 20q11.21. However, there were limitations of the GWAS including phenotypic heterogeneity of samples and sample size. The second GWAS of OM identified a SNP bordered by *CDCA7* and *SP3* on chromosome 2 as significantly associated, using the Minnesota COME/ROM Family cohort (Allen, Chen et al. 2013). Most recently, a study using this same cohort failed to replicate associations identified from the Raine cohort (Allen, Manichaikul et al. 2014). This further illustrates the need for the recruitment of larger, more diverse populations and the use of standardised inclusion criteria for OM studies.

1.5 Inflammatory processes

The immune system enables the human body to recognise and neutralise pathogens. All cells of the immune system are derived from pluripotent hematopoietic stem cells in the bone marrow. Once mature, these cells will circulate continuously between the blood and lymphatic vessels, and peripheral tissues of the body in order to perform their roles in identification and destruction of pathogens. The proper function of cellular responses to bacterial challenges and a controlled immune response are important in the resolution of some types of OM.

1.5.1 Innate

Innate immunity is the non-specific and immediate response of the immune system to pathogens. Its principal components include granulocytes, macrophages, mast cells, dendritic cells, natural killer cells and complement. When homeostasis is compromised through the invasion of the epithelial surfaces of the body by micro-organisms such as bacteria, recognition is made possible through binding of highly conserved epitopes, usually lipids, proteins and nucleic acids, found on the bacterial

cell wall. These pathogen associated molecular patterns (PAMPs) are identified by the pattern-recognition family of receptors. Upon recognition, mast cells and macrophages immediately begin to release cytokines and chemokines, matrix remodelling proteases, histamine and reactive oxygen species in order to induce the migration of additional leukocytes from the blood stream to the site of infection (de Visser, Eichten et al. 2006). This recruitment of additional granulocytes from the blood acts to propagate inflammation further in order to control and neutralise pathogens. Further, vascular and fibroblast responses are induced by mast cells and macrophages to help co-ordinate the destruction of invading pathogens (de Visser, Eichten et al. 2006). These vascular responses include the dilation and increased permeability of blood vessels during inflammation in addition to alterations in the adhesive properties of the endothelium. All these physiological responses have the net effect of maximising local blood flow, increasing fluid leakage from blood vessels and causing circulating leukocytes to bind endothelial cells of the blood vessel wall and migrate to infection sites. Subsequently, the flow of lymph is increased, and in turn increases the speed with which dendritic cells migrate to regional lymph nodes and activate the adaptive immune response.

1.5.2 Adaptive

The innate arm of the immune system acts to contain the infection, therefore allowing time for the adaptive immune response to generate a more specific and sophisticated response through the expansion of cells possessing specific antigen receptors which recognise specific pathogens. The two major types of lymphocytes which form the adaptive immune system are T and B lymphocytes. All T and B cells recognise a specific unique pathogen antigen due to possessing diverse antigen specific receptors; T-cell receptors on T cells, and immunoglobulins on B cells. Normally, T and B cells remain inactive in the blood stream, though once the specific

signals are received they can then generate sufficient numbers of antigen specific cells to counteract infection. Activation occurs through direct interactions of T cells with mature antigen presenting cells, such as dendritic cells. T cells are able to activate B cells directly, allowing the B cell antigen receptor, known as antibody, to be secreted.

1.5.3 Resolution of inflammation

The resolution of inflammation involves the controlled destruction of recruited cells through apoptosis and a halt in the recruitment of fresh cells from the blood (Khanna, Biswas et al. 2010). Additionally, once infection is resolved a small subset of B and T cells remain in the blood to form memory cells. This means that if the same antigen is encountered again the adaptive immune system can mount a quicker response and ultimately forms the basis of immunological memory.

Studies on human ME fluid have identified a number of inflammatory cells in OM (Palva, Häyry et al. 1980; Wong and Hawke 1983; Berger, Hawke et al. 1984; Sipilä 1984; Skotnicka, Stasiak-Barmuta et al. 2005), suggesting that OM can be considered as an inflammatory condition. In human OM, large numbers of lymphocytes and IgA secreting cells are recruited to the ME suggesting that immune system responses may help resolve infection (Sabirov, Hamilton et al. 2008). Additionally, increases in antibodies in both the ME mucosa and blood serum have been correlated with the resolution of AOM (Karjalainen, Koskela et al. 1991). It is still not understood whether the inflammation observed in OM is the cause of conductive hearing loss, or whether the inflammation occurs as a secondary event to resolve the primary insult causing OM. In recent years a number of mouse models for OM have been identified and used to help discover new signalling pathways and potential therapeutic targets in OM.

1.6 The mouse as a model

The mouse has become an important model in scientific research for a variety of human diseases. The ease of genetic manipulation, relatively short generation times and comparable physiology with humans make mice the ideal model to explore our understanding of disease mechanisms and help to develop cures. Furthermore, fully sequenced mouse (Mouse Genome Sequencing, Waterston et al. 2002) and human (Lander, Linton et al. 2001; Venter, Adams et al. 2001) genomes now exist, which have aided in the identification of genetic loci contributing to human disease. The environment they are housed in can be well regulated, allowing this effect to be examined or minimised so that the environment–gene interactions can be fully scrutinised. Similar diagnostic tests as those used in human patients have been established in the mouse, allowing phenotyping results to be at least partly comparative between mouse models and human studies, leading to stronger translatable results. Standardised protocols for mouse phenotyping are available, allowing researchers to characterise mouse models in a comparable way (EMPreSS (European Mouse Phenotyping Resource of Standardised Screens) (Brown, Chambon et al. 2005) and IMPReSS (International Mouse Phenotyping Resource of Standardised Screens)).

1.6.1 Genetic manipulation

Humans have been interested in breeding mice and their inheritance of traits over the last 3,000 years (Beck, Lloyd et al. 2000). Inbred mice are strains that have undergone at least twenty consecutive 'sister x brother' or 'parent x offspring' matings, where on average 98.6% of the loci in each mouse are homozygous. Using inbred strains in research minimises background effects allowing us to observe

phenotypic differences caused only by the desired genetic manipulations. Mouse mutagenesis can be divided into two main strategies (Brown, Wurst et al. 2009); either a gene-driven approach, for instance gene targeting mutagenesis that allows predesigned, site-specific modifications into the genome; or a phenotype-driven approach such as *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis that produces random point mutations. Spontaneous mutations also occur, but are only detected if there is a visible phenotype. With recent advances in site-specific mutagenesis, in particular the sophisticated CRISPR/Cas system, and the continued development of conditional mutagenesis methods, these technologies promise to expand the toolkit to manipulate the mouse genome and hence our ability to explore and alter the genome to help understand and treat disease.

1.6.1.1 Gene-driven mutagenesis

Targeted manipulation of the mouse genome has been possible for over 20 years (Capecchi 1989). Gene targeting involves the pre-planned insertion of a selectable marker into the genome of embryonic stem cells by homologous recombination (HR) (Thomas, Folger et al. 1986; Brown, Wurst et al. 2009). Specifically, insertion into an exon of the target gene (or the replacement of one or more exons) resulting in gene inactivation. This method can generate many sorts of genetic modification, including gene knockout, overexpression, point mutations, splice variants and conditional alleles. Conditional gene targeting allows researchers to selectively target gene inactivation to a specific tissue, cell type and/or developmental stage. This selective targeting avoids embryonic lethality, which can be common with the classical gene targeting approach. In a conditional mutant, the Cre/loxP system is the most frequently used method to attain gene inactivation (Rajewsky, Gu et al. 1996; Brault, Besson et al. 2007). Over a hundred Cre transgenic strains have been

generated with tissue-specific Cre expression (Nagy and Mar 2001), with many Cre lines accessible at The Jackson Laboratory [<http://cre.jax.org/index.html>].

These conventional gene targeting strategies have been very effective but the generation of mice is often time consuming. More recently zinc finger nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs) have proven to be useful additions for targeted manipulation of the mouse genome (Davies, Davies et al. 2013; Chen, Forsberg et al. 2014). ZFNs are artificial restriction enzymes that are capable of inducing double stranded DNA cleavage and can be engineered to target pre-planned DNA sequences. Similarly, TALENs can be generated to bind a particular DNA sequence and then cause a double stranded DNA cleavage. Homology-directed repair (HDR) or non-homologous end-joining (NHEJ) is stimulated and can then be utilised to introduce specific mutations into the mouse genome (Gaj, Gersbach et al. 2013). Unlike traditional HR, ZFNs and TALENs are quicker, simpler and can bypass the time consuming assembly of large constructs.

Alternatively, the bacterial CRISPR/Cas system has recently emerged as an efficient gene targeting technology in mammalian cells (Li, Qiu et al. 2013; Wang, Yang et al. 2013). The system works via RNA-guided DNA cleavage, whereby CRISPR RNAs direct sequence-specific cleavage of target DNA by Cas nucleases to introduce double stranded breaks that stimulate HDR or NHEJ at specific genomic locations (Gaj, Gersbach et al. 2013). A significant advantage of the CRISPR/Cas system over ZFNs and TALENs is that multiplex genes can be targeted simultaneously with multiple targeted CRISPR RNAs to enable simultaneous editing of several sites within the mammalian genome (Cong, Ran et al. 2013). The CRISPR/Cas system can provide an important gene targeting tool for generating suitable animal models for the functional testing of particular human SNP variants identified from GWAS of human disease.

1.6.1.2 Phenotype-driven mutagenesis

ENU is a powerful chemical mutagen, which randomly mutagenises the spermatogonial germ cells routinely causing single base-pair changes (Justice, Noveroske et al. 1999). The introduction of random point mutations can result in a variety of different alleles. It is used as a phenotype-driven approach where no *a priori* assumptions are made concerning the mutated gene involved and its function. ENU has been used successfully in many mutagenesis programmes worldwide to identify novel genes of human diseases (Nolan, Peters et al. 2000; Acevedo-Arozena, Wells et al. 2008). Mutagenised males are crossed with wild-type females to produce G_1 progeny (Figure 1.4). The G_1 progeny are then assayed using a variety of different phenotyping tests to screen for dominant mutations. In order to screen for recessive mutations, G_2 female animals are mated back to the original G_1 to homozygose mutations. The G_3 animals can then undergo the same phenotyping tests to be screened for recessive mutations. The major problem with ENU mutagenesis has always been the costly and labour intensive mapping of any mutations generated. With the emergence of low-cost bench top next-generation sequencing platforms this step is becoming considerably quicker, greatly facilitating mutation recovery from mutagenesis screens.

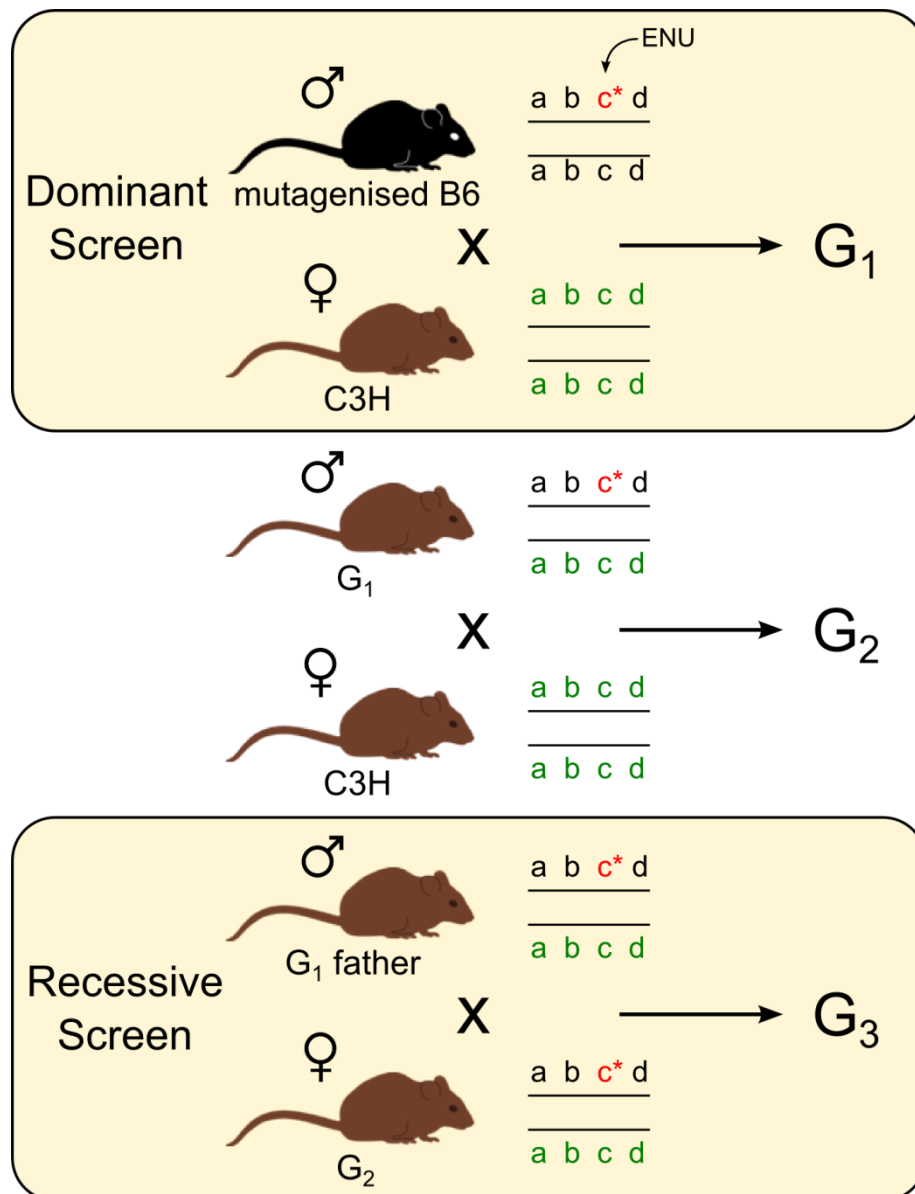


Figure 1.3| Breeding scheme for G₁ and G₃ pedigree generation in *N*-ethyl-*N*-nitrosurea (ENU) mutagenesis screens.

Male C57BL/6J mice are injected with the mutagen ENU and mated to wild-type C3H-HeH females (G₀). Their G₁ progeny enter the phenotyping pipeline for the dominant screen. To generate G₃ animals to screen for recessive mutations, G₂ females are generated and backcrossed back to the founder G₁ male. Modified from Hardisty-Hughes *et al.*, 2010.

1.6.2 Mouse Resources

Following on from the completion of sequencing the mouse genome, the International Knockout Mouse Consortium (IKMC) was established to systematically generate null mutant ES cells that provide both complete null and conditional null

alleles for every protein coding gene in the mouse genome (Austin, Battey et al. 2004; Skarnes, Rosen et al. 2011). The IKMC aims to make the generated resources readily available to the research community (Ringwald, Iyer et al. 2011). Exploiting the knockout mouse resource the International Mouse Phenotyping Consortium (IMPC) was established to generate mouse lines from each targeted ES cell and determine the phenotype of the mutant mice (Gates, Mallon et al. 2011; Brown and Moore 2012). With high-throughput phenotyping of each line functional information can be attached to every gene in the mouse genome (Brown and Moore 2012), providing novel genes and models for translational research for human diseases including OM.

1.7 Mouse models of OM

The functional and anatomical similarities between human and mouse ears make the mouse an ideal model for OM (Brown, Hardisty-Hughes et al. 2008). Mice have been used to model AOM by inoculation of the ME with human otopathogens via trans-bullar injection (Ryan, Ebmeyer et al. 2006) or pressure induced translocation from the NP (Stol, van Selm et al. 2009). Interest is also developing in the use of mice as a model for mucosal vaccination against OM (Sabirov and Metzger 2008). Models of COM have been identified from mouse mutagenesis programs, including gene-driven knockout approaches (Chen, Ingham et al. 2013; Tateossian, Morse et al. 2013) and phenotype-driven ENU mutagenesis screens (Hardisty, Erven et al. 2003; Parkinson, Hardisty-Hughes et al. 2006). As such, several murine models have now been described in the literature with mutations in a variety of pathways. Summaries of the published AOM and COM murine models can be found in Table 1.3 and 1.4 respectively. An introduction to some of these OM models which I consider the most interesting and informative are described below in detail.

Table 1.3| Summary of the current mouse models for acute OM.

KO, knockout; SNP, single nucleotide polymorphism.

Pathway	Locus	Chr	Model type	Background Strain	General Phenotype	OM Phenotype	References
Acute (induced) otitis media							
Complement	<i>Bf</i>	17	KO	C57BL/6	Mild immune defects	Delayed clearance of acute infection	Tong <i>et al.</i> , 2010
	<i>Bf/C2</i>	17	Double KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Tong <i>et al.</i> , 2010
	<i>C1qa</i>	4	KO	C57BL/6	Autoimmune disease, increased mortality, glomerulonephritis	Delayed clearance of acute infection	Tong <i>et al.</i> , 2010
Toll-like Receptors	<i>MyD88</i>	9	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Hernandez <i>et al.</i> , 2008
	<i>Tlr2</i>	3	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Leichtle <i>et al.</i> , 2009
	<i>Tlr4</i>	4	SNP or KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Hirano <i>et al.</i> , 2007; MacArthur <i>et al.</i> , 2006
	<i>Tlr9</i>	9	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Leichtle <i>et al.</i> , 2012
	<i>Ticam1 (Trif)</i>	17	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Leichtle <i>et al.</i> , 2009
TNF	<i>lpr</i>	19	SNP	MRL/MpJ	Autoimmune disease	Delayed clearance of acute infection	Rivkin <i>et al.</i> , 2005
	<i>TNFA</i>	17	KO	C57BL/6; 129S6	Immune defects	Spontaneous mild mucosal hyperplasia. Delayed clearance of acute infection	Leichtle <i>et al.</i> , 2010; Ebmeyer <i>et al.</i> , 2011
Miscellaneous	<i>Cyld</i>	8	KO	C57BL/6	Lymphoid hyperplasia, susceptibility to colitis and colonic adenocarcinoma	Enhanced inflammation	Lim <i>et al.</i> , 2007
	<i>Il-10</i>	1	KO	C57BL/6.129P2	Reduced size, anaemia, chronic enterocolitis	Reduced mucins in acute infection	Tsuchiya <i>et al.</i> , 2008
	<i>Il-17A</i>	1	KO	C57BL/6	Immune defects	Delayed clearance of acute infection	Wang <i>et al.</i> , 2014
	<i>Lyz2</i>	10	KO	C57BL/6.129Sv	Immune defects	Increased susceptibility to acute infection	Shimada <i>et al.</i> , 2008

Table 1.4| Summary of the current mouse models for chronic OM.

KO, knockout; SNP, single nucleotide polymorphism.

Pathway	Locus	Chr	Model type	Background Strain	General Phenotype	OM Phenotype	References
Chronic otitis media							
TGF- β	<i>Fbxo11</i>	17	SNP	C3H/HeN	Mild sensorineural hearing loss	Spontaneous chronic in heterozygote	Hardisty <i>et al.</i> , 2003; Hardisty-Hughes <i>et al.</i> , 2006
	<i>Tgif1</i>	17	KO	C57BL/6J	Smaller size, craniofacial defect	Spontaneous chronic	Tateossian <i>et al.</i> , 2013
NF- κ B	<i>Evi1</i> (<i>Mecom</i>)	3	SNP	C3H/HeN	Extra digits	Spontaneous chronic in heterozygote	Parkinson <i>et al.</i> , 2006
	<i>IkBaΔN</i>	9	Knock-in	C57BL/6	Defective epithelial appendages, lymph node formation, and macrophage function	Spontaneous chronic	Schmidt-Ullrich <i>et al.</i> , 2001
EYA	<i>Eya1</i>	1	KO	129/SvJ, C57BL/6 or Balb/C	Ossicular and inner ear defects, renal anomalies	Spontaneous chronic in heterozygote	Xu <i>et al.</i> , 1999
	<i>Eya4</i>	10	KO	CBA/J	Sensorineural hearing loss	Spontaneous chronic in heterozygote	Depreux <i>et al.</i> , 2008
Ciliary Defects	<i>Cby1</i>	15	KO	C57BL/6	Absence of motile cilia	Spontaneous chronic	Voronina <i>et al.</i> , 2009
	<i>Dnahc5</i>	15	Transgenic	C57BL/6; CBA/J	Immotile cilia, respiratory infection, left-right defect, hydrocephalus,	Spontaneous acute/chronic	Ibañez-Tallon <i>et al.</i> , 2002
	<i>Dnahc11</i>	12	SNP	C3H/HeH or mixed	Immotile cilia, sperm dysmotility, rhinitis, sinusitis	Spontaneous chronic	Lucas <i>et al.</i> , 2012
	<i>Dpcd</i>	19	KO	C57BL/6-Tyrc-Brd	Smaller size, hydrocephalus, situs invertus, sperm dysmotility, rhinosinusitis	Not well characterised	Vogel <i>et al.</i> , 2012
	<i>Kif27</i>	13	KO	C57BL/6-Tyrc-Brd	Smaller size, hydrocephalus, rhinosinusitis, reduced viability	Not well characterised	Vogel <i>et al.</i> , 2012
	<i>Odf2</i>	2	KO	C57BL/6	Dysfunctional cilia, sperm dysmotility, hydrocephalus, sinusitis	Not well characterised	Kunimoto <i>et al.</i> , 2012
	<i>Stk36</i>	1	KO	C57BL/6-Tyrc-Brd	Smaller size, hydrocephalus, craniofacial defects, reduced viability	Not well characterised	Vogel <i>et al.</i> , 2012
	<i>Ttll1</i>	15	KO	C57BL/6-Tyrc-Brd	Immotile cilia, sperm dysmotility, rhinitis, sinusitis	Not well characterised	Vogel <i>et al.</i> , 2010
	<i>Ulk4</i>	9	KO	C57BL/6-Tyrc-Brd	Shortened cilia, smaller size, hydrocephalus, rhinosinusitis, reduced viability	Not well characterised	Vogel <i>et al.</i> , 2012

Introduction

Lysosomal Disorders	<i>Gus</i>	5	Spontaneous transposition	C3H/HeOuJ	(MPS VII) Widespread lysosomal storage, dysostosis	Spontaneous chronic, lysosomal granules	Vogler <i>et al.</i> , 2001
	<i>Ids</i>	X	KO	C57BL/6.129	(MPS II) Widespread lysosomal storage.	Spontaneous chronic, lysosomal granules	Jung <i>et al.</i> , 2010; Hong <i>et al.</i> , 2012
	<i>Idua</i>	5	KO	C57BL/6	(MPS I) Widespread lysosomal storage, dysostosis	Spontaneous chronic, lysosomal granules	Schachern <i>et al.</i> , 2007
	<i>Naglu</i>	11	KO	C57BL/6	(MPS IIIB) Widespread lysosomal storage, neurological defects.	Spontaneous chronic, lysosomal granules	Heldermon <i>et al.</i> , 2007
Miscellaneous	<i>Chd7</i>	4	Spontaneous deletion	BALB/cByJ	Keratoconjunctivitis, circling behavior, growth retardation	Spontaneous chronic	Tian <i>et al.</i> , 2012
	<i>Dbh</i>	2	KO	C57BL/6; 129/SvCPJ	Altered metabolism, thermoregulation, motor function, learning	Spontaneous chronic	Maison <i>et al.</i> , 2010
	<i>E2f4</i>	10	KO	C57BL/6	Erythropoietic defects, chronic rhinitis	Spontaneous chronic	Humbert <i>et al.</i> , 2000
	<i>Fgfr1</i>	8	SNP	C3HeB/FeJ	Pinna and ossicle malformation, cochlear defects, shortened skull	Spontaneous chronic in heterozygote	Pau <i>et al.</i> , 2005; Calvert <i>et al.</i> , 2011
	<i>Isl1</i>	13	SNP	C3HeB/FeJ	Ossicular fusion	Spontaneous chronic in heterozygote	Hilton <i>et al.</i> , 2011
	<i>Lmna</i>	3	KO	C57BL/6J	Enlarged nuclear morphology of macrophages, hyperphosphatemia	Spontaneous chronic	Zhang <i>et al.</i> , 2012
	<i>McpH1</i>	8	KO	C57BL/6	Eye defects, erythrocyte defects, infertile	Spontaneous chronic	Chen <i>et al.</i> , 2013
	<i>Muc5b</i>	7	KO	C57BL/6	Impaired growth and survival, reduced mucociliary clearance, abnormal breathing, hypoxaemia	Not well characterised	Roy <i>et al.</i> , 2013
	<i>Nf2</i>	11	Conditional KO	FVB/N; C57BL/6	Schwannomas, osteosarcomas, other tumours, craniofacial defects	Spontaneous chronic	Giovannini <i>et al.</i> , 2000
	<i>Oxgr1</i>	14	KO	C57BL/6J-TyrC-Brd; 129 Sv/EvBrd	Not well characterised	Spontaneous chronic in homozygote	Kerschner <i>et al.</i> , 2013
	<i>p73</i>	4	KO	C57BL/6 or BALB/c	Lethal gastrointestinal and intracranial haemorrhage, neurological malformation	Spontaneous chronic	Yang <i>et al.</i> , 2000
	<i>Phex</i>	X	Spontaneous deletion	BALB/cAnBomUrd	Craniofacial, cochlear, and bone defects, endolymphatic hydrops, deformity of epithelial cilia	Spontaneous chronic	Megerian <i>et al.</i> , 2008; Han <i>et al.</i> , 2012
	<i>Plg</i>	17	KO	Mixed C57BL/6 & DBA/1J	Widespread fibrin deposition in organs	Spontaneous chronic, fibrin deposition	Eriksson <i>et al.</i> , 2006
	<i>Rpl38</i>	11	Spontaneous deletion	TsJ/LeJ.BlackSwiss	Shortened, kinked tail, reduced body weight, anaemia, skeletal anomalies	Spontaneous chronic in heterozygote, with crystal deposition	Noben-Trauth <i>et al.</i> , 2011
	<i>Sall4</i>	2	KO	C57BL/6J-TyrC-Brd; 129/SvEvBrd	Exencephaly, renal hypoplasia, anogenital defects	Not well characterised	Warren <i>et al.</i> , 2007
	<i>Sh3pxd2b</i>	11	Spontaneous deletion	B10.A-H2h4/ (4R)SgDvEg	Reduced growth, craniofacial and ocular defects, infertility	Spontaneous chronic	Mao <i>et al.</i> , 2009; Yang <i>et al.</i> , 2011
	<i>Tbx1</i>	16	Transgenic	C57BL/6.129	Cardiovascular defects, craniofacial and pharyngeal arch anomalies	Spontaneous chronic	Moraes <i>et al.</i> , 2005
	<i>Df1 (22q11DS)</i>	16	Transgenic	C57BL/6; 129S5 SvEvBrd	Cardiovascular defects, schizophrenia and pharyngeal arch anomalies	Spontaneous chronic in heterozygote	Fuchs <i>et al.</i> , 2013
	<i>MMU16 (Ts65Dn)</i>	16	Partial trisomy	C57BL/6EiC3Sn	Neurological deficits, craniofacial malformation	Spontaneous chronic	Han <i>et al.</i> , 2009

1.7.1 AOM models

AOM is usually a consequence of bacterial infection of the ME space. It is thought that ascension of commensal bacterial species normally resident in the NP occurs with an alteration in mucosal conditions. Certainly, an URTI precedes most AOM, and is thought to be the trigger for indeterminate mucosal changes that enable bacterial colonisation and proliferation within the ME. The relative contributions of the innate or adaptive immune systems to AOM susceptibility have not been ascertained. The innate immune system is easier to experimentally manipulate and to study, and so has been the focus for developing murine models of AOM.

1.7.1.1 Toll-like receptors

One of the most important families of the pathogen recognition receptors is the toll-like receptors (TLRs) (Akira and Takeda 2004). TLRs are present on the surface of cells prominent at mucosal surfaces and include epithelial, mast and dendritic cells. There are 10 different TLRs in humans and 12 in mice (Kawai and Akira 2010), with each TLR possessing distinct functional properties enabling the detection of a broad range of pathogens (Akira, Uematsu et al. 2006). Activation of TLRs results in the production of both pro- and anti-inflammatory cytokines including TNF α and IL-10 respectively. For most TLRs this happens via MyD88 activation of NF- κ B and/or MAP kinase dependent pro-inflammatory gene expression. However, TLR3 and TLR4 signal via a MyD88 independent pathway, acting via TRIF, leading to the production of type I IFNs. The activation of TLRs and their downstream signalling cascades results in the production of a broad range of cytokines and chemokines to induce the adaptive immune response in addition to innate inflammatory responses.

The deletion of various TLRs in mice, including TLR2 (Leichtle, Hernandez et al. 2009), TLR4 (Hirano, Kodama et al. 2007) and TLR9 (Leichtle, Hernandez et al. 2012), has provided important insights into the role these receptors play in mediating inflammation in response to *NTHi*. Compared to wild-type, *TLR2*^{-/-} and *TLR4*^{-/-} mice have a prolonged and persistent OM, characterised by reduced bacterial clearance, altered mucosal inflammation and leukocyte infiltration kinetics (Leichtle, Hernandez et al. 2009). *TLR9*^{-/-} mice also demonstrate a delay in inflammatory cell recruitment and bacterial clearance in the ME after *NTHi* challenge (Leichtle, Hernandez et al. 2012). Likewise, *MyD88*^{-/-} mice demonstrate prolonged ME mucosal thickening compared to wild-type mice after challenge with *NTHi*. They also demonstrate a delay in the recruitment of macrophages and neutrophils, with an attenuation of these cells' ability to internalise and phagocytose bacteria (Hernandez, Leichtle et al. 2008). While the absence of MyD88 does not result in a complete failure of *NTHi* eradication through potential MyD88-independent mechanisms, its presence is required to prevent AOM becoming persistent (Hernandez, Leichtle et al. 2008). The finding that *MyD88*^{-/-} mice have a more severe phenotype than TLR9, TLR2 and TLR4 knockouts alone has suggested functional redundancy between mammalian TLRs to compensate for their loss (Fritz and Girardin 2005).

1.7.1.2 Complement

Activation of complement in response to pathogens culminates in the cleavage of the central C3 protein. This produces the C3a and C5a components and formation of the membrane attack complex; all mediated via the classical, alternative and mannose binding lectin pathways. Analysis of paediatric ME with RAOM have demonstrated the presence of complement components from all three complement cascades (He, Scholes et al. 2013). Mice with knockout of complement factors C1qa, Factor B and Factor B/C2 demonstrate increased susceptibility to OM caused by *S. pneumoniae*

(Tong, Li et al. 2010). Defects in complement also prolong the time mice spend with OM (Bogaert, Thompson et al. 2010; Tong, Li et al. 2010).

1.7.2 COM models

Models of COM have been recovered from both gene and phenotype-driven mouse mutagenesis programs. Most of these models are syndromic and display a multitude of anatomical and functional deficits as well as ME inflammation, and mirror the phenotypes of some human syndromes. A smaller number of mouse models are non-syndromic, without the wide-ranging phenotypic effects of syndromic models. These non-syndromic models represent better models for non-syndromic human OM.

1.7.2.1 Syndromic

Children with certain congenital diseases have a higher prevalence of COM than the general population, with OM developing as one of a number of phenotypic abnormalities. The genetic polymorphisms that underlie such syndromes are becoming increasingly known.

In several syndromes, the susceptibility to COM is associated with craniofacial abnormalities, and this is reflected in mouse models of the same syndromes. For example, the *Eya1*^{+/-} mouse (Xu, Adams et al. 1999) is a model of Branchio-oto-renal Syndrome, the *Tbx1* mouse (Moraes, Nóvoa et al. 2005) is a model of 22q11.2 deletion syndrome, and the *Ts65Dn* mouse (Han, Yu et al. 2009) is a model of Down syndrome. Much of the existing literature suggests that the association of COM in craniofacial malformation is due to dysmorphism and consequent dysfunctional opening of the ET. However, whereas ET dysmorphology is probably important in aetiology, it may not be the only or main factor. Susceptibility to OM is more likely

complex, heterogeneous, and to some extent specific to each syndrome. A factor not often recognised is the co-existence of additional overt or subtle immunodeficiency in craniofacial malformation, which has for example been demonstrated in Chromosome 22q.11.2 deletion (Sullivan, Jawad et al. 1998; Aggarwal and Morrow 2008), Kabuki syndrome (Hoffman, Ciprero et al. 2005), CHARGE syndrome (Jyonouchi, McDonald-McGinn et al. 2009) and Down syndrome (Kusters, Verstegen et al. 2009; Ram and Chinen 2011). With an increased interest in using mouse models to unravel the pathobiology in Down syndrome, there is a growing resource of Down syndrome mouse models. A recent study by Bhutta *et al.*, screened the murine library of Down syndrome models to identify a critical region or set of loci underlying the COM seen in some models (Bhutta, Cheeseman et al. 2013). A two-locus model was proposed for the development of OM in Down syndrome mice, whereby loci from two chromosome 16 regions contribute to the onset of chronic ME inflammation.

Mucopolysaccharidoses (MPS) are a group of lysosomal storage diseases caused by the absence of or defects in 11 lysosomal enzymes required for the breakdown of glycosaminoglycans (GAGs). Accumulation of GAGs in the ME triggers chronic inflammation (Bhutta 2011). MPS I (Schachern, Cureoglu et al. 2007), MPS II (Jung, Park et al. 2010), MPS IIIB (Heldermon, Hennig et al. 2007) and MPS VII (Vogler, Levy et al. 2001) mouse models have been characterised, with similar pathologies as human disease. The MPS II mouse model is caused by knockout of iduronatesulfatase (IDS) with IDS-KO mice having increased hearing threshold with thickening of the tympanic membrane and ME exudates at 17 weeks compared to wild-type littermates (Hong, Chu et al. 2012). Enzyme replacement therapy with recombinant human IDS, resulted in prevention of the hearing deficits in the MPS II mouse model (Hong, Chu et al. 2012), highlighting the importance of OM mouse models when evaluating therapies.

The *Cby1* (Voronina, Takemaru et al. 2009), *Dnahc5* (Ibañez-Tallon, Gorokhova et al. 2002) and *Dnahc11* (Lucas, Adam et al. 2012) deficient mice are well characterised models of PCD that have ciliary defects and develop a spontaneous COM. In PCD there is a chronic ME effusion, but it is not clear if this is due to a simple failure of clearance of mucus or due to another mechanism. The evidence of disrupted ciliary function in non-syndromic COME is contradictory (Takeuchi, Saida et al. 1990; Wake and Smallman 1992; Agius, Wake et al. 1995; Ohashi, Nakai et al. 1995; Mason, Adam et al. 2002; Gurr, Stark et al. 2009), and where disruption has been observed, it is not clear whether this is cause or effect.

The relevance of some of these mouse models in understanding non-syndromic OM is debatable, as craniofacial syndromes, MPS, or PCD probably invoke mechanisms of disease that are unique to these syndromes. However, OM in humans can occur as part of a syndrome, so these models are relevant for elucidating the underlying molecular pathways and mechanisms of syndromic OM.

1.7.2.2 Non-syndromic

Mouse models of chronic spontaneous OM that do not have an association to a known human syndrome could be considered of greater importance in implicating potential roles for pathways involved in driving chronic inflammation.

1.7.2.2.1 EYA

In man, mutations in EYA are known to lead to middle or inner ear malformation or degeneration, but not inflammation (Wayne, Robertson et al. 2001; Pfister, Tóth et al. 2002; Hildebrand, Coman et al. 2007). However, a well-described non-syndromic mouse model of OM is the *Eya4*^{-/-} mutant, which develops spontaneous COM

(Depreux, Darrow et al. 2008). The *Eya4* gene encodes a transcriptional coactivator and expression of EYA4 has been localised to structures within both the inner ear and the middle ear during embryonic development, including the ET mucociliary epithelium (Borsani, DeGrandi et al. 1999; Depreux, Darrow et al. 2008). Analyses of craniofacial and anatomical structures of the *Eya4*^{-/-} mouse showed abnormal MEC and ET morphology, which may contribute to the development of OM leading to impaired clearance of ME fluid (Depreux, Darrow et al. 2008). However, murine EYA4 has been shown to play an important role in the innate immune response, which could provide an alternative explanation for the association of EYA4 deficiency with COM (Okabe, Sano et al. 2009).

1.7.2.2.2 NF-κB

The NF-κB family of transcription factors are involved in stress-induced, immune, and inflammatory responses (Baldwin 1996). The *Junbo* mutant (Parkinson, Hardisty-Hughes et al. 2006) and *IκBaΔN* knockin (Schmidt-Ullrich, Aebischer et al. 2001) mouse models suggest a possible role for NF-κB in COM. The *Junbo* mouse carries a mutation in *Evi1* and develops spontaneous COM usually by 54 days after birth (DAB). *EVI1* is a nuclear transcription factor that has interaction with several signalling pathways, such as TGF-β (Izutsu, Kurokawa et al. 2001; Alliston, Ko et al. 2005) and JNK (Kurokawa, Mitani et al. 2000). However, more recent work has suggested that EVI1 also acts as an inducible negative-feedback regulator of NF-κB by inhibiting its binding to DNA through inhibition of p65 acetylation (Xu, Woo et al. 2012). It is predicted that the mutation in *Junbo* results in over-expression of NF-κB, which most likely leads to the chronicity of inflammation in the ME.

Conversely, the *IκBaΔN* knockin mouse suppresses NF-κB and still spontaneously develops COM (Schmidt-Ullrich, Aebischer et al. 2001). The suppression of NF-κB in

these mice leads to diffuse embryonic disruption, with malformation of epithelial appendages and defective lymphatic development, including only rudimentary development of secondary lymphoid organs. It is likely that OM in this model is the regional manifestation of a more generalized defect in lymphocytes. Together, these models suggest that NF- κ B signalling cascades may play a pivotal role in the transition from acute to COM.

1.7.2.2.3 TGF- β

The *Jeff* mutant (Hardisty, Erven et al. 2003) and *Tgif1* knockout (Tateossian, Morse et al. 2013) mice provide strong evidence that TGF- β signalling also plays an important role in regulating chronic inflammation in the ME. TGF- β is a key regulator of inflammatory response in a number of organs (Li, Wan et al. 2006; Yoshimura, Wakabayashi et al. 2010). TGF- β acts through specific receptors activating multiple intracellular pathways, which in turn results in phosphorylation of receptor-regulated SMAD2/3 proteins that associate with SMAD4 (Heldin, Miyazono et al. 1997). These complexes translocate to the nucleus and bind DNA to regulate transcription.

COM spontaneously develops in the *Jeff* heterozygote mouse (Hardisty, Erven et al. 2003), which carries a mutation in *Fbxo11* (Hardisty-Hughes, Tateossian et al. 2006). FBXO11 is a member of the large F-box family of proteins, which are specificity factors for the SCF E3 ubiquitin ligases and are responsible for the targeted degradation of proteins (Hindley, McDowell et al. 2011). In *Jeff* homozygotes (*Fbxo11*^{Jf/Jf}), nuclear accumulation of phospho-SMAD2 (p-SMAD2) is observed and is significantly upregulated in epithelia. Even though mice heterozygous for *Jeff* and *Smad2* mutations recapitulate the phenotype of *Fbxo11*^{Jf/Jf} mice, a direct interaction between FBXO11 and SMAD2 was not observed. FBXO11 is also known to neddylate p53, and therefore inhibit its activity (Abida, Nikolaev et al.

2007). p53 is a transcriptional cofactor of SMAD2 (Cordenonsi, Dupont et al. 2003) and it is thought that the *Jeff* mutation impacts upon TGF- β signalling, possibly via p53, leading to its abundant activity. However, reduced rather than elevated levels of p53 are found in the *Jeff* mouse (Tateossian, Hardisty-Hughes et al. 2009), suggesting FBXO11 has a role in stabilising p53.

TGF- β promotes transcription of *Tgif1*, which in turn has been found to function through several paths as a negative regulator of TGF- β signalling. TGIF was initially identified as a SMAD2-binding protein and a co-repressor of TGF- β -induced transcription (Wotton, Lo et al. 1999), but it can also inhibit SMAD2 phosphorylation by an alternative mechanism due to its association with SMAD2 in partnership with c-Jun (Seo, Ferrand et al. 2006). Spontaneous COM is observed in the *Tgif1* knockout mouse, with onset at around 21 DAB (Tateossian, Morse et al. 2013). Both p-SMAD2 and p21 levels were lowered in the homozygous mutant, demonstrating a suppression of the TGF- β pathway.

Overall, the findings in the *Jeff* mutant and *Tgif1* knockout mouse models provide considerable support to the view that defects in the TGF- β /SMAD pathway impact on susceptibility to COM.

1.7.2.2.4 HIF-VEGF

Mouse models of OM have also been exploited to explore disease mechanisms and therapy. Within the chronically inflamed ME of the *Junbo* and *Jeff* mouse models, the mucosa and leukocytes have been shown to be hypoxic (Cheeseman, Tyrer et al. 2011). *Hif-1a* expression has been shown to be elevated in bulla fluid inflammatory cells in *Junbo* and *Jeff*, along with upregulation of its target genes including *Vegfa*. Targeting of HIF-VEGF pathways, with small molecule inhibitors of VEGFR signaling and destabilizing HIF by inhibiting its chaperone HSP90, can lead to resolution of

inflammation and improved hearing in *Junbo* mice (Cheeseman, Tyrer et al. 2011). The effectiveness of HIF-VEGF pathway inhibitors in suppressing OM in the *Junbo* model implicates HIF-mediated VEGF as playing a fundamental role in the pathogenesis of COM.

1.7.3 Mouse to man

A key advantage of studies using mouse models is that the findings can be used to examine the effects of these same genes in the human population. This is commonly achieved through candidate gene association studies. As mentioned earlier, genetic polymorphisms in the human population have been found for *IL10* (Alper, Winther et al. 2009; Sale, Chen et al. 2011), *TLR4* (Emonts, Veenhoven et al. 2007; Alpay, Etem et al. 2010; Sale, Chen et al. 2011; MacArthur, Wilmot et al. 2014), *TNFA* (Patel, Nair et al. 2006; Emonts, Veenhoven et al. 2007), *FBXO11* (Segade, Daly et al. 2006; Rye, Wiertsema et al. 2011), *SMAD2* and *SMAD4* (Rye, Wiertsema et al. 2011; MacArthur, Wilmot et al. 2014). Thus, findings from mouse models for a number of innate immune system and TGF- β pathway components have led to identification of their importance in human OM. However, results obtained using this approach requires caution in human studies due to the frequent non-replication of findings from genetic association studies (Tabor, Risch et al. 2002).

1.7.4 Advancements in OM mouse models

At present, the use of mouse models is the most promising method to identify candidate loci underlying susceptibility to OM. Mouse models have highlighted the role of TLRs in AOM, in particular the protection against commensal and pathogenic bacteria, and that persistent NF- κ B or TGF- β signalling could be two mechanisms leading to the overactive pro-inflammatory response seen in COM (Figure 1.3). On-

going mutagenesis screens will no doubt highlight new models and new pathways, and also elaborate further the pathways already implicated in the genetic susceptibility of OM. Such knowledge may in the future allow targeted or individualised molecular therapy for these diseases.

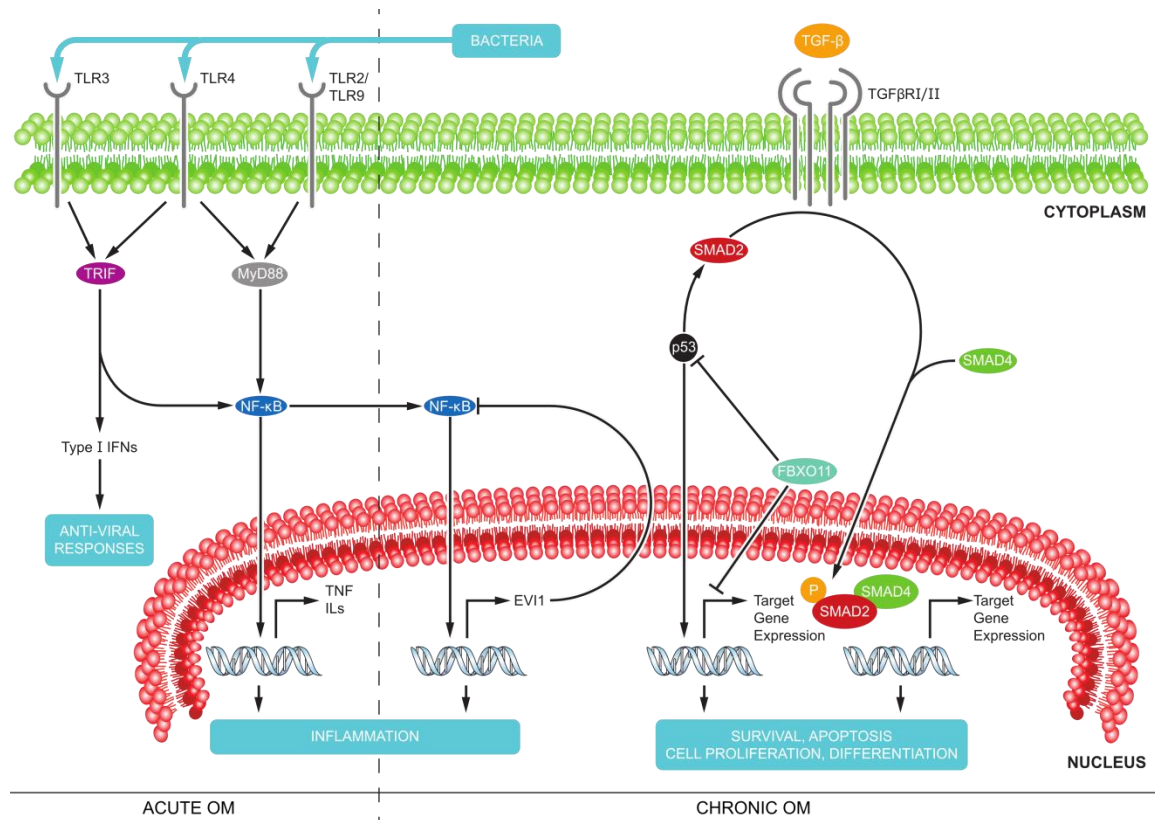


Figure 1.4| Pathways linked to OM proneness identified through mouse models.

Schematic indicating the potential roles of interacting pathways related to OM susceptibility identified through mouse models of acute and chronic OM. Bacteria such as *NTHi* can induce a signalling cascade through a number of TLRs, via TRIF and MyD88 to NF-κB. This results in downstream induction of TNF and ILs. Furthermore, TRIF mediated signalling leads to production of type I IFNs. NF-κB can be regulated by EVI1, which can act as an inducible negative-feedback regulator. OM susceptibility can also occur via changes in TGF-β signalling pathways. TGF-β induces SMAD2 phosphorylation, which complexes with SMAD4 and accumulates in the nucleus, where they regulate the expression of target genes. SMAD2 has a transcriptional cofactor, p53. FBXO11 can suppress p53 function and inhibits its transcriptional activity. See the text for a more detailed description of pathways. Image taken from Tyrer, Crompton and Bhutta, 2013.

1.8 *edison*: a novel model of OM

A new genetic mouse model of OM, *edison*, has recently been discovered by the mutagenesis program at MRC Harwell. Preliminary characterisation shows that *edison* develops a highly penetrant COM in the absence of any other significant pathology. Thus the *edison* mutant represents a powerful model for studying the genetic and pathophysiological bases of COM. The discovery of the gene underlying the *edison* mutant will highlight a new candidate gene for OM susceptibility in the human population and will provide more evidence towards understanding the aetiology of this complex childhood disease.

1.9 Project aims and objectives

This thesis aims to characterise the *edison* mouse mutant further and establish the role that it plays in COM. To achieve this, the following approaches have been taken:

- Identify the gene underlying the *edison* mutation and validate it as the causative gene.
- Determine the onset and progression of OM in *edison* mice by undertaking comprehensive phenotypic characterisation.
- Investigate the genetic and molecular roles of the causative gene to understand the development of the COM seen in *edison*.
- Explore the potential use of the *edison* mouse as a model for bacterial challenge with human otopathogens.

CHAPTER 2: Materials and methods

2.1 Mouse Lines

2.1.1 Animal care

Mice were housed in specific pathogen free (SPF) conditions in individually ventilated racks (Techniplast UK Ltd) containing grade 6 sawdust bedding (Datesand Ltd, UK), under a controlled 12 hour light/dark cycle at 21°C (± 2) and 55% (± 10) relative humidity. Mice were supplied with irradiated mouse diet (Special Diets Services, UK) and water (25 μ L/L of chlorine) *ad libitum*. All animal procedures were conducted in accordance with the appropriate UK Home Office Project and Personal licences; and under the guidelines issued by the Medical Research Council in "Responsibility in the use of animals in bioscience research: Expectations of the major research council and charitable funding bodies" (updated April 2013).

2.1.2 MRC Harwell ENU mutagenesis screen

The *edison* mouse was identified in a ENU mutagenesis vision screen at MRC Harwell (Thaung, West et al. 2002). Male C57BL/6J mice were mutagenized with ENU and mated with C3H.*Pde6b*⁺ mice (a C3H stock that does not carry the retinal degeneration allele *Pde6b*rd). G₃ offspring were screened for abnormalities using the modified-SHIRPA protocol.

2.1.3 Stock maintenance

Once identified, *edison* mice (*Nisch*^{*edsn*}) were outcrossed to C3H/HeH and were backcrossed for 10 generations and maintained as a congenic line on a C3H/HeH background. Mouse line B6.129S-*Itga5*^{*tm1Hyn*}/J (Yang, Rayburn et al. 1993) was sourced from the Jackson Laboratory (Stock No. 002274; Bar Harbour, Maine, USA). A description of how this construct was generated is outlined in Figure 2.1. This line

was re-derived and maintained on a C57BL/6J background through inter-crossing. It was additionally backcrossed to C3H/HeH, but due to time constraints was only backcrossed 5 generations to an incipient congenic. Mouse lines *Nisch*^{V33A}, *Nisch*^{L142Q} and *Nisch*^{T445I} were all re-derived from the ENU sperm archive at MRC Harwell and backcrossed to C3H/HeH.

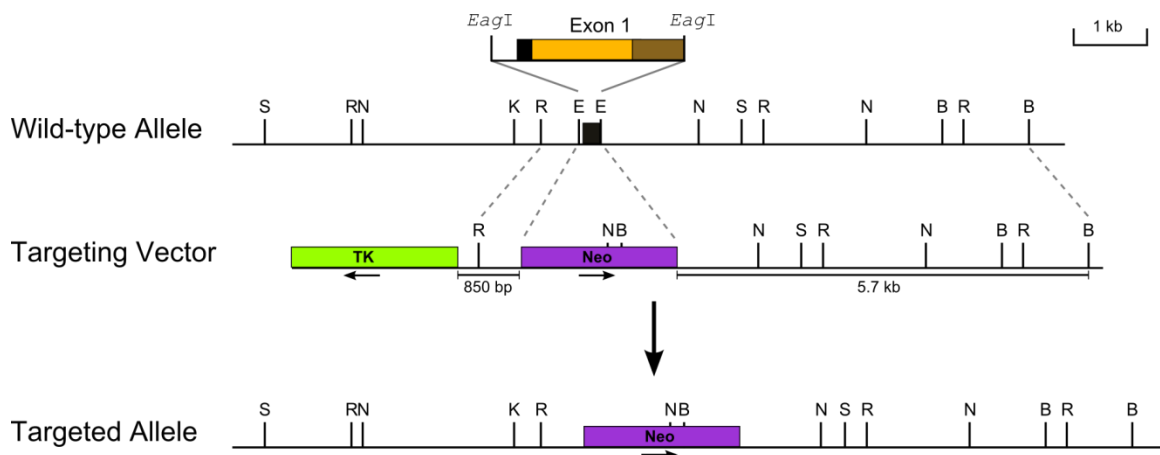


Figure 2.1 | Generation of the *Itga5*^{tm1Hyn} mouse line.

This diagram illustrates the published strategy to create a knockout allele of the *Itga5* locus. Disruption of the first coding exon of the *Itga5* gene, in ES cells, was caused by recombination at the *Itga5* locus with a targeting vector. Restriction maps of the wild-type allele, the targeting vector and the targeted allele of *Itga5* are shown. An *EagI* fragment containing most of the first coding exon is enlarged to illustrate the structure of the exon: 5' untranslated sequence (black); signal sequence (orange); sequence encoding the mature protein (brown). The PGK-neomycin resistance cassette and PGK-thymidine kinase cassette are designated as Neo (purple) and TK (green), respectively; the orientations of the genes are shown by arrows. The bars under the restriction map of the targeting vector indicate the sizes of the flanking sequences homologous to the wild-type *Itga5* locus. Restriction sites: B, *BamHI*; E, *EagI*; K, *KpnI*; N, *NcoI*; R, *EcoRI*; S, *SacI*. ES cells were injected into C57BL/6J blastocysts and chimeric males were bred to C57BL/6J females. Modified from Yang *et al.*, 1993.

2.1.4 *In vitro* fertilisation with cryopreserved spermatozoa

Re-derivation of mouse lines was undertaken by the Frozen Embryo and Sperm Archive (FESA) group at MRC Harwell. *In vitro* fertilisation was utilised to re-derive

selected mouse lines from cryopreserved spermatozoa, as described by Thornton *et al.* (Thornton, Brown *et al.* 1999).

2.1.5 Generation of compound mutant mice

The three *Nisch* mouse lines re-derived from the MRC Harwell ENU-DNA sperm archive (*Nisch*^{V33A}, *Nisch*^{L142Q} and *Nisch*^{T445I}) were crossed with *Nisch*^{edsn} mice, through crossing heterozygous mice. The following compound heterozygote mice were generated: *Nisch*^{V33A/edsn}, *Nisch*^{L142Q/edsn} and *Nisch*^{T445I/edsn}.

Through a series of crosses, as shown in Figure 2.2, *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice were generated. Double heterozygotes were generated by crossing *Itga5*^{tm1Hyn/+} mice with *Nisch*^{edsn/+} mice. *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} double heterozygote mice were then crossed again with *Nisch*^{edsn/+} mice. A cross between these two mouse lines resulted in a range of progeny, including *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice.

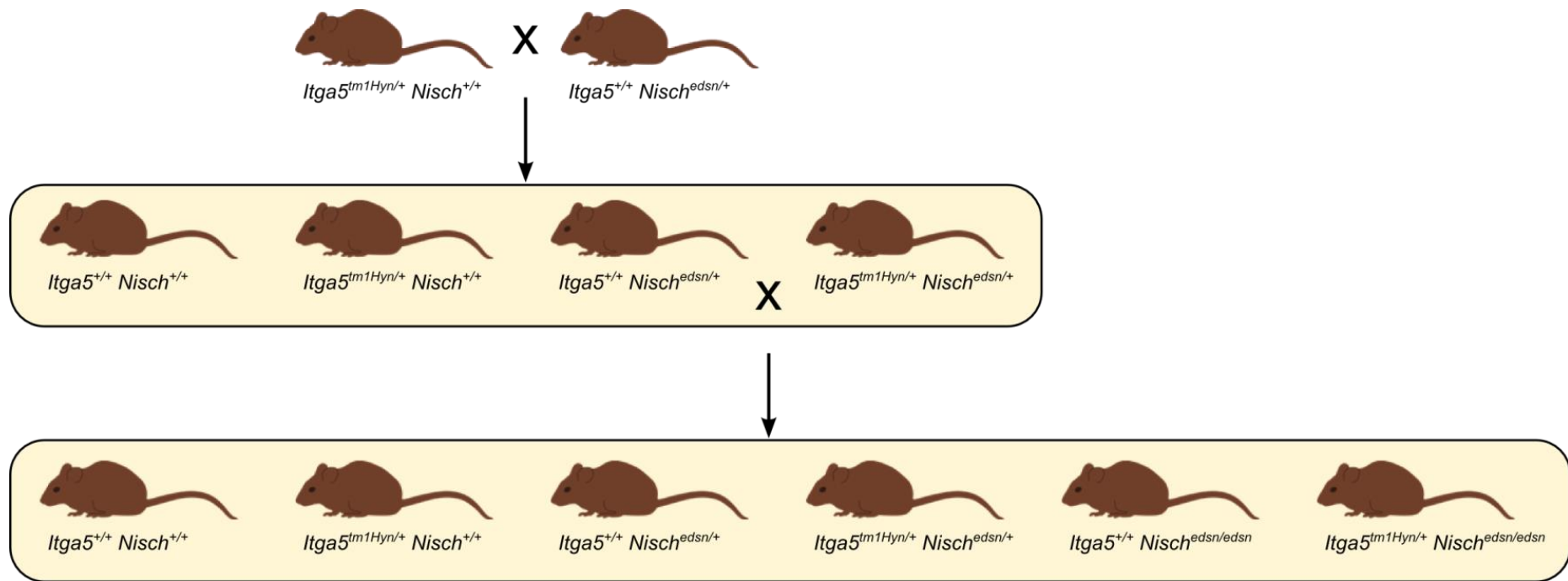


Figure 2.2| Generation of *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice.

Itga5^{tm1Hyn/+} Nisch^{+/+} mice (male or female) were mated with *Itga5^{+/+} Nisch^{edsn/+}* mice (male or female) to produce *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* progeny. *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice were further crossed to *Itga5^{+/+} Nisch^{edsn/+}* animals to produce mice of six different genotypes, including *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice.

2.2 Identification of *edison* causative mutation

2.2.1 DNA extraction for pyrosequencing using Viagen lysis solution

DNA, for whole genome scanning and further mapping, was extracted from ear clips or tails following a modified protocol outlined by Bioquote Ltd (York, UK). Individual ear clips or tails were incubated at 55°C overnight with 175 µL DirectPCR (mouse tail) lysis reagent (Viagen Biotech) and 5 µL Proteinase K solution (Qiagen). Samples were then incubated at 85°C for 45 minutes in a water bath and centrifuged briefly to pellet any hair. Crude lysates were stored short term at 4°C for up to a week after extraction and long term storage required freezing at -20°C. The crude lysates were used directly in PCR assays.

2.2.2 Whole genome scan and mapping

Genome-wide low-resolution mapping was performed by the Genotyping and Mutation detection Screens (GEMS) group at MRC Harwell, using DNA samples from mice that were identified as probable carriers of the *edison* mutation on the basis of their size and hearing phenotype. Genomic DNA samples were screened for a custom set of 63 SNP markers, spaced equidistantly across the genome, using the Pyrosequencing method. Samples were either homozygous C3H; heterozygous C3H-C57BL/6J; or homozygous C57BL/6J for each SNP.

For fine genetic mapping, further outcrosses of *edison* mice with C3H/HeH produced recombinant mice to help narrow the critical region. Pyrosequencing was again implemented using additional sets of SNP markers, evenly spaced across the critical chromosomal region identified.

2.2.3 PCR for whole genome scan and mapping

A variety of primers and cycling conditions were used to amplify genomic DNA (see Table 2.1 and 2.2). Primers for the whole genome scan and further mapping were designed by the GEMS core using Pyrosequencing Assay Design software (Biotage). Three primers were designed for each assay; consisting of a biotinylated PCR primer, a sequencing primer and an opposite PCR primer.

PCR amplification was carried out in 96 well Abgene PCR plates (ThermoScientific). Each well contained 10 µl of Qiagen *Taq* PCR master mix, 8.2 µl ddH₂O, 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM) and 1 µl DNA, with a final volume of 20 µl. Plates were sealed using peelable heat sealing foil sheets (ThermoScientific) and centrifuged briefly. DNA was then amplified using MJ Research PTC-225 thermocyclers. Full PCR cycling conditions for all assays can be found in Table 2.2.

Table 2.1| Primer sequences for SNP markers used for fine mapping of Chromosome 14.

All primers were sourced from Eurofins MWG Operon. Primer modifications: [Btn], Biotinylated.

Location (Mb)	RefSNP cluster ID	Primer type	Primer sequences (5' – 3')
11.02	rs31328715	Forward	[Btn]TGTTCTTTTGTCCCTACAG
		Reverse	AACTGGGCACTATTCTAGGC
16.48	rs3672910	Forward	TAATTTTAGGCTGTGTACCTCATC
		Reverse	[Btn]CCAAAGTAGAAATCTCTCTGTTGA
21.98	rs4230209	Forward	TCTGTAGCGGCTTTGTTTTTATTT
		Reverse	[Btn]CGGACCCGTGGTTCAAAAAGT
25.41	rs30822109	Forward	[Btn]GGATGAAAAAGGCAGAGACT
		Reverse	CCCTGTCTAGGTCACAGTAGC
26.37	rs38078209	Forward	TACGGACAACCTGAATCCAT
		Reverse	[Btn]CCTGGTGTGGTTGATATCTG
27.40	rs30778552	Forward	[Btn]ACAATCCTGTGGGCTCTT
		Reverse	AGTCTGCTACCCACACATACC
30.94	rs31536769	Forward	CAGCAGGGATTTCAGTTA
		Reverse	[Btn]CGTGAAGGTTTGGCTGTA
36.53	rs46823676	Forward	CTCTGGCACTCGTGTTAAAA
		Reverse	[Btn]CTCTGAGCTTTTGAATGAGTTT
47.07	rs30244168	Forward	CAGGATCTATCTCCATGACTCA
		Reverse	[Btn]CTGGTTTTGGCGTTACAC

Table 2.2| PCR cycling conditions for SNP markers used for fine mapping of Chromosome 14.

Cycling conditions for SNP markers located at (A) 16.48, 26.37 and 30.94 Mb; (B) 36.53 Mb; (C) 11.02, 21.98, 25.41 and 47.07 Mb; and (D) 27.40 Mb.

(A)			(B)		
Step	Temperature	Time	Step	Temperature	Time
1.	95 °C	5 min	1.	95 °C	5 min
2.	95 °C	15 sec	2.	95 °C	15 sec
3.	54 °C	30 sec	3.	56 °C	30 sec
4.	72 °C	15 sec	4.	72 °C	15 sec
5.	Go to step 2, 44 times		5.	Go to step 2, 44 times	
6.	72 °C	5 min	6.	72 °C	5 min
7.	15 °C	∞	7.	15 °C	∞

(C)			(D)		
Step	Temperature	Time	Step	Temperature	Time
1.	95 °C	5 min	1.	95 °C	5 min
2.	95 °C	15 sec	2.	95 °C	30 sec
3.	60 °C	30 sec	3.	62 °C	30 sec
4.	72 °C	15 sec	4.	72 °C	40 sec
5.	Go to step 2, 44 times		5.	Go to step 2, 44 times	
6.	72 °C	5 min	6.	72 °C	5 min
7.	15 °C	∞	7.	15 °C	∞

2.2.4 Pyrosequencing

Pyrosequencing is a sequence-based detection technology that enables rapid and accurate quantification of sequence variation by using “sequencing by synthesis”. The amplified DNA strand which is biotinylated serves as the Pyrosequencing template. After denaturation, the biotinylated single-stranded PCR amplicon is isolated and allowed to hybridize with a sequencing primer specific to each assay (see Table 2.3). The hybridized primer and single-stranded biotinylated template are incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase; as well as the substrates adenosine 5' phosphosulfate and luciferin; and deoxynucleotide triphosphates (dNTPs). Addition of dNTPs is performed sequentially. DNA polymerase catalyzes addition of the dNTP to the sequencing primer, if it is complementary to the base in the template strand. Each incorporation event is accompanied by the luciferase-mediated production of light detected by the pyrosequencer. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace generated.

Table 2.3| Sequencing primers used for analysing SNP markers by pyrosequencing.

All primers were sourced from Eurofins MWG Operon.

SNP location (Mb)	Sequencing primers (5' – 3')	C3H/HeH	C57BL/6J
11.02	AGGAACAAGCAGAACAG	T	A
16.48	GACATTGCCCCAGTC	T	A
21.98	ATTTATACTCTACAAGGGAT	A	G
25.41	GGTTCTGTTGGACTCCT	A	T
26.37	AAAGCCATCTCCTTGTA	A	C
27.40	CTTCACAGGGATGGG	G	A
30.94	TCAGCACAAGTCCCA	T	C
36.53	GGGACCAATCCAATGT	G	A
47.07	AGACAGGAACCACTACAA	C	A

PCR plates for analysis were spun at $1700 \times g$ for 3 minutes and 70 μ l of binding buffer master mix was added per sample; containing 38 μ l of PyroMark Binding Buffer, 30 μ l of ddH₂O and 2 μ l of streptavidin sepharose beads. Plates were covered with adhesive PCR film (ThermoScientific), and placed on a shaker for 5 minutes. The sequencing master mix was prepared, containing 11.5 μ l of PyroMark Annealing Buffer and 0.5 μ l of sequencing primer (Table 2.3) per sample, and 12 μ l was pipetted into the corresponding wells on PyroMark Q96 reaction plates. The PyroMark Q96 *vacuum* workstation was set up with troughs of 70% ethanol, PyroMark Denaturing Solution and PyroMark Wash Buffer. The Vacuum Prep Tool (VPT) was turned on and lowered into the plates, taken from the shaker, to pick up the Sepharose beads. The VPT was moved to the trough with 70% ethanol and the beads were washed for 5 seconds. The VPT was then moved to the denaturing solution trough for 5 seconds, followed by the trough containing the wash buffer for 5 seconds. The vacuum was shut off and the VPT was lowered and gently shaken into the PyroMark Q96 reaction plates to release the beads. The plates were covered with adhesive PCR film and heated in an MJ Research thermocycler at 80°C for 2 minutes. The PSQ 96MA cassette was filled with PyroMark Q96 reagents (Enzyme mix, Substrate mix and individual dNTPs). The PyroMark Q96 reaction plates were

placed into the pyrosequencing machine in the correct orientation and the pyrosequencing reactions started. The pyrosequencing results were analysed using the PyroMark Q96 ID system (Figure 2.3).

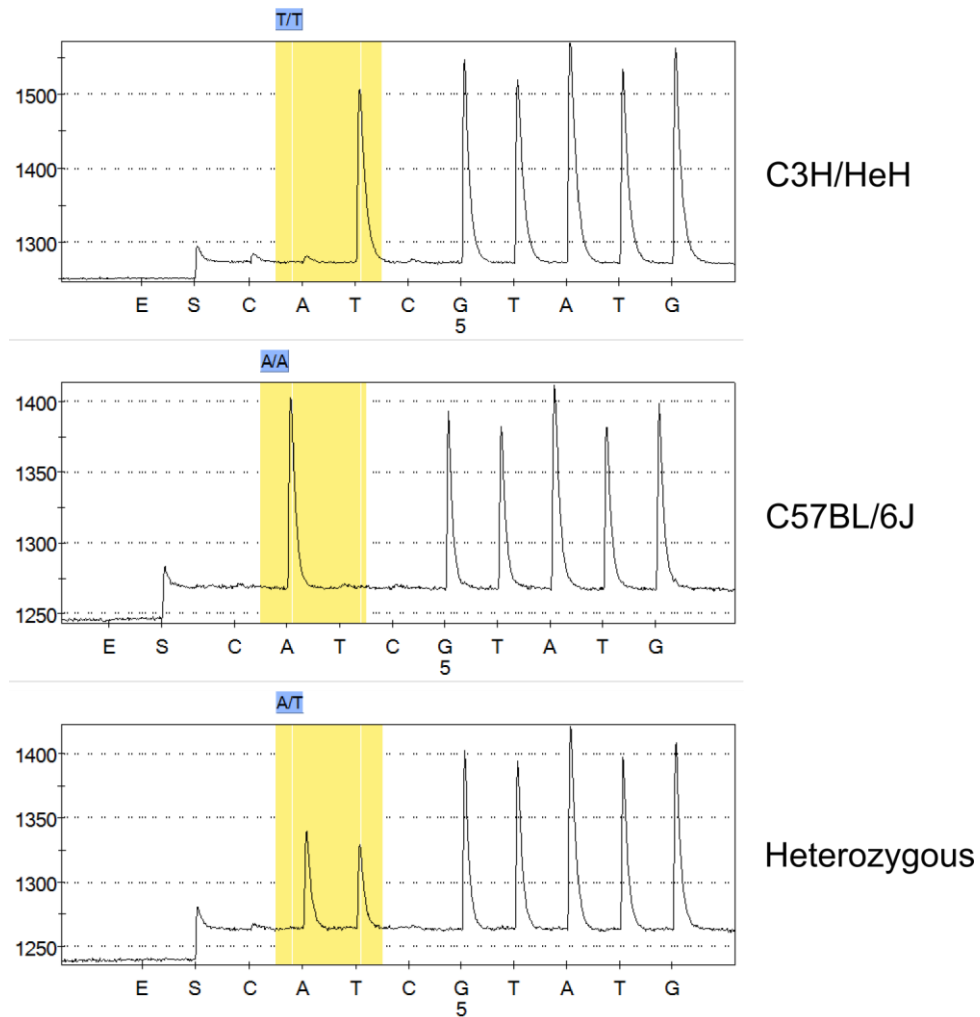


Figure 2.3| Example pyrogram output for pyrosequencing analysis of the SNP marker at 11.02 Mb on Chromosome 14.

2.2.5 Next-generation sequencing

Next-generation sequencing (NGS) was utilised to sequence the DNA from two *G₁₂ edison* mice homozygous C57BL/6J in the critical region. NGS was completed by the

High-Throughput Genomics group at the Wellcome Trust Centre for Human Genetics, using the Illumina Genome Analyser IIX platform. Identification, analysis and dissemination of sequence variant information identified through NGS were achieved with the tools provided by the MRC Harwell Biocomputing custom sequence analysis pipeline. The pipeline is based on existing packages (Bowtie, Samtools, CASAVA, nFold3) and custom developed components. The pipeline was used to align reads to the C3H/HeH reference sequence, automatically identify unique variants, populate a custom sequence database and identify low and high confidence single nucleotide variants (SNVs).

2.2.6 Sequencing to confirm putative mutations

To get sufficient PCR product for sequencing, three 20 µl PCR reactions were performed for each primer pair. To make sure that all three reactions worked with the same efficiency, a 5 µl aliquot was removed and run on 1% agarose gel. Three reactions were then combined and used for sequencing by Source BioScience.

Source BioScience carried out PCR purification, followed by high throughput Sanger sequencing using an Applied Biosystems 3730 DNA Analyser. PCR fragments and sequencing primers were sent by courier and sequences returned by e-mail. The result was analysed using NCBI blast and sequence alignment tools.

2.2.7 Sequence alignment and structural prediction

Nischarin genomic, transcript and peptide sequences were exported from Ensembl (<http://www.ensembl.org/>). Sequence alignment of Nischarin peptide sequences was performed using ClustalW2 (EMBL-EBI clustal). Pecan multiple alignment analysis (Paten, Herrero et al. 2008; Paten, Herrero et al. 2009) was used to observe the

evolutionary conservation among 19 amniota vertebrates. Prediction of putative protein domains and their boundaries for Nischarin were performed using the DomPred Protein Domain Prediction Server (<http://bioinf.cs.ucl.ac.uk/dompred/>) (Marsden, McGuffin et al. 2002; Bryson, Cozzetto et al. 2007).

2.2.8 Protein function prediction

SIFT (Sorting Intolerant From Tolerant), SNAP (Screening for Non-Acceptable Polymorphisms), and PROVEAN (Protein Variation Effect Analyzer) were utilised to predict whether an amino acid substitution had an impact on the biological function of a protein [SIFT: (Ng and Henikoff 2001; Ng and Henikoff 2002; Ng and Henikoff 2003; Ng and Henikoff 2006). SNAP: (Bromberg and Rost 2007). PROVEAN: (Choi, Sims et al. 2012)]. SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through PSI-BLAST. SIFT analysis scores variants with a score above 0.05 as "tolerated", and those below 0.05 "not tolerated". SNAP is a neural-network based method that uses *in silico* derived protein information in order to make predictions regarding functionality of mutated proteins, and results are marked as either "neutral" or "non-neutral" with no score provided. The PROVEAN algorithm provides a generalised approach to predict the functional effects of protein sequence variations by using the delta alignment score approach. PROVEAN variants with a score equal to or below -2.5 are considered "deleterious," and variants with a score above -2.5 are considered "neutral."

2.3 ENU-DNA sperm archive screen for additional *Nischarin* alleles

The MRC Harwell ENU-DNA sperm archive (Quwailid, Hugill et al. 2004) (http://www.har.mrc.ac.uk/services/dna_archive/) was screened for additional *Nischarin* alleles using a LightScanner system, which uses high resolution melting analysis for mutation scanning and genotyping (method described further in section 2.4.5). The ENU-DNA archive was made up of 10,464 samples when I carried out the screen. To accelerate mutation screening, four sets of samples were pooled together and stored in 96-well plates for high throughput screening. ENU archive DNA was amplified by PCR using Hotshot MasterMix and LCGreen (a fluorescent dye that binds to double stranded DNA). Amplified samples were then run on the LightScanner to assess melt curves. Wild-type DNA strands will anneal perfectly (homoduplex) and will emit the most fluorescence. DNA strands containing any SNVs will have a heterozygous mismatch between DNA strands (heteroduplex) leading to less fluorescence. Any samples showing a possible mutation from the pooled DNA plates were selected and repeated. If the interesting result was replicated, then individual DNA was screened to locate which DNA sample from the pools contained the possible mutation. Screened individual DNA samples which showed a positive result were sent for Sanger sequencing (see section 2.2.6), to identify the mutation causing the change in the LightScanner melt curve. Prediction software was utilised to assess whether the SNVs discovered would have an impact on the biological function of NISCH (as mentioned in section 2.2.8).

Nischarin is made up of 21 exons and I designed 28 primer sets, using LightScanner Primer Design software, to span the coding region of each exon (Table 2.4). A variety of cycling conditions were used to amplify genomic DNA from ten of these primer sets (Table 2.5 and 2.6).

Table 2.4| Primer sequences for ENU-DNA archive screening assays.

All primers were sourced from Eurofins MWG Operon.

Assay	Primer type	Primer sequences (5' – 3')
<i>Nisch</i> exon 1	Forward	GCCACTGGTTGTGCTGTCAATTA
	Reverse	GTCGAGAACTCCGGGTTCC
<i>Nisch</i> exon 2	Forward	CCTTGCAGCTATTGTGAGTT
	Reverse	CCCACCCCTTGAGATACAG
<i>Nisch</i> exon 3	Forward	GCTTCTTGGTCTGTGTTTGAT
	Reverse	TGGTGGCTGTGGGTTTCATA
<i>Nisch</i> exon 4	Forward	GATGGACAGGTAGGTGTCTAAC
	Reverse	CTGCTACCTACTAGATTAGGCAA
<i>Nisch</i> exon 5	Forward	CTGTACAACAGGCTGGGAC
	Reverse	GAACTAGAGCCAAGTGAGTGAC
<i>Nisch</i> exon 6	Forward	GTGCAGATGGGGAAGGTAT
	Reverse	ACACAGACATTAGGTGCAAG
<i>Nisch</i> exon 7	Forward	TGAGGCTTCAAGATGCCATTTTC
	Reverse	TCACTCACAAAGGCTGTGC
<i>Nisch</i> exon 8	Forward	ATGTTCCACTCAGTGTGCGC
	Reverse	TAACAGGCAATCCTAGGGTCT
<i>Nisch</i> exon 9	Forward	GCCAGCCTCCTTGATTAATGATA
	Reverse	CACCTAGAGAGCAAGTCCT
<i>Nisch</i> exon 10	Forward	TGACCTCCCTGGAGGACTAA
	Reverse	AAGGTCTAGCAACCCTAGAT
<i>Nisch</i> exon 11	Forward	GTCTGTTGCTGAGGTGTTAG
	Reverse	TTTCCTTGCCAGTCTCTCT
<i>Nisch</i> exon 12	Forward	CTCCTGCCTTCTCCATACTGTAA
	Reverse	TCCCATGGCTCAGTTTCCC
<i>Nisch</i> exon 13	Forward	GTCTGTTCTTCCCGCTCTTT
	Reverse	GAGCTGAGCAACTCCACTG
<i>Nisch</i> exon 14	Forward	TTCTTGAACAGCTGCTCTCC
	Reverse	TCAGACAGCAGCAGCATGA
<i>Nisch</i> exon 15	Forward	CCAGTGAGTGCTGAGGAAC
	Reverse	CTACACTGTCTGCTCGGTC
<i>Nisch</i> exon 16 amplicon 1	Forward	GGATAGAATGTCCCTGTGTCTCTGA
	Reverse	TCCCATTTCAAAGTAGCGGT
<i>Nisch</i> exon 16 amplicon 2	Forward	AGGAAGAGGAGGATGTTGC
	Reverse	AAGAGCGTGTTCTCACTGT
<i>Nisch</i> exon 16 amplicon 3	Forward	TGTGTCTGGTGCTCAAGGT
	Reverse	GGTTGAAGTCGGCCTTGAT
<i>Nisch</i> exon 16 amplicon 4	Forward	CTCACATCCCAGCATCTCA
	Reverse	GACCATACCTAAGCCAACC

<i>Nisch</i> exon 17 amplicon 1	Forward	TACAGGTGTCCACCACACT
	Reverse	GGAGCCTCTGCTGAAGTTAG
<i>Nisch</i> exon 17 amplicon 2	Forward	GTACAGGCCCCAGGAGAGGA
	Reverse	TTAGGCGCTCACTTGGGTACT
<i>Nisch</i> exon 17 amplicon 3	Forward	CTGGCACAAGCAGAAGTCC
	Reverse	CCAACACAAACTATACCCACCC
<i>Nisch</i> exon 18	Forward	AAGTGTGGTCCAGGAGGAA
	Reverse	CAAGTCCACAGTTGGCTCTTA
<i>Nisch</i> exon 19	Forward	CTGCCGGACATGTTTAACC
	Reverse	GCCTTCACCCAAACTGGGA
<i>Nisch</i> exon 20	Forward	GGTCCTGGTTTTCTGTGGG
	Reverse	GTTAGGCAGAGACAAGGGAAAG
<i>Nisch</i> exon 21 amplicon 1	Forward	GGGCTGCTGTTTCTTTTGA
	Reverse	TCGTGACCCTGTGTGTCAT
<i>Nisch</i> exon 21 amplicon 2	Forward	TACCCACAGGCCCTCACTC
	Reverse	CGCTAACAGCACAAGGACG
<i>Nisch</i> exon 21 amplicon 3	Forward	CCTAACGCTGGGAAGACAT
	Reverse	CCTTTTGGGACCCCAGTTC

Table 2.5| PCR reaction mix details for ENU-DNA archive assays.

PCR mix details for (A) *Nisch* exon 2, 4, 5, 8, 12, 17 (amplicon 3) and 19; (B) exon 15; and (C) exon 7 and 9.

	Reagent	Volume (μl)	Total volume (μl)
(A)	HotShot MasterMix	5	
	LCGreen dye	1	
	ddH ₂ O	1.8	
	Forward primer (20 μM)	0.1	
	Reverse primer (20 μM)	0.1	
	DNA	2	10
(B)	HotShot MasterMix	5	
	LCGreen dye	1	
	ddH ₂ O	1.6	
	DMSO	0.2	
	Forward primer (20 μM)	0.1	
	Reverse primer (20 μM)	0.1	
	DNA	2	10
(C)	HotShot MasterMix	5	
	LCGreen dye	1	
	ddH ₂ O	1.3	
	DMSO	0.5	
	Forward primer (20 μM)	0.1	
	Reverse primer (20 μM)	0.1	
	DNA	2	10

Table 2.6| PCR cycling conditions for ENU-DNA archive assays.

PCR cycling conditions for amplification of (A) *Nisch* exon 2; (B) exon 4, 5, 7, 9, 15 and 19; (C) exon 8 and 17 (amplicon 3); and (D) exon 12.

(A)			(B)		
Step	Temperature	Time	Step	Temperature	Time
1.	95 °C	2 min	1.	95 °C	2 min
2.	95 °C	30 sec	2.	95 °C	30 sec
3.	57 °C	30 sec	3.	58 °C	30 sec
4.	72 °C	30 sec	4.	72 °C	30 sec
5.	Go to step 2, 44 times		5.	Go to step 2, 44 times	
6.	72 °C	4 min	6.	72 °C	4 min
7.	95 °C	30 sec	7.	95 °C	30 sec
8.	25 °C	30 sec	8.	25 °C	30 sec
9.	10 °C	∞	9.	10 °C	∞

(C)			(D)		
Step	Temperature	Time	Step	Temperature	Time
1.	95 °C	2 min	1.	95 °C	2 min
2.	95 °C	30 sec	2.	95 °C	30 sec
3.	60 °C	30 sec	3.	62 °C	30 sec
4.	72 °C	30 sec	4.	72 °C	30 sec
5.	Go to step 2, 44 times		5.	Go to step 2, 44 times	
6.	72 °C	4 min	6.	72 °C	4 min
7.	95 °C	30 sec	7.	95 °C	30 sec
8.	25 °C	30 sec	8.	25 °C	30 sec
9.	10 °C	∞	9.	10 °C	∞

2.4 Genotyping

2.4.1 DNA extraction for genotyping using Viagen lysis solution

For routine genotyping of *edison* mice, DNA was extracted from ear clips or tails using Viagen lysis solution (Viagen Biotech) as described in section 2.2.1.

2.4.2 DNA extraction using Qiagen DNeasy blood and tissue kit

DNA extractions from ear clips of mouse lines re-derived by IVF from the MRC Harwell ENU-DNA sperm archive were achieved by using the DNeasy Blood and Tissue kit (Qiagen). Ear clips were incubated overnight with 180 μ L Buffer ATL and 20 μ L Proteinase K (Sigma) at 56°C. Samples were then vortexed and 200 μ L Buffer AL and 200 μ L (96-100%) ethanol added per sample. Samples were vortexed and passed through a DNeasy mini spin column in a 2 mL collection tube at 6,000 $\times g$ for 1 minute to bind DNA to the column. The DNeasy spin column was placed in a new 2 mL collection tube and 500 μ L Buffer AW1 added to the column. The column was spun for 1 minute at 6,000 $\times g$ and the column placed in a new 2 mL collection tube. 500 μ L Buffer AW2 was added to the column and the column spun for 3 minutes at 20,000 $\times g$. The column was placed in a fresh 1.5 mL microcentrifuge tube and DNA was eluted by adding 100 μ L Buffer AE, with incubation for 1 minute at room temperature, and centrifugation for 1 min at 6,000 $\times g$. DNA concentrations were determined using a Nanodrop 8000 and diluted to 5 ng/ μ L with nuclease free water where required and stored at 4°C.

2.4.3 DNA extraction using Taqman Sample-to-SNP kit

For genotyping of *Nisch*^{V33A}, *Nisch*^{L142Q} and *Nisch*^{T445I} mice, DNA was extracted using the Taqman Sample-to-SNP kit (Applied Biosystems). Ear clips or tails were incubated at 95°C for 3 minutes with 50 μ L of Lysis Solution. Samples were cooled at room temperature for 30 seconds before adding 50 μ L of DNA Stabilizing Solution and mixing the solutions. For long term storage, sample lysates were stored at -20°C. Lysates were diluted 1:10 with ddH₂O before PCR.

2.4.4 PCR for routine genotyping

A variety of primers and cycling conditions were used to amplify genomic DNA (see Table 2.7 - 2.10). The gel based genotyping assay for *Itga5^{tm1Hyn}* was provided by the Jackson Laboratory.

For LightScanner-based genotyping, PCR amplification was carried out in FrameStar plates (Clontech Life Science) in a 10 µl volume; containing HotShot or Kapa *Taq* master mix, LCGreen dye, ddH₂O, forward primer, reverse primer, probe if required and DNA. Samples were overlaid with 20 µl mineral oil (Sigma) and centrifuged briefly before DNA was amplified on a G-storm thermocycler (GRI).

For gel-based genotyping, PCR amplification assays were conducted in 96 well Abgene PCR plates (ThermoScientific) with a final volume of 20 µl, containing Qiagen *Taq* PCR master mix, ddH₂O, forward primer, reverse primer and DNA. Plates were sealed using peelable heat sealing foil sheets (ThermoScientific) and centrifuged briefly. DNA was then amplified using G-storm thermocyclers. Full PCR mix details for all assays can be found in Table 2.9.

Table 2.7| Primer and probe sequences for LightScanner based PCR assays.

All primers were sourced from Eurofins MWG Operon. Primer modifications: [SpC3], C3 Spacer (blocks 3' end of probe by adding a three carbon spacer to the 3' terminus).

Assay	Primer type	Primer sequences (5' – 3')
<i>Nisch^{edsn}</i>	Forward	GGCAGCACAAAGATGGCGGTAAC
	Reverse	AACTGCCGCAACCGCAACA
<i>Nisch^{V33A}</i>	Forward	GACTGAGTACCTTGCAGCTA
	Reverse	CTGTAACGGTGTGTTGATCGTC
	Probe (Forward)	CCCTTTAGGCTTATGTCATCCAGGTTAC-[SpC3]
<i>Nisch^{L142Q}</i>	Forward	GCTGTACAACAGGCTGGGA
	Reverse	GCAGCTGTTCTGTGATAGCATA
	Probe (Forward)	TTACAGGAGAGCAACTTCAAGGAGCC-[SpC3]
<i>Nisch^{T445I}</i>	Forward	ACTGTAAGCTACTTTCTGGACT
	Reverse	TTGACATCTTTGGCTTTCTGAATTG
	Probe (Reverse)	GTCCAGCTCTTTCTCTATGGTTGCGA-[SpC3]

Table 2.8| Primer sequences and PCR product sizes for agarose gel based PCR assays.

All primers were sourced from Eurofins MWG Operon.

Assay	Primer type	Primer sequences (5' – 3')	Band type	Product size (bp)
Wild-type	Forward	CGTTGAGTCATTCGCCTCT	Wild-type	227
<i>Itga5^{tm1Hyn}</i>	Reverse	CTACCGCGTCTAGGTTGAAGC		
Mutant	Forward	GACAATCGGCTGCTCTGA	Mutant	495
<i>Itga5^{tm1Hyn}</i>	Reverse	GCAGGCATCGCCATG		

Table 2.9| PCR reaction mix details for routine genotyping assays.

PCR mix details for (A) the *Itga5*^{tm1Hyn} allele; (B) the *Nisch*^{edsn} allele; and (C) the *Nisch*^{V33A}, *Nisch*^{L142Q} and *Nisch*^{T445I} alleles.

Assay	Reagent	Volume (μl)	Total volume (μl)
<i>Itga5</i>^{tm1Hyn}	Qiagen <i>Taq</i> PCR master mix	10	20
	ddH ₂ O	6	
	Forward primer (20 μM)	1	
	Reverse primer (20 μM)	1	
	DNA	2	
<i>Nisch</i>^{edsn}	HotShot MasterMix	5	10
	LCGreen dye	1	
	ddH ₂ O	1.6	
	DMSO	0.2	
	Forward primer (20 μM)	0.1	
	Reverse primer (20 μM)	0.1	
	DNA	2	
<i>Nisch</i>^{V33A}	KAPA <i>Taq</i> master mix	5	10
	LCGreen dye	1	
	ddH ₂ O	0.9	
	Forward primer (20 μM)	0.1	
	Reverse primer (20 μM)	0.5	
	Probe (Forward) (20 μM)	0.5	
	DNA	2	
<i>Nisch</i>^{L142Q}	KAPA <i>Taq</i> master mix	5	10
	LCGreen dye	1	
	ddH ₂ O	0.9	
	Forward primer (20 μM)	0.1	
	Reverse primer (20 μM)	0.5	
	Probe (Forward) (20 μM)	0.5	
	DNA	2	
<i>Nisch</i>^{T445I}	KAPA <i>Taq</i> master mix	5	10
	LCGreen dye	1	
	ddH ₂ O	0.9	
	Forward primer (20 μM)	0.5	
	Reverse primer (20 μM)	0.1	
	Probe (Reverse) (20 μM)	0.5	
	DNA	2	

Table 2.10| PCR cycling conditions for routine genotyping assays.

PCR cycling conditions for amplification of (A) the *Itga5*^{tm1Hyn}, (B) *Nisch*^{edsn}, (C) *Nisch*^{V33A}, *Nisch*^{T445I} and (D) *Nisch*^{L142Q} alleles.

(A)			(B)		
Step	Temperature	Time	Step	Temperature	Time
1.	94 °C	3 min	1.	95 °C	2 min
2.	94 °C	30 sec	2.	95 °C	30 sec
3.	62 °C	1 min	3.	64 °C	30 sec
4.	72 °C	1 min	4.	72 °C	30 sec
5.	Go to step 2, 35 times		5.	Go to step 2, 44 times	
6.	72 °C	2 min	6.	72 °C	4 min
7.	10 °C	∞	7.	95 °C	30 sec
			8.	25 °C	30 sec
			9.	10 °C	∞

(C)			(D)		
Step	Temperature	Time	Step	Temperature	Time
1.	95 °C	2 min	1.	95 °C	2 min
2.	95 °C	30 sec	2.	95 °C	30 sec
3.	60 °C	30 sec	3.	58 °C	30 sec
4.	72 °C	30 sec	4.	72 °C	30 sec
5.	Go to step 2, 55 times		5.	Go to step 2, 55 times	
6.	95 °C	30 sec	6.	95 °C	30 sec
7.	25 °C	30 sec	7.	25 °C	30 sec
8.	10 °C	∞	8.	10 °C	∞

To distinguish between wild-type and *edison* homozygous (*Nisch*^{edsn/edsn}) mice the same method was used, however wild-type DNA was mixed in a 1:1 ratio with genomic DNA from samples being tested to convert the *Nisch*^{edsn/edsn} samples into false heterozygous samples.

2.4.5 LightScanner

LightScanner plates for analysis were spun at 1,700 × *g* for 3 minutes and then analysed using the LightScanner HR/96 (Idaho Technology) in conjunction with

LightScanner software Call-It 2.0. The LightScanner method utilises LCGreen dye which binds double stranded DNA. PCR is performed in the presence of the double stranded DNA binding dye LCGreen. After PCR samples are then heated on the LightScanner and the fluorescence emitted by bound LCGreen is monitored. As the DNA melts the LCGreen is released and so the fluorescence decreases until all the DNA has melted and all LCGreen is unbound.

Unlabelled probe genotyping was utilised for *Nisch*^{V33A}, *Nisch*^{L142Q} and *Nisch*^{T445I} assays (Figure 2.5 - 2.7), to distinguish between different homozygote samples at a given SNV. Here a 3' blocked oligonucleotide (LunaProbe) was designed that sits directly over the SNV. Asymmetric exhaustive PCR was performed using five times the amount of probe and opposite primer (see section 2.4.4). This creates two products, one is the full PCR product between the normal primers and the other is the probe that is bound to the opposite strand. When the products are melted the probe melts at a lower temperature and by focussing analysis on this section, homozygous, heterozygous and wild-type samples can be resolved.

Scanning analysis genotyping was utilised for the *Nisch*^{ed^{sn}} assay. The region where the SNV in *edison* was located was very GC-rich and a suitable LunaProbe could not be designed. Traditional PCR was performed, where wild-type and homozygous DNA strands in the *edison* assay will anneal perfectly and will emit the most fluorescence. Heterozygous DNA strands will have a mismatch between DNA strands leading to less fluorescence. To distinguish between wild-type and homozygous DNA the same method was used, however wild-type DNA was mixed in a 1:1 ratio with genomic DNA from samples being tested to convert the homozygous samples into false heterozygous samples (Figure 2.4).

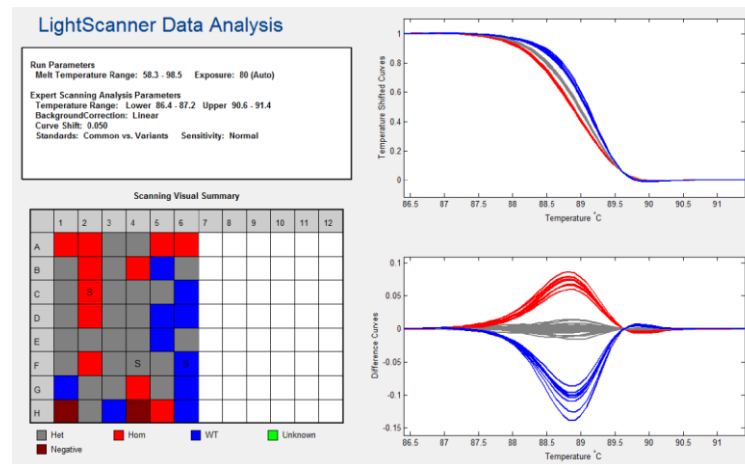


Figure 2.4| Example LightScanner output for *Nisch*^{edsn} genotyping.

Scanning report summary showing *Nisch*^{+/+} (blue), *Nisch*^{edsn/+} (grey) and *Nisch*^{edsn/edsn} (red).

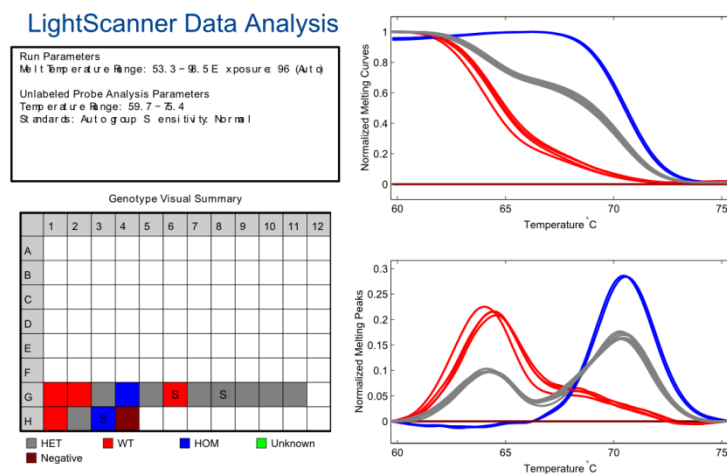


Figure 2.5| Example LightScanner output for *Nisch*^{V33A} unlabelled probe genotyping.

Scanning report summary showing *Nisch*^{+/+} (red), *Nisch*^{V33A/+} (grey) and *Nisch*^{V33A/V33A} (blue).

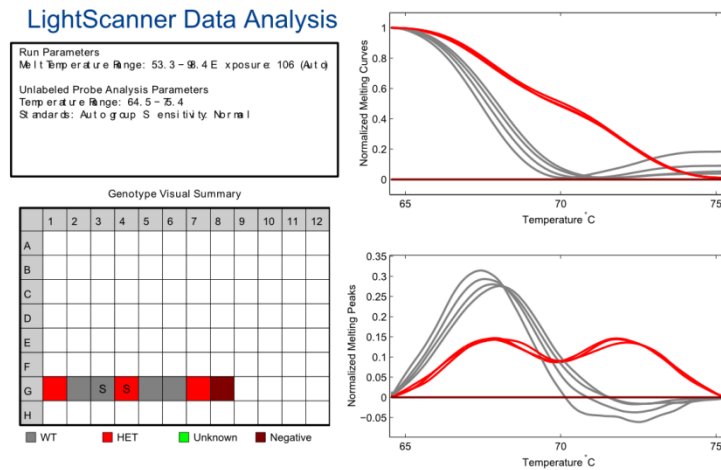


Figure 2.6| Example LightScanner output for *Nisch*^{L142Q} unlabelled probe genotyping. Scanning report summary showing *Nisch*^{+/+} (grey) and *Nisch*^{L142Q/+} (red).

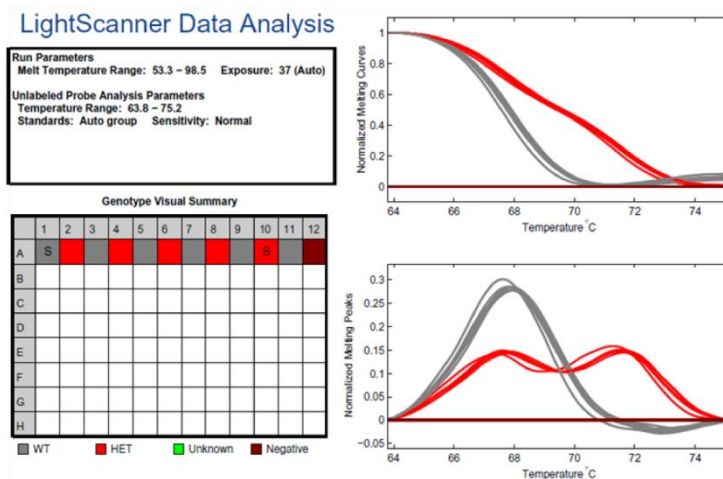


Figure 2.7| Example LightScanner output for *Nisch*^{T445I} unlabelled probe genotyping. Scanning report summary showing *Nisch*^{+/+} (grey) and *Nisch*^{T445I/+} (red).

2.4.6 Agarose gel electrophoresis

PCR reactions requiring analysis using gel electrophoresis utilised 1.5% agarose gels (Sigma) made up in 1X TAE buffer. Ethidium bromide was used to visualise PCR products, by adding 0.5 µg/ml directly to agarose gels. Samples were centrifuged briefly and Orange G loading buffer added to the sample before loading onto the gel. PCR products were separated by gel electrophoresis at 150 V for 20 minutes. PCR

product sizes were compared to a 100 bp standard molecular weight marker (New England Biolabs) due to the expected band size (Figure 2.8). Gels were viewed using an ultra-violet gel doc system and Quantity One v7-6.3 software (Bio-Rad).

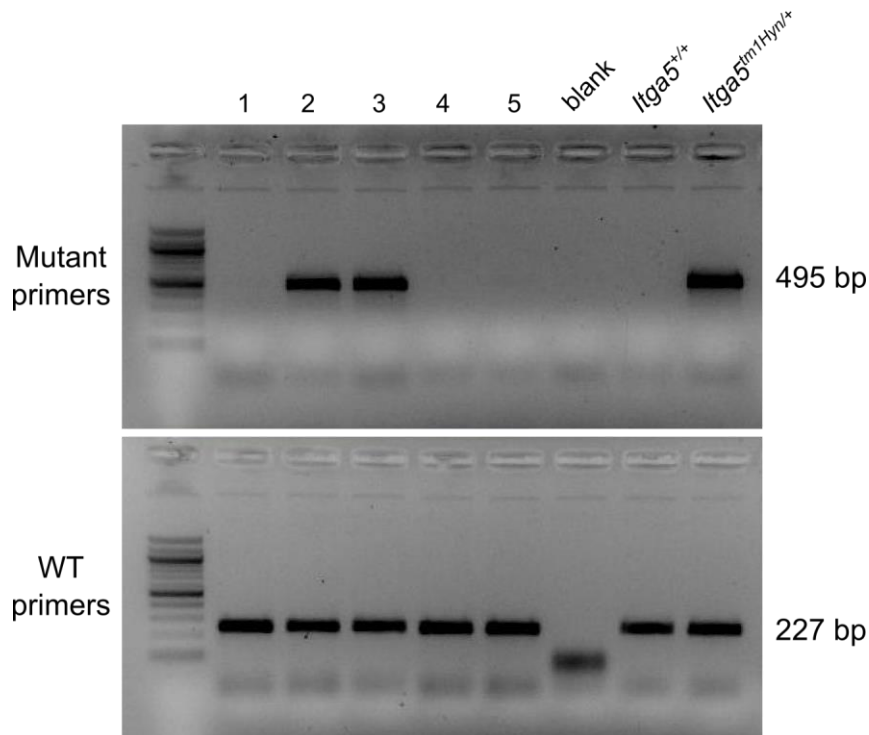


Figure 2.8| Example gel image for *Itga5*^{tm1Hyn} genotyping.

A single upper band indicates a homozygous animal, with a lower band representing a wild-type. Samples with both an upper and a lower band indicate a heterozygote. Higher molecular weight bands are 495 bp with lower bands at 227 bp. PCR products were run on a 1.5% agarose gel.

2.5 Auditory brainstem response (ABR)

The assessment of hearing thresholds in mice was determined using a click-evoked or frequency-specific auditory brainstem response (ABR) as described by Hardisty-Hughes *et al.*, 2010. Here, a broad spectrum stimulus is played into the ear of the mouse and the decibel level of the stimulus is then reduced until no replicable response can be detected giving an estimation of the hearing threshold of the

subject, as a measure of sound pressure level (dB SPL). Each stimulus was delivered in free field at stimulation intensity starting from a maximum of 90 dB SPL at decreasing steps of 5 dB. Minimum intensity stimulation was reached at 5 dB SPL.

Mice were weighed and anaesthetised by intraperitoneal (IP) injection of a mix containing 1 ml Ketamine (100 mg/ml; Ketaset®, Fort Dodge), 0.5 ml Xylazine (20 mg/ml; Sedaxylan®, Eurovet Animal Health) and 8.5 ml sterilised water (Dechra) at a dose of 0.1 ml/10g. Mice were kept in a heated cage at 37°C and eye lubricant (0.2 mg/g carbomer; Viscotears®, Novartis AG) was administered to both eyes to prevent drying out during the procedure. Mice were transferred to a heated mat in an auditory sound booth and three electrodes positioned subcutaneously into the body: (1) below the right pinna (ground electrode), (2) into the muscular mass below the left ear (reference electrode) and (3) on the top of the head between the two pinnae (active electrode). A speaker directly facing the auditory canal was positioned at a distance of 1.5 cm from the ear. Tucker Davis Technologies (TDT) equipment and TDT SigGen RP/RZ software is used to play a short tone or click stimulus and the electrical response of the auditory pathway is measured via the electrodes. The auditory response file was recorded and analysed in the TDT BioSig RP/RZ software through the generation of a graphical trace (Figure 2.9) containing five peaks; each representing a different part of the neuronal circuit responsible for processing sounds. Mice were recovered after sub-cutaneous injection of 0.1 ml of a reversal agent containing 0.1 ml Atipamezole (5mg/ml; Antisedan®, Orion Pharma) and 9.9 ml sterilised water. Once fully recovered, mice were returned to their home cage.

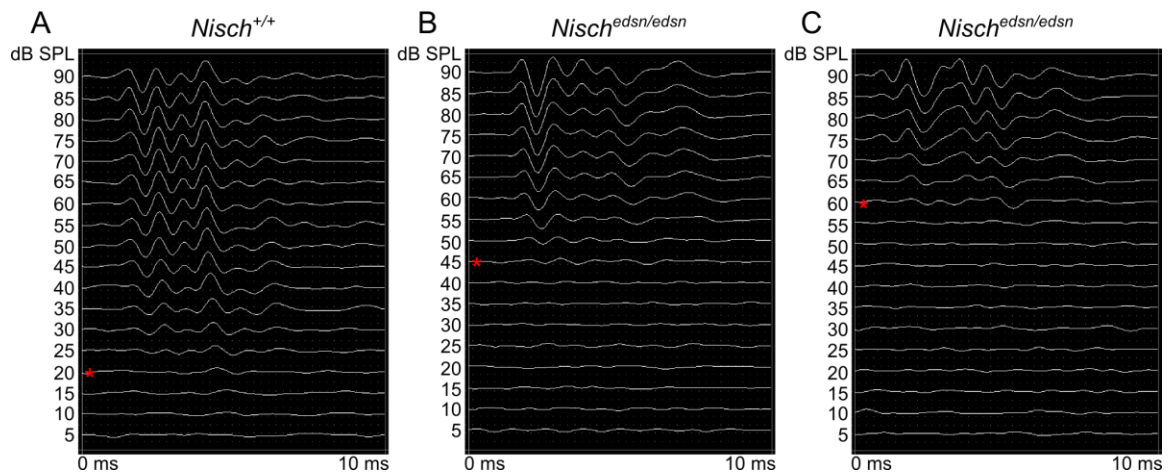


Figure 2.9| Auditory brainstem response (ABR) testing.

Typical response traces to a click-evoked stimulus from 90 dB sound pressure level (SPL) to 5 dB SPL in **(A)** *Nisch^{+/+}* and **(B and C)** *Nisch^{edsn/edsn}* mice. Hearing threshold marked with red asterisk.

2.6 Morphological analysis

Body mass was measured each week on scales to 0.01g for the longitudinal weight study. Craniofacial measurements were taken from X-ray analysis of skulls from mice 20 weeks of age. Radiography was performed using a Faxitron digital X-ray system at 26kV with average exposure time of 3s. Digital images were obtained by using a fitted camera with a field of view 2x4 inches and 10pixel/mm resolution. The Specimen program on the computer accompanying the Faxitron machine enabled instant viewing of X-ray images as well as storage of images as digital files. ImageJ 1.44p software was used to measure the craniofacial features of the X-ray images taken. Measurements were then normalised against a 20mm block placed parallel to the skull during X-ray. The following distances on the skull were measured: skull length, nasal bone length, frontal bone length, parietal bone length and skull width. These measurements were adapted from Richtsmeier *et al.*, 2000.

2.7 Alizarin stained skulls of adult mice

The skulls of adult *edison* mice were prepared using a rapid method for high quality alizarin staining as described by Selby 1987. Briefly, mice were overdosed with IP administration of sodium pentobarbital (Pentoject, Animalcare Ltd). With the head removed and skinned, the eyes were removed and the fat behind them. The skulls were placed in solution A, 1% (w/v) aqueous potassium hydroxide (KOH), for one day. On day 1 the solution was poured away, the bottle and skull rinsed (all rinses are with tap water), and covered again with solution A. On day 3 the solution was poured out, the bottle and skull rinsed, and covered with solution B, 2% (w/v) aqueous KOH. On day 7 the solution was poured out, the bottle and skull rinsed, and covered with solution B again. On day 8 the solution was poured out, the bottle and skull rinsed, and covered with solution C, 1.9% (w/v) aqueous KOH containing 0.04g alizarin red S per litre. On day 10 the solution was poured out, the bottle and skull rinsed, and covered with solution E, clearing solution (2:1:2 of Glycerol:Benzyl alcohol:70% ethanol). On day 13 the solution was poured out, with no rinse, and covered with glycerol. The time in solution C should always be 2 days; times in solutions A, B, and E can be varied to accommodate working schedule. Stained skulls were then imaged and ET tube angles were measured using ImageJ software.

2.8 Collection of embryos

To analyse lungs and palate between mutant and wild-type *edison* mice, embryos at 16.5 dpc and 18.5 dpc were harvested from pregnant females. Females were sacrificed through cervical dislocation and embryos removed and placed in ice cold PBS. Embryos were decapitated and heads and bodies placed in 10% neutral buffered formaldehyde (Surgipath Europe). Tails were taken for genotyping.

2.9 Histology

All processing steps for the decalcification, wax embedding and sectioning of heads were conducted by the MRC Harwell necropsy and histology teams. The F4/80 immunohistochemistry (section 2.15), H&E and PAS staining were also conducted by the histology team.

2.9.1 Decalcification, wax embedding and sectioning

Heads were fixed for 48 hours in 10% neutral buffered formaldehyde (Surgipath Europe) and then decalcified by placing fixed specimens in D.F.B decalcifying agent (Kristenson; Pioneer Research Chemicals) for 48 hours. Samples were then rinsed with water and placed in an automated ThermoShandon Pathcentre machine (Thermoscientific) to dehydrate samples by submersion in a series of graded industrial methylated spirits (70, 90 and 100%) (Chemix). Specimens were then cleared with Sub-X (xylene substitute clearing agent) (Surgipath Europe) and coated in paraffin wax (Surgipath Europe). The samples were transferred to a tissue-embedding centre (R.A. Lamb) where sections were embedded in a fresh paraffin wax block and left to solidify. The paraffin wax block was next transferred to a microtome (Finesse ME; ThermoShandon) and sections of the desired thickness cut using an S35 feather disposable microtome blade (Surgipath Europe). Cut sections were floated on clean distilled water at 50°C to remove creases and then transferred to electrostatically charged slides (Surgipath Europe).

2.9.2 Hematoxylin and Eosin (H&E) staining

Wax embedded sections were placed in a Shandon Varistain 24 (Thermoscientific) which automates solution changes. Briefly, paraffin was removed from slides by

submerging samples in Sub-X, before rehydrating samples by passing them through a graded series of isopropanol (70 and 100%) (Fisher Scientific) and running water. The slides were then immersed in Hematoxylin (Shandon Gill 3, ThermoScientific) for 6 min to stain basophilic structures (for instance the nucleus). Slides were rinsed in running water before being immersed in 1% acid alcohol (containing hydrochloric acid diluted to 1% in 70% methylated spirits) to remove any background staining. The slides were rinsed in running water and then immersed in 1% lithium carbonate (Fisher Scientific) to turn cell nuclei blue. The slides were rinsed in water again and then submerged in eosin (Shandon Eosin Y (aqueous), Thermo Electron) for 3 min to counterstain the eosinophilic structures pink (for instance the cytoplasm). The slides were rinsed again and dehydrated by several isopropanol changes (70% and 100%). Sections were covered with a coverslip (Fisher Scientific) and mounted with Clearium (Surgipath Europe).

2.9.3 Periodic Acid-Schiff (PAS) staining

Wax embedded sections were placed in a Shandon Varistain 24 (ThermoScientific) which automates solution changes. Briefly, paraffin was removed from slides by submerging samples in Sub-X, before rehydrating samples by passing them through a graded series of isopropanol (70 and 100%) (Fisher Scientific) and deionised water. The slides were then immersed in Periodic Acid Solution (ThermoScientific) for 5 min to oxidise various tissue moieties to aldehydes. Slides were then rinsed in several changes of deionised water before being immersed in Schiff's Reagent (ThermoScientific) for 15 min, which bonds to the induced tissue-aldehyde in its colourless form. The slides were rinsed in running water for 10 min, which removes the sulphurous acid restoring the Schiff Reagent to its rose-coloured form. The slides were then counterstained in Hematoxylin (Shandon Gill 3, ThermoScientific) for 1 min to stain basophilic structures (for instance the nucleus). Slides were rinsed in

running water and then immersed in 1% lithium carbonate (Fisher Scientific) to turn cell nuclei blue. The slides were rinsed again and dehydrated by several isopropanol changes (70% and 100%). Sections were covered with a coverslip (Fisher Scientific) and mounted with Clearium (Surgipath Europe).

2.10 Histological Analysis

2.10.1 ME mucosa thickness

Heads were fixed for 48 hours in 10% neutral buffered formalin and then decalcified with Formical (Decal Corp) for 72hr. Wax embedded transverse sections of the ME were stained with H&E and then scanned using the NanoZoomer 2.0 slide scanner (Hamamatsu). Evaluation was carried out using NanoZoomer digital pathology software version 1.1.27 (Hamamatsu). For blinded assessment of mucosal thickness measurements, a standard 1000 μm length of ME mucosa parallel to the tympanic membrane (avoiding the cochlea and the region close to the ET) was measured and 5 equidistant points used to determine the average mucosal thickness. Comparable regions of ME were measured for each ear assessed.

2.10.2 Lung morphology

To study the lung phenotype of *edison* mice, H&E stained sections from adult and embryonic lungs were viewed using a Zeiss Axiostar Plus bright-field microscope and analysed using cell^B imaging software (Olympus). The numbers of airways in three randomly selected $6.5 \times 10^5 \mu\text{m}^2$ regions of lungs were counted to calculate the mean number of airways. In these three randomly selected areas, thirty random airways were measured to calculate the mean airway diameter.

2.11 Quantitative Real-time PCR (RT-qPCR)

2.11.1 Collection of ME exudates

Mice were overdosed with IP administration of Sodium pentobarbital (Pentoject, Animalcare Ltd). Ears were examined under a dissecting microscope (Leica MZ6), the outer surface of the ear drum cleaned with fine forceps and the ossicles extracted. Measured volumes of ear exudates were collected, added to microcentrifuge tubes containing 20 μ l RNase free water and stored on dry ice. Ear exudate samples from left and right ears were pooled for each mouse and samples stored at -80°C until further processing.

2.11.2 Collection and RNA extraction of mouse blood

0.5 ml of blood for RNA extraction was collected through retro-orbital bleeding after overdose of mice (section 2.11.1). Blood was added to RNA/ater tubes (Ambion) and mixed before storage at -20°C. RNA extractions from blood were conducted using a Mouse RiboPure-Blood RNA Isolation Kit (Ambion) following the manufacturer's instructions with some protocol modification. Blood samples were thawed and centrifuged for 3 min at 15,000 x *g* and the supernatant discarded. Lysis solution was then added to the pellet and samples vortexed vigorously to re-suspend the pellet and lyse the blood cells. The lysed blood sample was added to a 15 ml tube and 200 μ l 3M Sodium Acetate added to the sample. Lysis solution was added to bring the sample to a total volume of ~3.8 ml, then vortexed until homogenous. 1.5 ml acid-Phenol:Chloroform was added to the sample and the tube shaken vigorously for 30 sec. Samples were stored at RT for 5 min and then centrifuged for 10 min at 2,000 x *g*. The aqueous (upper) phase was removed and transferred to a fresh 15 ml tube. A half volume of 100% ethanol was added to this aqueous phase and mixed

through pipetting (For example, if 4 ml of aqueous phase was recovered, 2 ml of 100% ethanol was added). Samples were then loaded into the filter cartridge utilising vacuum filtration to pass the sample through. The filter cartridge was transferred to a collection tube and 350 μ l Wash solution 1 added to the column. Samples were centrifuged for $\sim$ 5-10 sec at $8,000 \times g$ and the flow-through discarded. 78 μ l DNase reagent, containing buffer RDD and DNaseI (Qiagen), was added to the filter cartridge and samples left to stand for 15 min at room temperature before addition of another 350 μ l Wash solution 1. Samples were again centrifuged for $\sim$ 5-10 sec at $8,000 \times g$ and the flow-through discarded. 750 μ l Wash solution 2/3 was applied to the filter cartridge and centrifuged for $\sim$ 5-10 sec at $8,000 \times g$. The flow-through was discarded and another 750 μ l Wash solution 2/3 added to the cartridge. The sample was then spun for 1 min at $11,000 \times g$ to remove residual fluid from the filter. RNA was eluted from the filter through the addition of $2 \times 125 \mu$ l of heated water (80°C), separated by a 1 min room temperature incubation step and centrifugation at $8,000 \times g$ for 1 min. RNA concentrations were determined using a Nanodrop 8000 (Thermo Scientific) and samples stored at -20°C .

2.11.3 RNA extraction from pooled ear exudates

RNA extractions of ear exudates were conducted using a NucleoSpin RNA/Protein Isolation Kit (Macherey-Nagel) following the manufacturer's instructions with some protocol modification. Ear exudate samples were thawed on ice, and four individual samples were pooled to yield enough RNA for experiments. 5 μ l carrier RNA (4 ng/ μ l, QIAGEN) was added before isolation of each pool to improve the recovery of total RNA. 350 μ l Buffer RP1 and 3.5 μ l β -mercaptoethanol were added to the pooled samples before being vortexed vigorously. 350 μ l of 70% ethanol was added and mixed by pipetting before transferring to a NucleoSpin® RNA/protein column placed in a collection tube. Samples were centrifuged for 30 sec at $11,000 \times g$. Column with

bound RNA was placed in a new collection tube, with 350 μ l MDB (membrane desalting buffer) placed on the membrane. Samples were centrifuged for 1 min at 11,000 $\times g$ to dry the membrane and flow-through was discarded. 95 μ l of rDNase reaction mixture (10 μ l rDNase and 90 μ l reaction buffer for rDNase) was added directly onto the centre of the silica membrane of the column and incubated for 15 min at RT. 200 μ l Buffer RA2 was then added to the column and centrifuged for 30 sec at 11,000 $\times g$. The filter column was then placed in a new collection tube and 600 μ l Buffer RA3 added to the column. Samples were centrifuged for 30 sec at 11,000 $\times g$, afterwards the flow-through was discarded and the column placed back in the same collection tube. 250 μ l buffer RA3 was then added to the column and centrifuged for 2 min at 11,000 $\times g$ to dry the membrane. The NucleoSpin RNA/protein column was then transferred to a RNase free collection tube and RNA eluted in 2 $\times$ 20 μ l separated by a centrifugation step for 1 min at 11,000 $\times g$. RNA concentrations were determined using a Nanodrop 8000 (Thermo Scientific) and samples were stored at -80°C.

2.11.4 cDNA synthesis

cDNA was generated following manufacturer's instructions using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). RNA was reverse transcribed into cDNA through firstly adding 250 ng total RNA in 10 μ l to 10 μ l 2X RT master mix (2 μ l 10X RT Buffer, 0.8 μ l 25X dNTP mix (100 mM), 2 μ l 10X RT Random Primers, 1 μ l MultiScribe Reverse Transcriptase and 4.2 μ l nuclease-free H₂O). Finally, tubes were placed on a thermal cycler and cDNA synthesised according to the programme provided by the manufacturers: 25°C for 10 min; 37°C for 120 min; 85°C for 5 min; hold at 4°C. Samples were either used immediately in RT-qPCR reactions or stored long term at -80°C.

2.11.5 RT-qPCR using murine TaqMan assays

TaqMan Fast Universal PCR Master Mix (Applied Biosystems) was used in conjunction with TaqMan Gene Expression Assays (Applied Biosystems) for RT-qPCR analysis of synthesised cDNA. Reaction volumes were made up of 5 µl cDNA (2.5 ng/µl), 10 µl 2X Fast Universal PCR Master Mix, 1 µl 20X TaqMan Gene Expression Assay and 4 µl nuclease-free water to make a final volume of 20 µl. Samples were aliquoted into a MicroAmpR Fast Optical 96-well reaction plate and sealed with a MicroAmp optical adhesive film (Applied Biosystems). Real-time amplification was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) with a Fast programme (cycling conditions: 95°C for 20 sec; 40 cycles at 95°C for 3 sec; 60°C for 30 sec). Each TaqMan assay was performed in triplicate for each sample. *Ppia* was used as the endogenous control in all RT-qPCR experiments as the cycling threshold (C_t) value of this gene does not change with chronic inflammation (as previously shown in our lab). Murine TaqMan gene expression assays used in this thesis were *Hif-1a* (Mm01283756_m1), *Il-1 β* (Mm01336189_m1), *Tnfa* (Mm00443258_m1), *Vegfa* (Mm00437304_m1), *Src* (Mm00436785_m1), *Evi1* (Mm00491303_m1), *Fbxo11* (Mm01227499_m1) and *Ppia* (Mm02342429_g1).

2.11.6 RT-qPCR analysis

TaqMan assays are formed of three target-specific oligonucleotides consisting of two primers to amplify the cDNA and a probe which contains a 5' FAM fluorophore and a 3' quencher. The probe is designed to anneal to the cDNA between the two primers. During PCR, cDNA is amplified by *Taq* polymerases and results in the degradation of the probe and a release of the fluorophore. The separation of the fluorophore from the quencher results in the detection of fluorescence by the thermocycler. The amount of

fluorescence is directly correlated with the amount of cDNA present in the sample, with RT-qPCR measuring the number of cycles that are required to reach a specific threshold at which fluorescence is detected. This is known as the cycle threshold (C_t). Samples with more abundant mRNA (and therefore cDNA) will reach this specific threshold more quickly and have lower C_t values. Analysis of results was performed using Applied Biosystems software (v2.0.6).

To calculate the fold change of the samples of interest against the calibrator sample:

$$1) \Delta C_t = C_t \text{ (target)} - C_t \text{ (endogenous)}$$

$$2) \Delta\Delta C_t = \Delta C_t - \Delta C_t \text{ (reference)}$$

3) The fold change (RQ) can then be calculated: $2^{-\Delta\Delta C_t}$ which gives the relative expression levels with respect to the reference sample.

Error bars are then calculated as the RQ min and max through calculation of the standard deviation (STDEV) of each ΔC_t value which enables derivation of the standard error of the mean. RQ min and max can then be calculated using the defined formulae, where t is equal to the degrees of freedom using 95% confidence in a student's t-test.

$$1) SE\Delta C_t = \sqrt{(STDEV \text{ sample}^2 / n) + (STDEV \text{ calibrator}^2 / n)}$$

$$2) RQ \text{ min} = 2^{-[av\Delta\Delta C_t + (SE\Delta C_t \times t)]}$$

$$3) RQ \text{ max} = 2^{-[av\Delta\Delta C_t - (SE\Delta C_t \times t)]}$$

2.12 Scanning electron microscopy (SEM)

2.12.1 Dissection

Mice were sacrificed through cervical dislocation, the head and skin removed and the head bisected down the midline. The brain and extraneous tissue from around the ear was removed, leaving the inner ear and surrounding middle ear bulla. The bulla was lifted away from the inner ear and the stapes removed by opening up the fluid space in the inner ear. A hole was also made in the apex of the cochlea by gently scratching the bony shell away to facilitate penetration of fixative. Inner ears were placed in glass specimen jars containing 5 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer. Inner ears were fixed between 4 hours and overnight at 4°C. Inner ears were washed three times in 0.1 M phosphate buffer for 15 min and decalcified in a solution of 4.3% EDTA in 0.1 M phosphate buffer at 4°C for 48 hours. Inner ears were then washed three times in 0.1 M phosphate buffer and stored in buffer at 4°C until fine dissection.

Fine dissections of inner ears were conducted using a Zeiss Discovery v.20 stereomicroscope (Zen 2011). For fine dissection, inner ears were pinned to a sylguard filled petri dish using a 30G needle and the bony shell of the inner ear removed to reveal the cochlear duct. The stria vascularis was then removed using fine watchmakers forceps (size 5) to reveal the hair cell rows beneath. Cochlea samples were returned to 0.1 M phosphate buffer until Osmium-Thiocarbohydrazide-Osmium-Thiocarbohydrazide-Osmium (OTOTO) processing.

2.12.2 OTOTO processing

OTOTO processing is a post-fixative step. Here, dissected inner ears were placed in a specimen holder and incubated in 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer for 1 hour at room temperature. OsO_4 was removed and samples

washed six times with distilled water, with each wash lasting 3 min. Specimens were then incubated with 1% thiocarbohydrazide for 30 min. This solution was removed and samples washed six times with distilled water, with each wash lasting 3 min. Samples were then incubated again with OsO_4 for 1 hour, followed by the wash steps outlined above totalling three soaks in OsO_4 separated by two soaks in 1% thiocarbohydrazide. In order to maintain cell structure, fixed tissues were dehydrated in alcohol to replace water lost in cells, as air drying causes collapse and shrinkage of tissues. To do this, samples were then transferred into glass jars and specimens were dehydrated through increasing strength ethanol solutions (25%, 40%, 60%, 80%, and 95%) for 45 min each at 4°C. Specimens were then incubated a further three times in 100% ethanol for 30 min at 4°C. Samples were left in the final ethanol solution at 4°C and for long term storage samples were placed in 100% acetone.

2.12.3 Critical point drying

Inner ear samples were dried in a critical point drier (Agar Scientific) with two soak and purge steps. Critical point drying uses liquid carbon dioxide to replace solvents. The carbon dioxide is then removed in a supercritical state so that no liquid is present within the sample. The dry specimen was then mounted using an adhesive button and silver paint, with the cochlea facing upwards.

2.12.4 Sputter coating and SEM

Inner ear samples were sputter coated twice with platinum (Quorum Q150RS). Sputter coating makes the sample conductive for the SEM procedure, allowing the sample to be grounded and also prevents damage from the electron beam in the

SEM. Samples were analysed using a scanning electron microscope (SEM) (Jeol JSM-6010LV).

2.13 Respiratory function test using plethysmography

Functional assessment of airway responsiveness in *edison* mice was determined by pulmonary function test. Airway reactivity was assessed using an unrestrained whole-body plethysmograph system (Buxco) and a combined hypoxia and methacholine challenge protocol. Enhanced pause (Penh) was measured as an indirect assessment of airway resistance and responses were recorded by Biosystem XA v1.5.4 software (Buxco).

Conscious animals were placed in the individual plethysmograph chambers, and activity was allowed to stabilize for 5 min, while the mice acclimatised to the chambers. Following acclimatisation, activity was recorded unchallenged for 5 min as a baseline for the methacholine challenge. The baseline period of activity for the hypoxia challenge was then recorded for 5 min with 21% oxygen gas at a flow rate of 10 litres per minute. Gas was switched to 10% oxygen and 3% carbon dioxide to measure the hypoxia responses for 5 min, followed by a 5 min recovery period with 21% oxygen gas. For the methacholine challenge, animals were then exposed to nebulised PBS for 2 min, and activity was recorded for 5 min to establish a baseline for vehicle. Methacholine (MCh) was dissolved in PBS to concentrations of 12.5, 25 and 50 mg/ml. Animals were exposed to increasing doses of nebulised MCh for 2 min and activity was monitored continuously for 5 min following each dose of MCh. Sensitisation was confirmed by a left shift in the dose response curve (airway resistance changes to methacholine).

2.14 Immunohistochemistry

2.14.1 Blocking

Paraffin was removed from slides with three changes of Sub-X (xylene substitute) clearing medium (Leica Microsystems) and the dewaxing solution was removed by passing through a graded series of alcohols. Samples were incubated in a Coplin jar with 3% H₂O₂ (VWR) in isopropanol for 30 min to block endogenous peroxidase activity. Sections were then washed once with tap water and twice with PBS.

2.14.2 Antigen retrieval

Where required an antigen retrieval step was conducted after deparaffinising and rehydrating the tissue sections. Antigen retrieval was performed to unmask antigens. Heat retrieval using citrate buffer was used for the anti-Nisch, anti-ITGA5 and anti-phospho-PAK1 antibody. The slides were placed in a chamber and microwaved (900W, 50% power) in 10 mM citrate buffer for 7 min. Distilled water was added to the chamber so samples did not dry out and slides were microwaved for a further 7 min. Sections were left to cool at room temperature and washed once in PBS. Trypsinisation was used as an antigen retrieval method for the F4/80 antibody. Here, slides were incubated in 0.1% calcium chloride (pH 7.8) for 30 min at 37°C and then washed once in PBS.

2.14.3 Incubation with antibodies

After blocking and antigen retrieval, if required, all tissue sections were drawn around using a PAP pen before being incubated with normal goat or rabbit blocking serum for 30 min. The blocking serum used depended on which species the secondary antibody was made. Sections were incubated overnight in a humidified

chamber at room temperature with primary antibody (Table 2.11) diluted in normal blocking serum. Negative control sections received no primary antibody. Slides were rinsed with PBS using a wash bottle and washed twice with PBS on a rocker. Sections were then incubated for 30 min with biotinylated secondary antibody solution (Vector). Biotinylated goat anti-rabbit or rabbit anti-rat antibody was used depending on the species in which the primary antibody was made. Sections were then washed twice with PBS and incubated with VECTASTAIN Elite ABC Reagent (Vector) for 30 min.

Table 2.11| Antibodies used in immunohistochemistry

Primary antibody	Manufacturer	Species	Concentration	Antigen retrieval
Anti-F4/80	Serotec	Rat	1:100	Yes
Anti-Nisch	Santa-Cruz	Rabbit	1:200	Yes
Anti-ITGA5	Santa-Cruz	Rabbit	1:200	Yes
Anit-Rac1	Santa-Cruz	Rabbit	1:400	No
Anti-phospho-PAK1	Santa-Cruz	Rabbit	1:50	Yes
Anti-phospho-SMAD2	Chemicon	Rabbit	1:200	No

2.14.4 Detection and analysis

Slides were washed twice in PBS after incubation with antibodies. A peroxidase-based immunohistochemical method utilising diaminobenzidine (DAB) was used to detect staining. After incubation with the substrate solution (Dako) sections were placed in PBS and counterstained with Hematoxylin, cleared and mounted by the histology team at MRC Harwell. Sections were imaged using a Zeiss Axiostar Plus bright-field microscope and Cell^B software (Olympus) used to record images.

2.15 Bacterial challenge experiments

All reagents used for bacterial culture were autoclaved prior to use and aseptic techniques used at all times.

2.15.1 *NTHi* strains

Genomically sequenced non-typeable *Haemophilus influenzae* (*NTHi*) strain #162 or streptomycin resistant #162 (#162^{SR}) were used in all bacterial challenge experiments. *NTHi* #162 is a non-encapsulated isolate derived from ME of children undergoing tympanocentesis during a Finnish pneumococcal vaccine study that took place between 1994 and 1995. The *NTHi* strain #162 was kindly provided by Juhani Eskola and the Finnish Otitis Media Study Group. The *NTHi* strain #162^{SR} was generated and supplied by Dr Derek Hood, MRC Harwell. Strain #162^{SR} was derived by introducing a streptomycin resistance cassette into the HI0227 gene of #162. This disrupts the open reading frame and results in a deletion of HI0227. *NTHi* #162^{SR} has no obvious difference in phenotype compared to the wild-type #162 with respect to cell phenotype, growth or LPS synthesis.

2.15.2 Preparation of inoculums

To culture *NTHi* from frozen stocks, a 1 µl loop of frozen *NTHi* #162 or #162^{SR} was streaked onto brain heart infusion-levinthal's (BHI-LEV) agar plates and incubated at 37°C, 5% CO₂ overnight. The following day, a 1 µl loop of bacteria was removed from the plate and added to liquid culture containing 4 ml BHI and 400 µl LEV. The liquid culture was agitated at 37°C, 5% CO₂ for 3 hr. 1 ml of this liquid culture was used to determine optical density (A_{490} OD) using a spectrophotometer (Jenway). The machine was blanked using an equivalent volume of uninoculated culture media.

Sample was then spun at 16,000 x g for 3 min and the pellet re-suspended with an appropriate volume of PBS containing 1% gelatine to make an inoculum containing 1×10^{10} bacteria per ml using the described equation $(OD/0.4) \times 2 \times 10^9$. The inoculum was titered onto BHI-LEV plates (with or without streptomycin depending on the *NTHi* strain used) to check the purity and number of viable bacteria or colony forming units (cfu). This was performed before mice were inoculated and also after the inoculation was complete to establish the viability of the bacteria in the time elapsed between inoculum preparation and mouse inoculation.

2.15.3 Intranasal challenge using *NTHi*

Mice for challenge were placed in an anaesthetic chamber containing Isoflurane (vaporizer to setting 3; oxygen flow rate of 3 litres/min). Once anesthetised, mice were removed from the chamber and 5 µl inoculum placed on each nostril. As mice are obligate nose breathers, the bacterial inoculum is inhaled with normal inspiration. Mice were then placed in their home cage to recover.

2.15.4 Determination of middle ear colonisation with *NTHi*

At specific time points, mice were sacrificed through IP overdose (as previously described), the heads skinned and a measured volume of each ear fluid was collected into 500 µl PBS. Any ears that had clear tympanic membranes were rinsed with 2 µl PBS. The tube containing sampled ear fluids was then mixed thoroughly until a homogenous suspension was obtained. After this, 10-fold serial dilutions of 10^{-1} and 10^{-2} were made for each sample and 50 µl aliquots of each dilution spread onto BHI-LEV agar plates (with or without streptomycin depending on the *NTHi* strain). Plates were then sealed in plastic bags and placed in a 37°C incubator overnight. The following day, BHI-LEV plates were examined for growth of *NTHi* and

the number of colonies counted. Representative colonies were picked from agar plates and wet mounts made in PBS on charged slides to examine the bacteria using phase contrast microscopy. Infection rates were calculated from the number of *NTHi* positive bulla cultures divided by the total number of *NTHi* positive plus *NTHi* negative cultures. Those ears with no fluid and after rinsing with PBS did not yield *NTHi* cultures were omitted from the calculation, as were plates which were overgrown with the swarming commensal *Proteus* which rendered the plate unreadable for the presence of *NTHi*. The *NTHi* titer in bulla fluids was calculated from colony counts relating to recovered volumes of collected ear fluid and dilution of the primary samples. Colonies from plates were also sampled and added to BHI media before storage at -20°C.

2.15.5 Nasopharyngeal sampling

In some experiments it was necessary to determine the nasopharyngeal carriage of bacteria. To do this nasopharyngeal lavage samples were obtained from the skinned head after the removal of the mandible. This dissection exposes the opening of the NP on the midline of the hard palate. The opening of the NP was cannulated and flushed with 200 µl of PBS and lavage fluid collected in a microcentrifuge tube. A 50 µl aliquot of this lavage fluid was spread on BHI-LEV agar plates with Streptomycin for quantitative culture of *NTHi* #162^{SR}. The antibiotic inhibits the growth of contaminant bacteria such as *Proteus* which are normal commensals of the NP.

2.15.6 Confirming *NTHi* culture from ME

A 1 µl loop was obtained from a cultured colony and added to 7 µl PBS on a microscope slide. The preparation was covered with a microscope slide and viewed at x1000 magnification with an oil immersion objective using a phase contrast illumination.

2.16 Statistics

All data are given as unadjusted mean $\pm$ SEM (standard error of the mean) unless stated otherwise. Data were analysed to establish normal distribution. Where data was normally distributed an ANOVA or Student's *t*-test were conducted. If data was not normally distributed the non-parametric equivalents of these tests were used (Kruskal-Wallis One Way Analysis of Variance on Ranks or Mann-Whitney Rank Sum Test) to establish if data were significant. The Holm-Sidak method (ANOVA) or Dunn's method (Ranks) was used for multiple comparisons versus a control group. Results with values of $P < 0.05$ were considered statistically significant. SigmaPlot 11.0 software was used to perform all statistical analysis.

2.17 Stock solutions

Tris-acetate-EDTA (TAE) buffer: 1X working solution contained 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA

Orange G: 110 ml glycerol, 90 ml TE pH 8.0, Orange G powder

Ethidium bromide: 10mg Ethidium bromide in 1 ml ddH₂O. Solution was placed on a roller, protected from light until dissolved

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄

0.1M Phosphate buffer: 4.44g sodium phosphate monobasic, 17.04g sodium phosphate dibasic, 789 ml ddH₂O

Fixative for cochlea: 2.5% glutaraldehyde in 0.1 M phosphate buffer

Decalcification solution for cochlea: 4.3% EDTA in 0.1 M phosphate buffer

1% Thiocarbohydrazide: 0.3 g Thiocarbohydrazide in 30 ml distilled water

1% osmium tetroxide: 2 x 5 ml OsO₄ ampoules in 30 ml sodium cacodylate

0.2 M sodium cacodylate: Add sodium cacodylate to 900 ml distilled water, pH 7.2 using 1 M hydrochloric acid. Make up to 1 litre with distilled water.

10 mM citrate buffer: 10 mM citric acid, 0.05% Tween 20, pH 6.0. Mix 1.92 g of anhydrous citric acid in 1000 ml of distilled water. Adjust the pH to 6.0 with 1N NaOH and then add 0.5 ml of Tween 20.

Solution A for skull staining: 1% aqueous KOH

Solution B for skull staining: 2% aqueous KOH

Solution C for skull staining: 1.9% aqueous KOH + 0.04 g/L Alizarin red S

Solution E for skull staining: Clearing solution (20 mL glycerol, 10 mL benzyl alcohol, 20 mL 70% ethanol)

Brain heart infusion (BHI) liquid medium: 37 g BHI per 1 litre deionised water

Levinthals (LEV): Add 200 ml of horse blood (TCS Bioscience) to 1 L of liquid BHI and heat at 95°C for 45 min. After cooling to 50°C, 1 g of nicotinamide adenine dinucleotide (NAD) (Roche) was added and mixed by stirring. The contents were then centrifuged at 4°C, at 3000 rpm for 30 min to pellet insoluble blood components. The supernatant was aliquoted and stored at -20°C until use.

BHI-LEV Agar plates (with or without streptomycin): 148 g of BHI was diluted in 4 L of ddH₂O and 40 g of agar (Sigma-Aldrich) added. The mixture was stirred until dissolved. The mix was autoclaved for 15 min at 121°C and then left to cool to approximately 45°C. 40 ml of LEV was then added to 400 ml BHI Agar and poured onto plates (BHI-LEV plates). When required 400 µl of 10 mg/ml Streptomycin was added to the cooled down liquid BHI agar giving a final concentration of 10 µg/ml.

CHAPTER 3: Identifying the putative mutation in *edison*

3.1 Introduction

3.1.1 ENU mutagenesis screening

N-ethyl-*N*-nitrosourea (ENU) is a potent mutagen (Russell, Kelly et al. 1979), which is used to efficiently introduce single nucleotide polymorphisms (SNPs), also known as point mutations, into mouse spermatogonial germ cells (Justice, Noveroske et al. 1999). Transfer of its ethyl group to nucleophilic nitrogen or oxygen sites on any of the four deoxyribonucleotides leads to mispairing of the base which results in a point mutation (Justice, Noveroske et al. 1999). ENU predominantly (in 87%) affects AT base pairs, with a preference for A-T/T-A tranversions (Justice, Noveroske et al. 1999). Point mutations are induced at random throughout the genome affecting one nucleotide every 1-2 Mb. The exact rate of mutation is dependent upon the dose of ENU and the mouse strain (Kile and Hilton 2005). The dose of ENU used at MRC Harwell results in one mutation every 1.01 Mb of which 1 in 1.82 Mb are potentially functional (Quwailid, Hugill et al. 2004).

The random nature of ENU-induced mutations facilitates a phenotype-driven approach to mutation detection. Phenotype-driven screens make no assumptions about the nature of the genetic change underlying observed phenotypes, and thus provide a powerful tool for identifying novel genes and pathways contributing to disease pathogenesis. ENU has been used successfully in many mutagenesis programs worldwide to identify novel genes of human diseases (Nolan, Peters et al. 2000; Acevedo-Arozena, Wells et al. 2008).

3.1.1.1 ENU-induced OM mouse mutants

Large scale phenotype-driven ENU mutagenesis screens undertaken at MRC Harwell have previously been used to identify two novel mouse mutants, *Jeff* and *Junbo*, that develop a conductive hearing loss characterised by ME fluid and mucosal

inflammation (Hardisty, Erven et al. 2003; Parkinson, Hardisty-Hughes et al. 2006). The *Jeff* mouse has a mutation in the F-box protein, FBXO11, on chromosome 2 that is involved in the regulation of the TGF- β signalling pathway by regulating the levels of p-SMAD2 in the epithelial cells of embryonic mice (Hardisty-Hughes, Tateossian et al. 2006; Tateossian, Hardisty-Hughes et al. 2009). Excitingly, polymorphisms in the human homologue of this gene have been associated with COME/ROM in two clinical studies of OM (Segade, Daly et al. 2006; Rye, Wiertsema et al. 2011). The causative mutation in the *Junbo* mouse maps to chromosome 3 in a gene encoding for the transcription factor EVI1. This DNA-binding zinc finger protein can repress the TGF- β signalling pathway by interacting with SMAD3 and can antagonize the growth-inhibitory effect of the pathway (Kurokawa, Mitani et al. 1998). The identification of these mutants has focused studies on the role of TGF- β signalling in the development of COM (Tateossian, Morse et al. 2013) and the interaction of this pathway with other molecular and cellular changes occurring in the inflamed ME (Cheeseman, Tyrer et al. 2011). Hence, these ENU induced models of OM have proved to be critical in elucidating the genetic and pathophysiological mechanisms that predispose to COM.

3.1.2 Detection of an ENU induced mutation

To study the function of an ENU induced mutation, it is essential to identify the underlying mutated gene causing the phenotype of interest. As ENU induces random point mutations throughout the genome, sequencing the whole genome alone will not reveal the causative mutation linked to the phenotype. With the assumption that each pedigree founder carries approximately 30 loss-of-function mutations (Kennedy and O'Bryan 2006), it is essential to segregate the mutation linked to the phenotype under study from those mutations not involved and localise the causal mutation.

The recovery of ENU-induced mutations entails outcrossing the affected male to a different inbred strain. The progeny are then backcrossed or intercrossed. Several differences exist between the genome of different inbred strains, thereby allowing the use of polymorphic DNA markers to map the mutation. By analysing mouse strain specific SNPs distributed throughout the entire genome, a chromosomal region containing the causal gene can be identified. Further backcrossing and additional SNP mapping, to narrow down this region, can then be done using more closely spaced polymorphisms. Traditionally, once linkage to a chromosomal region is identified, candidate gene sequencing can be carried out. However, this can be difficult with mutations in novel or relatively unknown genes. If candidates are not evident, then recent advancements in next-generation sequencing (NGS) technologies have made sequencing the exons of hundreds of genes within a chromosomal region feasible (Zhang, Alpert et al. 2009; Sabrautzki, Rubio-Aliaga et al. 2012; Sun, Mondal et al. 2012). In such studies, *in silico* prediction tools for coding variants are used to provide assessments on the impact of the genetic variants on protein function (Caruana, Farlie et al. 2013).

3.1.3 *edison*: an ENU-induced mutant exhibiting reduced startle response

The *edison* mutant was identified in a phenotype-driven ENU G₃ recessive screen, carried out at MRC Harwell, to identify mutants with visual defects (Thaung, West et al. 2002). A standard MRC Harwell design was employed using C57BL/6J and C3H mice to generate G₃ pedigrees. The C57BL/6J strain is utilised for ENU mutagenesis as this strain can tolerate the largest ENU dose of all mouse strains and still maintain fertility, ultimately resulting in researchers being able to uncover more mutations. C3H carries the retinal degeneration 1 (*rd1*) mutation of the retinal specific β sub-unit of cGMP phosphodiesterase gene (*Pde6b*), which would confound assessment of eye phenotypes when homozygous. Pedigrees generated in the vision screen used a

C3H strain carrying a wild-type allele at the *rd1* (*Pde6b*) locus, C3H-*Pde6b*. Briefly, male C57BL/6J mice were mutagenised with ENU and were outcrossed to C3H-*Pde6b* females to produce G₁ progeny. Male G₁ progeny were further outcrossed to C3H-*Pde6b* females to produce G₂ progeny. G₂ female animals are mated back to the original G₁ father to produce homozygous mutations in the G₃ animals (Figure 3.1).

G₃ animals were examined for phenodeviants. Mutant phenotypes were identified by using the modified-SHIRPA protocol that identifies a wide range of phenotypes and is a sensitive screen for mouse neurological, neuromuscular and behavioural mutants [<http://empress.har.mrc.ac.uk/>]. A mutant line exhibiting reduced startle response was identified in pedigree PEDV/7. The hearing mutant was outcrossed to C3H/HeH, and maintained on this strain with further backcrossing. The line displaying reduced startle response was named *edison*.

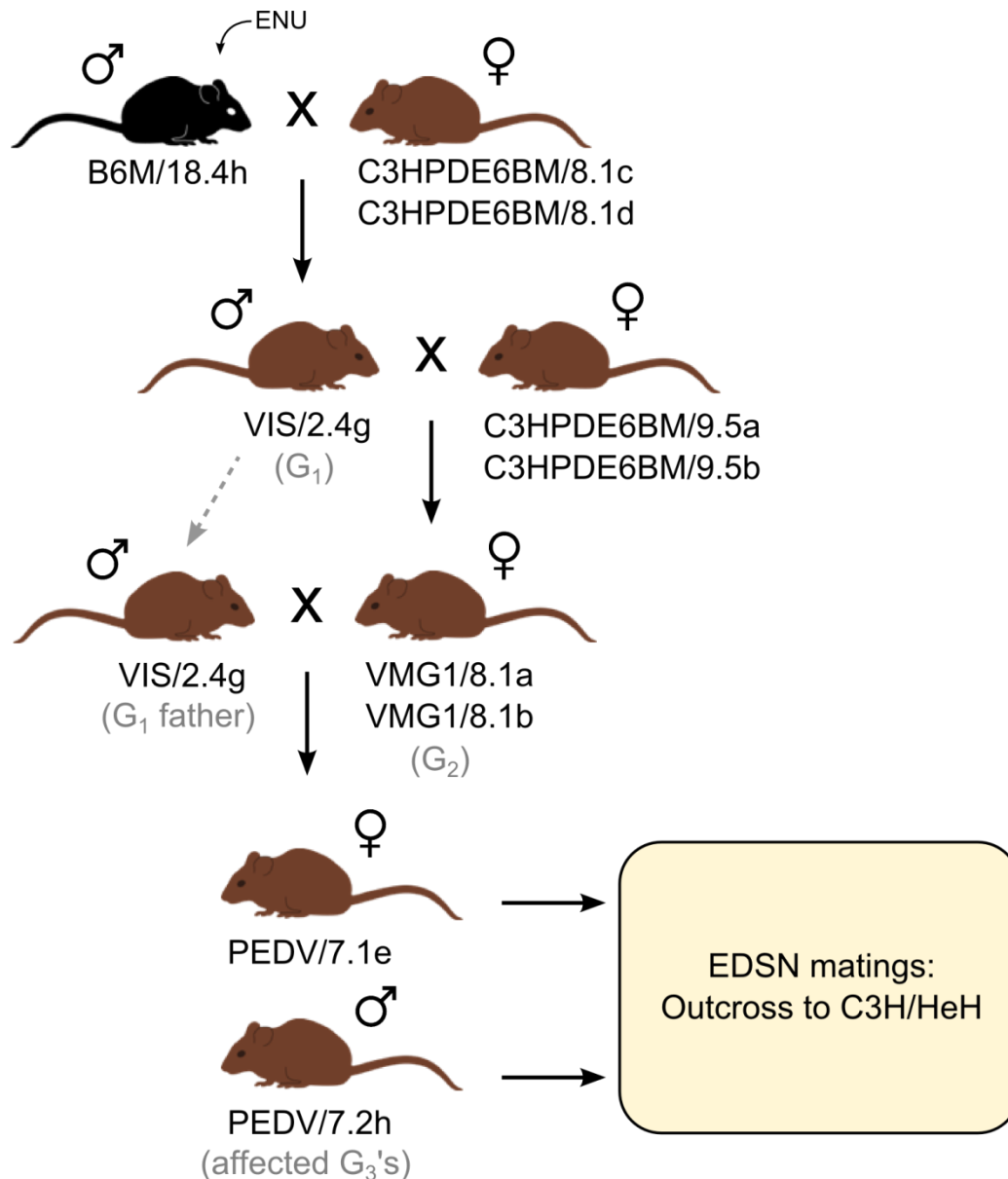


Figure 3.1| Generation of *edison* mouse line from affected G₃ in ENU vision screen.

B6M/18.4h, a C57BL/6J male, was injected with the chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) and mated with C3H-*Pde6b* females. VIS/2.4g, a G₁ male, was again mated with C3H-*Pde6b* females to produce G₂ litters. G₂ females were mated with the G₁ father, VIS/2.4g, to produce G₃ offspring. PEDV/7.1e and PEDV/7.2h had reduced startle response and were outcrossed to C3H/HeH to generate the *edison* mouse line (EDSN).

To determine the position of the mutation affecting *edison* within the genome, genomic DNA samples from thirteen mice with reduced startle response were given to the GEMS core facility at MRC Harwell for a SNP-based whole genome scan. Since the C57BL/6J strain was ENU mutagenised and backcrossed to C3H/HeH, a panel of

sixty three SNP makers that distinguished between C57BL/6J and C3H/HeH was used to determine genetic location. The initial genome-wide scan showed the *edison* mutation had a strong linkage to Chromosome 14 (Figure 3.2A; see Appendix, Figure 10.1 for individual results of genome-wide scan); with a large critical interval between the start of chromosome 14 and the marker at position 59 Mb. Additional SNP markers helped reduce the chromosomal region (Figure 3.2B), with the critical interval lying between the markers at positions 21.98 Mb and 47.07 Mb. This chapter focuses on the identification and confirmation of the underlying mutation in the ENU-induced hearing mutant, *edison*.

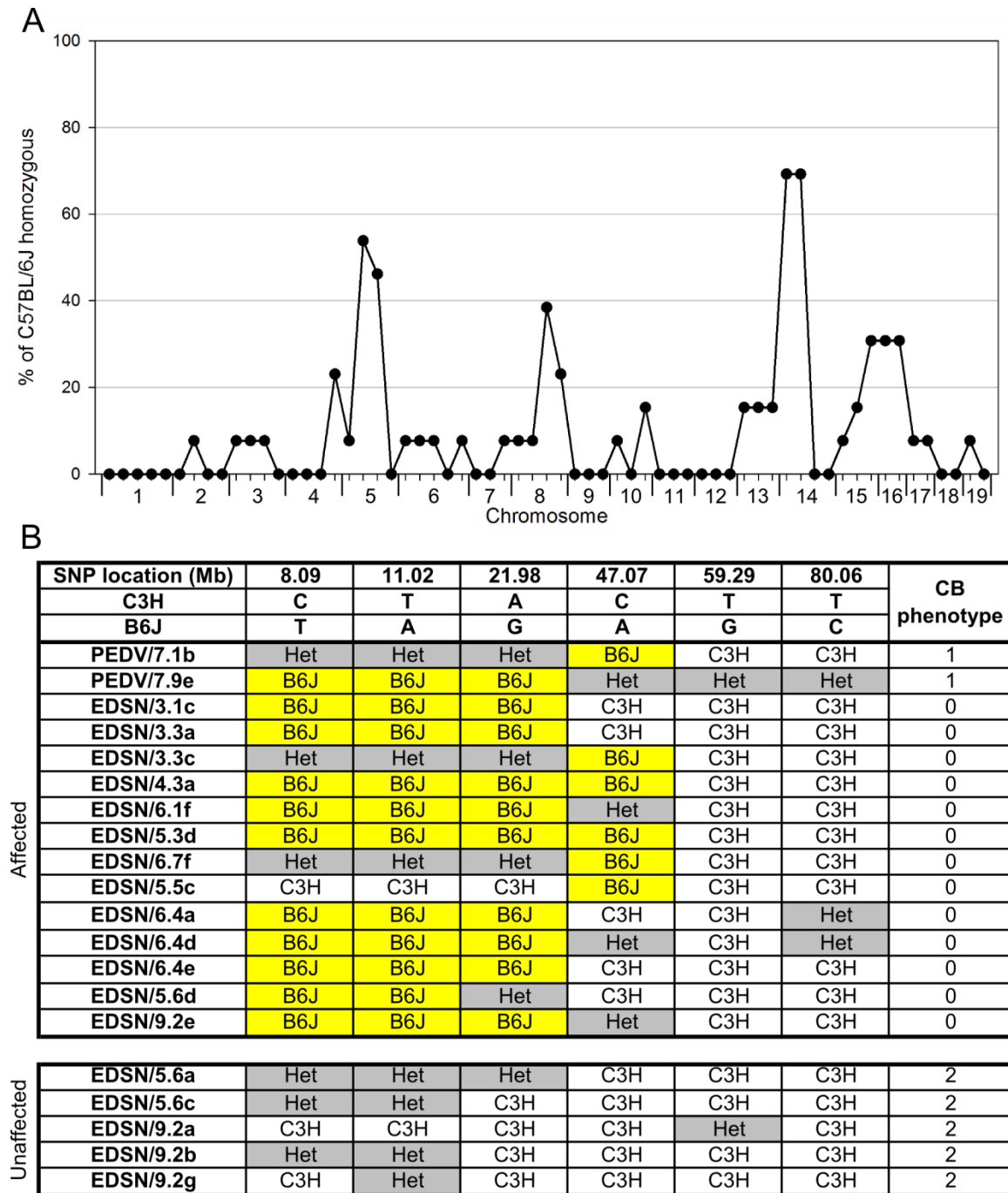


Figure 3.2| *edison* carries a mutation on Chromosome 14 within a 29.05 Mb region.

(A) Result from whole genome scan using 63 SNP markers and 13 mice with reduced startle response, showing percentage of mice genotyped as homozygous C57BL/6J at each marker. *edison* shows a strong linkage to Chromosome 14. **(B)** Preliminary genetic mapping data for Chromosome 14 in *edison* mice. Grouped into affected and unaffected mice according to hearing phenotype. Click box (CB) data; 2 = normal, 1 = reduced startle response, 0 = no startle response. C3H = C3H/HeH, Het = C3H/HeH;C57BL/6J, B6J = C57BL/6J.

3.2 Results

3.2.1 Genetic mapping of *edison*

The critical interval was very large (25.09 Mb), lying between the markers at positions 21.98 Mb and 47.07. This was an extremely gene rich region of the mouse genome, containing 409 genes. To further refine the genetic interval likely to contain the locus, I designed six additional SNP markers evenly spaced across the chromosomal region. This reduced the critical interval to a 9.13 Mb region delineated by rs30778552 and rs46823676, markers at 27.4 Mb and 36.53 Mb respectively (Table 3.1). The mutation showed a 100% linkage to rs31536769 at 30.94 Mb. No more informative SNP markers between C57BL/6J and C3H/HeH were identified in the region; hence further narrowing of this critical interval was not possible. This 9.13 Mb region on Chromosome 14 of the mouse genome was still gene rich, containing 119 genes. Of the 119 genes, there were no obvious candidate genes to select for gene sequencing.

Table 3.1| Further fine mapping of Chromosome 14 in *edison* mice.

Grouped into affected and unaffected mice according to hearing phenotype. Click box (CB) data; 2 = normal, 1 = reduced startle response, 0 = no startle response. C3H = C3H/HeH, Het = C3H/HeH;C57B6/J, B6J = C57B6/J.

	SNP location (Mb)	11.02	16.48	21.98	25.41	26.37	27.40	30.94	36.53	47.07	CB phenotype
	C3H	T	T	A	A	A	G	T	G	C	
	B6J	A	A	G	T	C	A	C	A	A	
Homozygous	EDSN/49.8b	B6J	B6J	B6J	B6J	B6J	B6J	B6J	C3H	C3H	0
	EDSN/44.1a	Het	B6J	Het	B6J	B6J	B6J	B6J	B6J	C3H	0
	EDSN/54.2a	Het	Het	Het	Het	Het	Het	B6J	Het	C3H	1
	EDSN/56.2f	Het	Het	Het	Het	Het	B6J	B6J	B6J	C3H	1
	EDSN/57.1a	Het	Het	Het	C3H	C3H	B6J	B6J	C3H	C3H	0
	EDSN/57.2e	C3H	C3H	C3H	C3H	C3H	B6J	B6J	Het	C3H	1
	EDSN/58.1e	C3H	C3H	C3H	Het	B6J	B6J	B6J	B6J	C3H	1
	EDSN/58.2d	Het	Het	Het	Het	B6J	B6J	B6J	Het	C3H	0
	EDSN/81.1d	C3H	C3H	Het	Het	Het	Het	B6J	C3H	C3H	1
EDSN/81.5d	Het	Het	Het	Het	Het	Het	B6J	C3H	C3H	1	
Heterozygous	EDSN/50.4a	Het	B6J	Het	C3H	Het	Het	Het	Het	C3H	2
	EDSN/50.7f	Het	Het	Het	Het	Het	Het	Het	Het	C3H	2
	EDSN/57.2a	C3H	C3H	C3H	C3H	C3H	Het	Het	Het	C3H	2
	EDSN/60.1a	C3H	C3H	Het	C3H	C3H	Het	Het	C3H	C3H	2
	EDSN/91.4f	C3H	C3H	C3H	C3H	C3H	C3H	Het	C3H	C3H	2
Wild-type	EDSN/50.7c	Het	Het	C3H	C3H	C3H	C3H	C3H	Het	C3H	2
	EDSN/50.7e	Het	Het	C3H	C3H	C3H	C3H	C3H	C3H	C3H	2
	EDSN/54.2b	C3H	Het	C3H	C3H	C3H	C3H	C3H	C3H	C3H	2
	EDSN/74.3c	C3H	C3H	Het	C3H	C3H	C3H	C3H	C3H	C3H	2
	EDSN/86.3a	C3H	C3H	C3H	C3H	C3H	C3H	C3H	C3H	C3H	2

3.2.2 Identification of mutation in *Nischarin*

In combination with high density SNP analysis, next-generation sequencing (NGS) was utilised to accelerate the process of mutation detection. Using the Illumina Genome Analyser IIX platform, the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics carried out NGS with genomic DNA from two G_{12} *edison* mice homozygous in the critical interval. Identification, analysis and dissemination of sequence variant information identified through NGS were achieved with the tools provided by the MRC Harwell Biocomputing custom sequence analysis pipeline (Figure 3.3). The pipeline is used to align reads to the C3H/HeH reference sequence, automatically identify unique variants, populate a custom sequence database and identify low, medium and high confidence single nucleotide variants (SNVs).

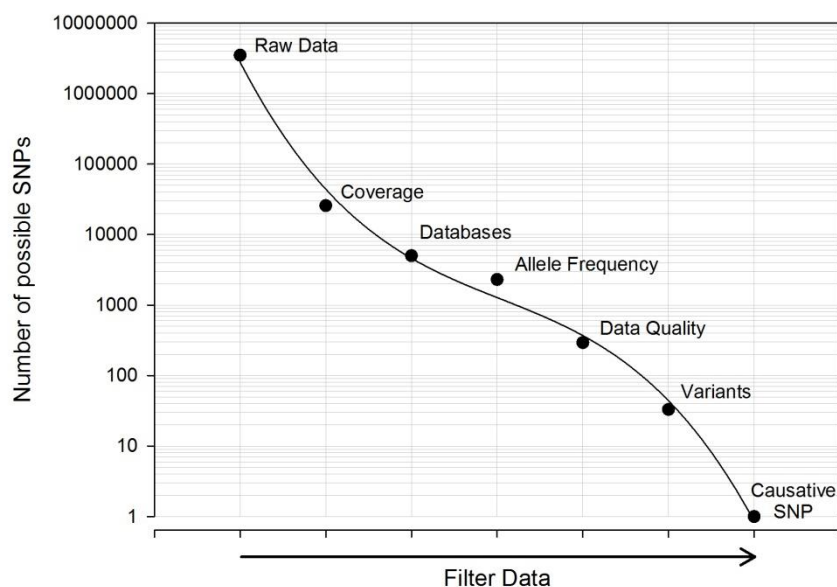


Figure 3.3| Bioinformatics pipeline to filter whole genome next-generation sequencing (NGS) data for identifying possible putative variants in *edison*.

From the raw data, reads are aligned to the C3H/HeH reference sequence. Data from the critical region is then filtered, to identify unique variants, by comparing against all known SNPs in NCBI and 17 genomes database and all known inbred strain SNPs. It is then filtered by allele frequency, with homozygotes being classed as having an allele frequency greater than 66%. Data quality (Phred) and read depth is used to identify low and high confidence SNVs. These unique variants are then analysed to find the putative SNV in the mutant.

Within the critical region, the NGS dataset had a mean read depth of 8.17 and the read coverage was 99.64%. The gaps in the read coverage were located within intronic and intergenic regions of the genome only, 0.19% and 0.17% respectively. After applying the filtering strategy, used to detect *de novo* ENU-induced mutations, sixty SNVs were identified in the 9.13 Mb critical interval in *edison* (Table 3.2). Thirty five low, fifteen medium and ten high confidence SNVs. Considering the high confidence SNVs; five were defined as intergenic (more than 5 kb either upstream or downstream of a transcript), three were intronic (located in the intron of a gene), one was classified as up/downstream (within 5 kb up/downstream of the 5'/3' end of a transcript), and importantly only one was identified as non-synonymous coding (changes a coding sequence such that an amino acid change occurs in the encoded protein sequence). No other non-synonymous sequence changes were identified

within the critical interval, with 82% (49/60) of the SNVs identified classified as intergenic or intronic.

Table 3.2| Possible ENU-induced single nucleotide variants (SNVs) located in 9.13 Mb critical interval identified by NGS.

NGS annotation definitions are listed in the Appendix (Table 10.1). UTR, untranslated region.

Functional class	High	Medium	Low	Total
3'/5' UTR	0	0	2	2
Intergenic	5	6	10	21
Intronic	3	7	18	28
Non-synonymous coding	1	0	0	1
Synonymous coding	0	0	1	1
Upstream/Downstream	1	1	3	5
Within non-coding region	0	1	1	2
Total	10	15	35	60

From the SNVs detected from the NGS dataset, the one non-synonymous variant identified was a 3079T>C substitution (open reading frame of NCBI RefSeq transcript NM_022656.2) in exon 16 of *Nischarin* (*Nisch*), causing a Leu972Pro mutation. To verify this variant, I designed a new primer pair to sequence part of exon 16 from genomic DNA. This again showed a single nucleotide substitution, T to C, causing a Leu972Pro mutation. DNA from mice that were wild-type (C3H/HeH;C3H/HeH), heterozygous (C3H/HeH;C57BL/6J) and homozygous (C57BL/6J;C57BL/6J) at rs31536769 showed complete association with the 3079T>C substitution identified (Figure 3.4A). The parental strains (C57BL/6J and C3H/HeH) were also sequenced in this region and all strains tested had the 'T' nucleotide. This provided evidence that the identified change was unique to the *edison* mutant. Furthermore, the *edison* mice are genotyped directly using the SNP and the phenotype and the genotype show a 100% correlation.

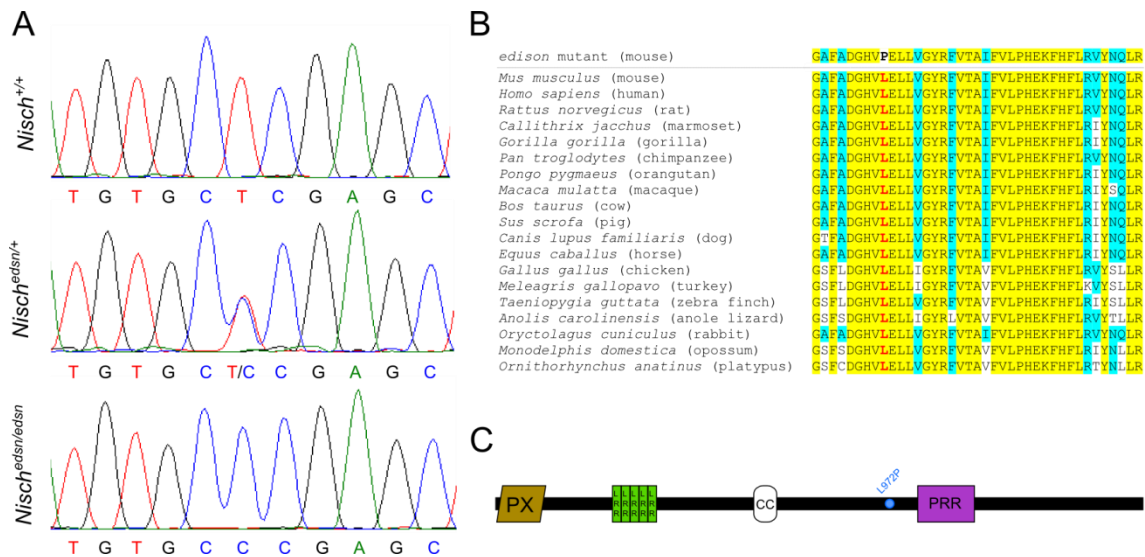


Figure 3.4| Confirmation and location of putative mutation in *Nischarin* (*Nisch*).

Putative mutation in *edison* mice was discovered to be a c.3079T>C, resulting in a non-synonymous amino acid change, p.972L>P. **(A)** Sanger sequencing data confirms the mutation is present across *edison* genotypes. **(B)** Evolutionary conservation of the leucine amino acid residue, found to be changed in *edison* mice, among 19 amniota vertebrates. Yellow indicates positions which have a single, fully conserved residue across all species listed. Blue indicates positions where the consensus has a conserved residue. **(C)** Protein structure of NISCH showing a phox-homology (PX) domain, five leucine-rich repeats (LRR), a coiled-coil (CC) domain and a proline-rich region (PRR). *edison* has an amino acid substitution, L972P, between the CC domain and PRR.

Pecan multiple alignment analysis was used to observe the evolutionary conservation of NISCH among 19 amniota vertebrates. NISCH is a highly conserved protein across mammalian species and the leucine residue mutated in *edison* lies within a block of amino acids highly conserved throughout evolution (Figure 3.4B). When the mutation found in *edison* was annotated onto the Nischarin protein structure, interestingly it did not reside within any known domains. The amino acid substitution, L972P, was located between the coiled-coil (CC) domain and the proline-rich region (PRR) of Nischarin (Figure 3.4C). Computational analysis using the DomPred Protein Domain Prediction Server showed that the amino acid change observed would result in a significant change in the predicted structures of wild-type

NISCH and *edison* NISCH (Appendix, Figure 10.2). In addition, *in silico* prediction software is available to evaluate the effects of single amino acid substitutions on protein function. Three *in silico* prediction tools widely used in publications for predicting effects on protein function are SIFT (Sorting Intolerant From Tolerant), SNAP (Screening for Non-Acceptable Polymorphisms), and PROVEAN (Protein Variation Effect Analyzer). These three tools were utilised to predict whether the L972P substitution found in *edison* would have an impact on the biological function of a NISCH. SIFT analysis predicted that the L972P substitution was “not tolerated”, SNAP predicted that it was “non-neutral”, and PROVEAN predicted that it was “deleterious” (Table 3.3). All three predicted that an L972P substitution in NISCH would have a significant impact on its biological function.

Table 3.3| NISCH Leu972Pro amino acid substitution in *edison* predicted to affect protein function.

Three universally used *in silico* prediction tools were used to predict whether the L972P amino acid substitution has an impact on the biological function of NISCH. SIFT (Sorting Intolerant From Tolerant); SNAP (Screening for Non-Acceptable Polymorphisms); PROVEAN (Protein Variation Effect Analyzer).

Sequence variant	SIFT	Score	SNAP	Score	PROVEAN	Score
Leu972Pro	Not tolerated	0.00	Non-neutral	-	Deleterious	-3.567

3.3 Discussion

3.1.4 Summary

The genetic insults responsible for many human diseases remain largely unknown. The use of mouse models in studying genetic disease provides a valuable resource for understanding the aetiology and molecular mechanisms contributing to such congenital defects. Large-scale, phenotype-driven ENU mutagenesis screens are useful for identifying novel genes and pathways that contribute to disease. The ENU-induced mouse model, *edison*, was discovered in a recessive G₃ mutagenesis screen at MRC Harwell and displays a reduced startle response. A novel allele associated with this hearing phenotype has been identified.

3.3.1 *edison* carries a mutation in *Nisch*

Through SNP-based genetic linkage analysis, the *edison* mutation was mapped to a 9.13 Mb critical interval on Chromosome 14. The traditional method to identify the putative mutation involves additional breeding for recombination mapping and further narrowing of the interval within which to select candidates for Sanger sequencing. This method can sometimes be labour intensive and costly, therefore whole genome sequencing was implemented to eliminate the need to perform further breeding and reduce the time taken to identify the putative causal mutation in *edison*. Within the 9.13 Mb critical interval, the NGS dataset had a read coverage of 99.64%, with 100% coverage in exonic regions. There was a very small percentage of sequencing gaps in the intronic (0.19%) and intergenic (0.17%) regions of the critical interval where mutations could have been missed. However, to date the majority of causative ENU-induced mutations have been shown to be located in exonic regions of the genome (Justice, Noveroske et al. 1999). Of the SNVs detected by NGS analysis, only one missense mutation was identified in the critical interval and it was located in *Nisch*. The mutation in exon 16 of *Nisch*, gave rise to a non-

synonymous Leu972Pro substitution. The leucine 972 residue in NISCH is highly conserved in amniota vertebrate species from platypus to human and its substitution to proline was predicted to have a biological impact on the protein function. Sanger sequencing confirmed the presence of this putative mutation and its complete association with the hearing deficit in *edison* mice.

3.3.2 Nischarin is a novel intracellular protein with many binding partners

NISCH is a highly conserved protein across mammalian species, however it lacks significant homology with any known protein with a well-described function. NISCH is a large protein of 1593 amino acids, consisting of an N-terminal phox homology (PX) domain, five putative leucine-rich repeats (LRRs), a predicted coiled-coil (CC) domain, a proline-rich region (PRR) and a long C-terminal region. The PX domain, found most commonly among sorting nexins, has been shown to mediate phosphoinositide binding (Xu, Seet et al. 2001). PX domain-containing proteins demonstrate central functions as both cargo adaptors in cell trafficking and scaffolds regulating signal transduction (Teasdale and Collins 2012). The primary function of LRRs is not known and might be for the provision of a versatile structural framework for the formation of protein-protein interactions (Kobe and Kajava 2001). The CC structural motif mediates subunit oligomerization for a wide variety of proteins (Burkhard, Stetefeld et al. 2001). CC domains can interact with each other to form homotypic oligomers, or with other CC domains to form heterotypic oligomers. PRRs are likely to have binding as a major function (Williamson 1994). These regions can bind rapidly and non-specifically to a range of other proteins, acting as 'sticky arms'.

In Ensembl Genome Browser (Genome assembly: GRCm38), *Nisch* was predicted to have twenty three transcripts (splice variants), of which twelve were classified as protein coding. Three of these transcripts contain leucine 972 which is substituted to

proline in *edison* mice. ENSMUST00000022469 produces the full length protein described above; ENSMUST00000168206 is predicted to produce an isoform of 1348 amino acids with a shortened N-terminal, hence no PX domain (first 245 amino acids removed); and ENSMUST00000163552 is predicted to produce an isoform of 242 amino acids containing part of exon 16 and the PRR. The mouse and human homologue of NISCH are highly conserved throughout, with 88% homology between protein coding (Figure 3.5). Less homology is observed in the PRR of each homologue.

The mouse homologue of Nischarin was first discovered in a yeast two-hybrid screen to detect novel proteins that interact with integrins and was identified as a novel intracellular protein that preferentially binds to the cytoplasmic domain of the integrin $\alpha 5$ (ITGA5) subunit (Alahari, Lee et al. 2000). NISCH not only interacts with the ITGA5 subunit, but also with PAK1 (p21-activated kinase 1) (Alahari, Reddig et al. 2004), LIMK1 (LIM domain kinase 1) (Ding, Milosavljevic et al. 2008), Rac1 (Ras-related C3 botulinum toxin substrate 1) (Reddig, Xu et al. 2005; Kuijl, Pilli et al. 2013), Rab14 (Kuijl, Pilli et al. 2013) and LKB1 (liver kinase B1) (Jain, Baranwal et al. 2013). The amino acids identified as crucial for all of these interactions are summarised in Figure 3.6. The various interacting partners predominantly bind to amino acids 246-1047 of NISCH. However, recent studies have shown that some interacting partners bind specifically to the carboxyl-terminus (1190–1593 aa). It is not clear how so many proteins bind to the same region; hence it is thought NISCH may function as a scaffold protein. Tissue distribution of NISCH mRNA in the adult mouse shows a ubiquitous expression pattern and analysis of a mouse embryo RNA blot indicated the presence of NISCH mRNA as early as 7 days of development (Alahari, Lee et al. 2000). Similarly, in the adult rat a ubiquitous expression pattern for NISCH mRNA and protein is also observed (Ding, Zhang et al. 2013).

Human	-M A T A R T F G P E R E A E P A K E A R V V G S E L V D T Y T V Y I I Q V T D G S H E W T V K H R Y S D F H D L H E K L V A E R K I D K N L L P P K K	75
Mouse	M A A A T L S F G P E R E A E P A K E A R V V G S E L V D T Y T V Y I I Q V T D G N H E W T I K H R Y S D F H D L H E K L V A E R K I D K S L L P P K K	76
Human	I I G K N S R S L V E K R E K D L E V Y L Q K L L A A F F G V T P R V L A H F L H F H F Y E I N G I T A A L A E E L F E K G E Q L L G A G E V F A I G	150
Mouse	I I G K N S R S L V E K R E K D L E V Y L Q T L L T T F P D V A P R V L A H F L H F H L Y E V N G V T A A L A E E L F E K G E Q L L G A G E V F A I R	151
Human	P L Q L Y A V T E Q L Q Q G K P T C A S G D A K T D L G H I L D F T C R L K Y L K V S G T E G P F G T S N I Q E Q L L P F D L S I F K S L H Q V E I S	225
Mouse	P L Q L Y A T T E Q L Q Q G K P T C A S G D A K T D L G H I L D F T C R L K Y L K V S G T E G P F G T S N I K E Q L L P F D L S I F K S L H Q V E I S	226
Human	H C D A K H I R G L V A S K P T L A T M S V R F S A T S M K E V L V P E A S E F D E W E P E G - T T L E G P V T A V I P T W Q A L T T L D L S H N S I	299
Mouse	H C D A K H I R G L V T S K P T L A T M S V R F S A T S M K E V L A P E A S E F D E W E P E G T A T L G G P V T A I I P T W Q A L T T L D L S H N S I	301
Human	S E I D E S V K L I P K I E F L D L S H N G L L V V D N L Q H L Y N L V H L D L S Y N K L S S L E G L H T K L G N I K T L N L A G N L L E S L S G L H	374
Mouse	C E I D E S V K L I P K I E Y L D L S H N G L R V V D N L Q H L Y N L V H L D L S Y N K L S S L E G V H T K L G N V K T L N L A G N L E S L S G L H	376
Human	K L Y S L V N I D L R D N R I E Q M E E V R S I G S L P C L E H V S L L N P L S I I P D Y R T K V L A Q F G E R A S E V C L D D T V T T E K E L D T	449
Mouse	K L Y S L V N I D L R D N R I E Q L D E V K S I G S L P C L E R L T L L N P L S I I P D Y R T K V L S Q F G E R A S E I C L D D V A T T E K E L D T	451
Human	V E V L K A I Q K A K V K S K L S N P E K K G G E D S R L S A A P C I R F S S S P P T V A P A S A S L P Q P I L S N Q G I M F V Q E E A L S S L S	524
Mouse	V E V L K A I Q K A K V K S K L S N T E K K A G E D F R L P P A P C I R F G G S P P - A A P A S A S L P Q P I L S N Q G I M F V Q E E A L S S L S	525
Human	S T D S L T P - F H Q P I A Q C S D S L E S I P A G Q A S D D L R D V P G A V G G A S E F H A E P E V Q V V P G S G Q I I F L P F T C I G Y T A T	598
Mouse	S T D S L P P E F H R P I A R C S D S L E S I P A G Q V A S D D L R D V P G A V G G V S F D H A E P E V Q V V P G S G Q I I F L P F T C I G Y T A T	600
Human	N Q D F I Q R L S T L I R Q A I E R Q L P A W I E A A N Q R E E G Q G E Q G E E D E E E E E E D V A E N R Y F E M G P P D V E E E G G Q G E E	673
Mouse	N Q D F I Q R L S T L I R Q A I E R Q L P A W I E A A N Q R E E A H G E Q G E E E - E E E E E E D V A E N R Y F E M G P P D A E E E G S G Q G E E	674
Human	E E E E E D E E A E E E R L A L E W A L G A D E D F L E H I R I L K V L W C F L I H V Q G S I R Q F A A C L V L T D F G I A V F E I P H Q E S R G	748
Mouse	E E E E E - D E E A E E E R L A L E W A L G A D E D F L E H I R I L K V L W C F L I H V Q G S I R Q F A A C L V L T D F G I A V F E I P H Q E S R G	748
Human	S S Q H I L S S L R F V F C F P H G D L T E F G F L M P E L C L V L K V R H S E N T L F I I S D A A N L H E F H A D L R S C F A P Q H M A M L C S P I	823
Mouse	S S Q H I L S S L R F V F C F P H G D L T E F G F L M P E L C L V L K V R H S E N T L F I I S D A A N L H E F H A D L R S C F A P Q H M A M L C S P I	823
Human	L Y G S H T S L Q E F L R Q L L T F Y K V A G G C Q E R S Q G C F P V Y L V Y S D K R M V Q T A A G D Y S G N I E W A S C T L C S A V R R S C C A P S	898
Mouse	L Y G S H T T L Q E F L R Q L L T F Y K V A G G S Q E R S Q G C F P V Y L V Y S D K R M V Q T P A G D Y S G N I E W A S C T L C S A V R R S C C A P S	898
Human	E A V K S A A I P Y W L L L T P Q H L N V I K A D F N P M P N R G T H N C R N R N S F K L S R V P L S T V L L D P T R S C T Q P R G A F A D G H V L E	973
Mouse	E A V K S A A I P Y W L L L T S Q H L N V I K A D F N P M P N R G T H N C R N R N S F K L S R V P L S T V L L D P T R S C T Q P R G A F A D G H V L E	973
Human	L L V G Y R F V T A I F V L P H E K F H F L R V N Q L R A S L Q D L K T V V I A K T P -	1017
Mouse	L L V G Y R F V T A I F V L P H E K F H F L R V N Q L R A S L Q D L K T V V I S K N P S A K P R N Q P A K S R A S A E Q R L Q E T P A D A P A P A A	1048
Human	- -	1062
Mouse	V P P T A S A P A P A E A L A P D L A P V Q A P G E D R G L T S A E A P A A A E A P A A A E A P A A A E A P A A A E A P A A A E A P A A A	1123
Human	P A A A S A S G P A K I P A P A E A S T S A L V P E E T P V E A P A P - P P A E A P A Q Y P S E	1109
Mouse	E A P A A A E A P A A E A P A A A E A P A A A E A P A S A E A P A P N Q A P A P A R G P A P A R G P A P A G G P A P A E A L A Q A E V P A Q Y P S E	1198
Human	S L I Q T S E E N Q I P S H L P A C P S L R H V A S L R G S A I I E L F H S S I A E V E N E E L R H L W S S V V F Y Q T P G L E V T A C V L L S T	1184
Mouse	S L I Q T S E E N Q I P S H L P V C P S L Q H I A R L R G R A I I D L F H N S I A E V E N E E L R H L W S S V V F Y Q T P G L E V T A C V L L S S	1273
Human	K A V Y F V L H D G L R R Y F S E P L Q D F W H Q K N T D Y N N S P F F H S Q C F V L K L S D L Q S V N V G L F D Q H F R L T G S T P M Q V V T C L T	1259
Mouse	K A V Y F I L H D G L R R Y F S E P L Q D F W H Q K N T D Y N N S P F F H S Q C F V L K L S D L Q S V N V G L F D Q Y F R L T G S S P T Q V V T C L T	1348
Human	R D S Y L T H C F L Q H L M V L S S L E R T P S P E P V D K D F Y S E F G N K T T G K M E N Y E L I H S S R V K F T Y P S E E E T G D L T F T V A Q	1334
Mouse	R D S Y L T H C F L Q H L M V L S S L E R T P S P E P V D K D F Y S E F G D K N T G K M E N Y E L I H S S R V K F T Y P S E E E T G D L T Y T V A Q	1432
Human	K M A P E K A P A L S I L L Y I Q A F Q V V T E H L G R G R G L R P K T L L L T S S E I F L L D E D C V H Y P L P E F A K E P P Q R D R Y R L D D	1409
Mouse	K M A P A K N P A L S I L L Y I Q A F Q V V T E H L G R G R G L R P K T L L L T S S E I F L L D E D Y I H Y P L P E F A K E P P Q R D R Y R L D D	1498
Human	G R R V R D L D R V L M G Y Q T Y P Q A L T L V F D D V Q G H D L M G S V T L D H F G E V P G G P A R A S Q G R E V Q W Q V F V P S A E S R E K L I S	1484
Mouse	G R R V R D L D R V L M G Y Y F Y P Q A L T L V F D D T Q G H D L M G S V T L D H F G E V P G G P R V G Q G R E V Q W Q V F V P S A E S R E K L I S	1573
Human	L L A R Q W E A L C G R E L P V E L T G	1504
Mouse	L L A R Q W E A L C G R E L P V E L T G	1593

Figure 3.5| Human and mouse alignment of NISCH peptide sequence.

The two protein sequences are highly conserved throughout (88% Homology between protein coding, ENSP00000339958 and ENSMUSP00000022469). Yellow indicates positions that have a single, fully conserved residue. Green indicates conservation between groups of strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix) and blue indicates conservation between groups of weakly similar properties (scoring ≤ 0.5 in the Gonnet PAM 250 matrix). Black text indicates non-similar amino acid residues.

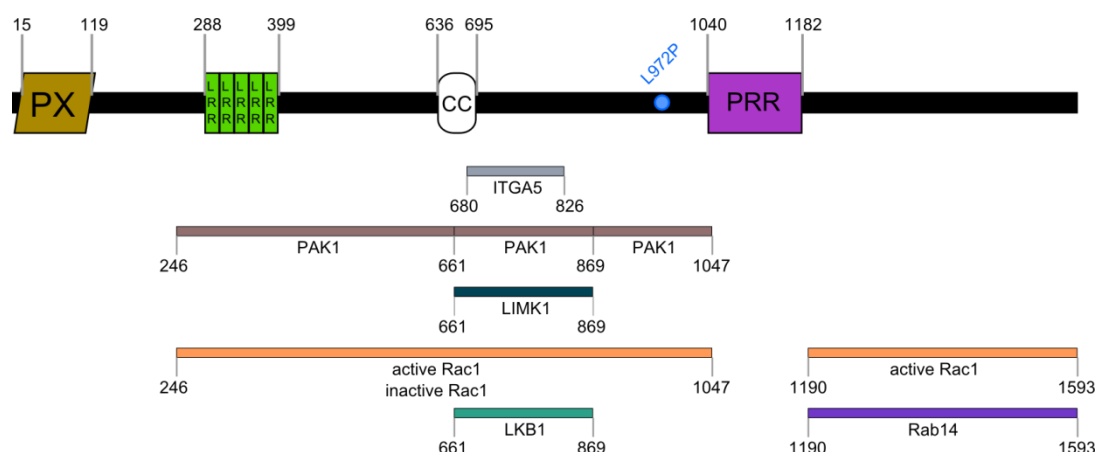


Figure 3.6| Summary of NISCH structure and known binding partners.

Protein structure of NISCH showing a phox-homology (PX) domain, five leucine-rich repeats (LRR), a coiled-coil (CC) domain and a proline-rich region (PRR). ITGA5, PAK1, LIMK1, Rac1, Rab14 and LKB1 have all been identified as NISCH interacting proteins. Schematic shows the region of NISCH that is sufficient for interaction with each protein to occur. Numbers indicate corresponding amino acid residue. ITGA5, integrin $\alpha 5$; PAK1, p21-activated kinase 1; LIMK1, LIM domain kinase 1; Rac1, Ras-related C3 botulinum toxin substrate 1; Rab14, member RAS oncogene family; LKB1, liver kinase B1 or Serine/threonine kinase 11 (STK11).

As mentioned above, NISCH was originally identified as a binding partner for ITGA5 and therefore the fibronectin receptor, $\alpha 5\beta 1$ integrin. Overexpression of NISCH markedly reduces $\alpha 5\beta 1$ -dependent cell migration (Alahari, Lee et al. 2000). Interestingly, neither the minimal ITGA5 binding region (680-826 aa) nor the full amino acid sequence between 246-1047 aa of NISCH is sufficient for inhibition of cell migration on its own, and full length NISCH is required (Alahari, Lee et al. 2000; Alahari, Reddig et al. 2004). This indicates that Nischarin's ability to inhibit cell migration is not exclusively through binding to integrins and blocking their function. NISCH also has been shown to selectively inhibit Rac1 driven signalling cascades that affect migration through PAK1 (Alahari 2003). However, in addition to regulating the PAK1 pathway of RAC1 signaling, NISCH can also regulate PAK1-independent RAC1 signal transduction (Reddig, Xu et al. 2005). Interaction with NISCH strongly inhibits the ability of PAK1 to phosphorylate its substrates (Alahari, Reddig et al. 2004). Two distinct regions of NISCH interact with Rac1 in its activated state; the region between 246-1047 aa and the carboxyl-terminus of NISCH (1190-1593 aa) (Reddig,

Xu et al. 2005). This interaction represses Rac1 signal transduction pathways. LIMK1 is a downstream effector of PAK1. Similarly, NISCH has been shown to be an interacting partner with LIMK, binding specifically to both the PDZ domain and the kinase domain (Ding, Milosavljevic et al. 2008). This association with NISCH regulates cell invasion through negative modulation of the LIMK-Cofilin pathway. More recently, a functional interaction between LKB1 and NISCH has been shown to regulate PAK-LIMK-Cofilin pathways to inhibit cell migration (Jain, Baranwal et al. 2013).

The human homologue of NISCH was first isolated through a screen to clone an imidazoline-1 receptor and was originally named IRAS (Imidazoline receptor antisera-selected) (Piletz, Ivanov et al. 2000). In the human homologue, a combined action of PX domain-mediated interaction with phosphatidylinositol 3-phosphate (PI(3)P) and homo-oligomerization mediated by its coiled-coil region has been shown to be necessary for association of NISCH with PI(3)P-enriched endosomal membranes (Lim and Hong 2004). Elevated levels of NISCH were shown to mediate the translocation of ITGA5 from the cell membrane to endosomes. Furthermore, the mouse homologue has recently been shown to interact with PI(3)P, along with Rac1 and Rab14, and play an important role in the maturation of endosomes (Kuijl, Pilli et al. 2013). Depletion of NISCH, Rac1 or Rab14 reduced this vesicle maturation.

Association of NISCH with this multitude of interacting partners has been shown to play a key role in regulating cell motility, cell invasion, vesicle maturation (Figure 3.7), and has also been identified as a tumour suppressor (Alahari, Lee et al. 2000; Ding, Milosavljevic et al. 2008; Baranwal, Wang et al. 2011; Kuijl, Pilli et al. 2013). The mutation in NISCH is in a highly conserved region and the L972P substitution was predicted to impact upon the proteins biological function. This would likely affect interaction, of some or all, of the many interacting partners of NISCH. Therefore, the

edison mutation could drive downstream signalling causing the *edison* phenotype (see Chapter 6).

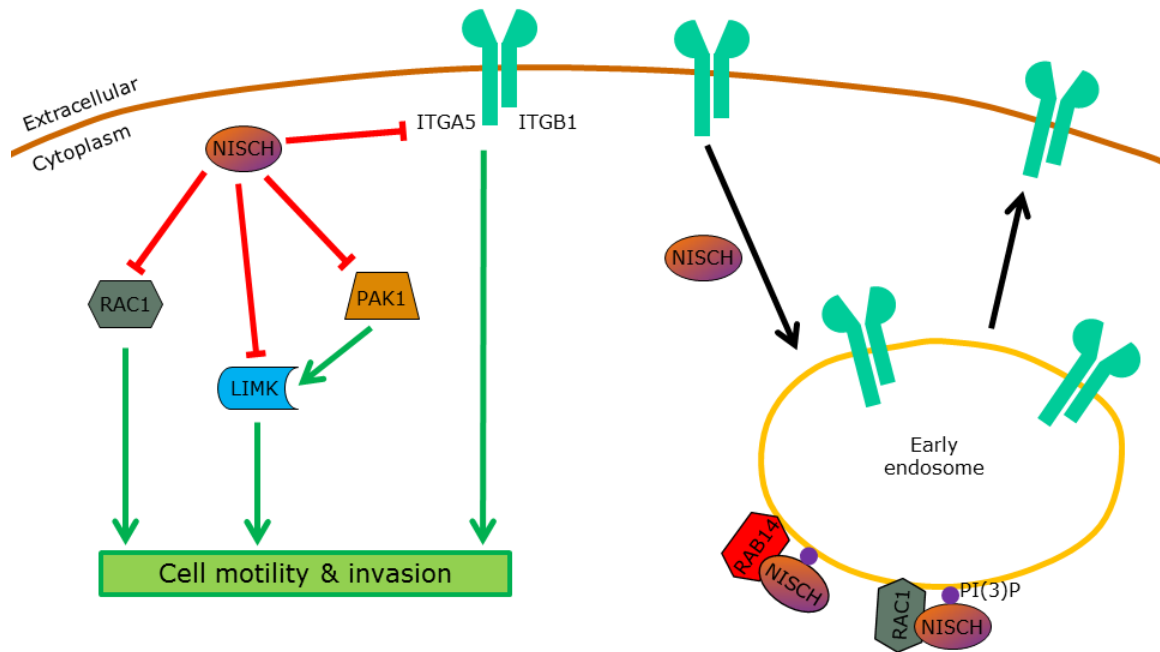


Figure 3.7| Summary of NISCH molecular functions and role in biological processes.

NISCH has been shown to play a key role in regulating cell motility and cell invasion, through interactions with ITGA5, PAK1, RAC1 and LIMK1. Interaction of NISCH with PI(3)P, along with RAC1 and RAB14, plays an important role in the maturation of endosomes. Regulation of ITGA5 is thought to act through the redistribution of surface ITGA5 to intracellular endosomal structures. ITGA5, integrin $\alpha 5$; ITGB1, integrin $\beta 1$; PAK1, p21-activated kinase 1; LIMK1, LIM domain kinase 1; RAC1, Ras-related C3 botulinum toxin substrate 1; RAB14, member RAS oncogene family; PI(3)P, phosphatidylinositol 3-phosphate.

3.3.3 Conclusion

The associations between phenotype and genotype, which can be identified without prior gene bias using ENU mutagenesis, are invaluable in providing insight into the mechanisms that underlie normal development and human congenital disease. Having identified the putative gene as *Nisch*, elucidating in detail the molecular mechanisms by which the ENU-induced mutant, *edison*, causes a reduced startle response will contribute to our understanding of the pathogenic processes involved in hearing loss.

CHAPTER 4: Pathobiology of the *edison* mutant

4.1 Introduction

This chapter focuses on the phenotypic characterisation of OM development in the ENU-induced *Nischarin* mutant, *edison*, congenic on the C3H/HeH background. I started working on the *edison* mutant after some initial preliminary characterisation had been undertaken by Rachel Hardisty-Hughes, Greg Ball and Richard Gale, as members of the Molecular Genetics and Pathobiology of Deafness group at MRC Harwell (summarised below). Comprehensive phenotypic characterisation revealed the *edison* mutant spontaneously displayed a progressive COM with good resemblance to the human disease. In addition to ME disease, phenotypic characterisation revealed an associated emphysema-like lung phenotype in *edison* mice that displayed a bilateral phenotype (OM in both ears). Characterisation of the pathobiology of *edison* has uncovered another powerful model for studying the genetic and pathophysiological bases of COM.

4.1.1 Preliminary characterisation of *edison*

As the *edison* mutant was discovered in a phenotype-driven ENU-mutagenesis screen, all littermates were put through a phenotyping pipeline based on a modified SHIRPA method, which included; trunk curl, limb grasping, vocalisation, contact righting reflex, reaching response and swim test. As part of the modified SHIRPA mice are also screened for elevated hearing thresholds based upon click-box assessment of the Preyer reflex, a startle response to a loud high frequency noise (90 dB, 20 KHz, 30 cm from the pinna). The founder mice for the *edison* line were discovered from the G₃ pedigree PEDV/7, which produced twelve litters consisting of seventy pups. Fourteen mice were noted as being small in size with a short face, where five of these mice had a reduced startle response (CB = 1) and four exhibited no startle response at all (CB = 0) (Table 4.1). During the swim test, circling was observed in one mouse with an absent startle response, indicating a possible

vestibular defect. All other SHIRPA tests were recorded as a normal wild-type phenotype for all seventy littermates. The proportions of affected mice produced from PEDV/7 suggested the abnormal hearing phenotype was recessive. PEDV/7.1e and PEDV/7.2h were selected as the founder mice to set up the *edison* mutant mouse line and were maintained on C3H/HeH.

Table 4.1| Preliminary SHIRPA phenotyping in affected PEDV/7 littermates.

Fourteen PEDV/7 littermates were small in size and some had a noticeably shortened face (n = 70). Affected mice were found for the startle response and swim test. All other SHIRPA tests showed a normal wild-type phenotype (data not shown). Startle response: 0 = No response; 1 = Preyer reflex only (backwards flick of the pinnae, no startle); 2 = Preyer reflex and startle. Swim test: 0 = swims (mouse body is elongated and the tail propels in a flagella-like motion); 1 = Irregular swimming (Includes: vertical swimming, swimming in a circle, swimming on side). *, mice used to set up EDSN matings.

Mouse ID	Startle response	Swim	Sensory shirpa comments
PEDV/7.1b	0	0	wonky ears
PEDV/7.1e	0	0	small, short face
PEDV/7.1f	1	0	small
PEDV/7.1j	1	0	small
PEDV/7.2h	0	1	small, short face, circling during swim
PEDV/7.5b	2	0	small
PEDV/7.5k	1	0	small, short face
PEDV/7.8a	2	0	small, tail stripes
PEDV/7.9e	0	0	small
PEDV/7.9f	2	0	short face
PEDV/7.11a	1	0	small
PEDV/7.11b	1	0	small
PEDV/7.12a	2	0	small
PEDV/7.12f	2	0	short face

The absence of Preyer reflex may be a good marker of the often severe hearing loss associated with cochlea deformity or malfunction, however because this is a supra-threshold response the test often fails to detect mild or moderate hearing impairment (Jero, Coling et al. 2001). Hearing thresholds may be further assessed using Auditory Brainstem Response (ABR) (Hardisty-Hughes, Parker et al. 2010). Once the *edison* colony had been established, in order to further assess the

reduced/absent startle response observed in the *edison* founders, mice were tested for auditory function by ABR. G_{11} *edison* mice were tested using frequency specific toneburst-evoked ABR tests at 12 weeks of age (wk) (Figure 4.1). Mice were anesthetised and hearing thresholds determined using specific frequencies of 8, 12, 20 and 26 kHz, ranging from 90 dB SPL to 5 dB SPL in decreasing 5 dB increments. ABR results were grouped into affected and unaffected mice based on startle response. There was a hearing deficit observed at each frequency, where affected *edison* mice had a significantly increased threshold ($P < 0.001$ for each frequency).

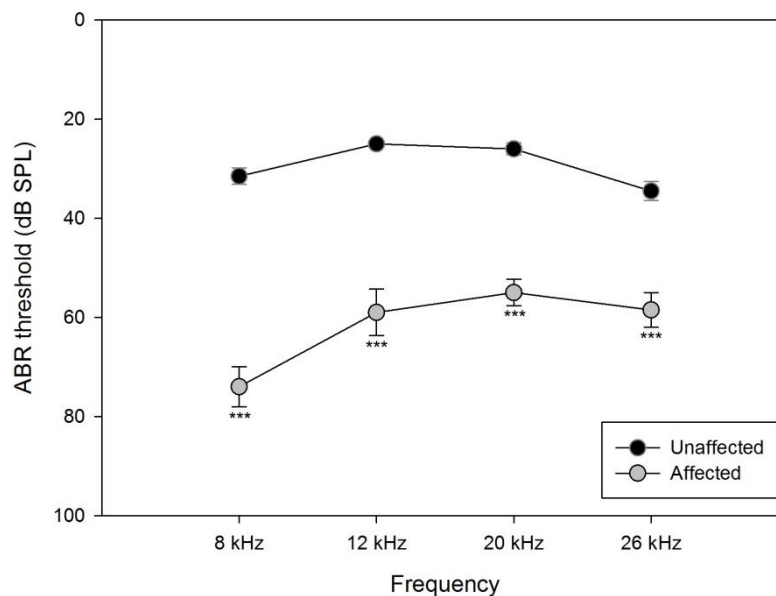


Figure 4.1| Preliminary frequency-specific auditory brainstem response (ABR) thresholds at 12wk for affected *edison* mice.

Wild-type ABR thresholds are shown as data recorded from unaffected littermates. Affected mice have elevated hearing thresholds ("worse hearing"). $n = 10$ for both groups. *** $P < 0.001$. Statistics were conducted using Mann Whitney Rank Sum tests.

4.2 Results

4.2.1 Genotypic ratio analysis reveals reduced numbers of *Nisch*^{edsn/edsn} progeny

The breeding scheme to generate *edison* mice for experimental cohorts is outlined in detail in chapter 2 of this thesis. Both heterozygous (*Nisch*^{edsn/+}) and homozygous (*Nisch*^{edsn/edsn}) *edison* mice were shown to be viable and fertile, however one initial observation was that the expected genotypic ratios from the *edison* crosses were not occurring in the anticipated Mendelian frequency.

4.2.1.1 *Nisch*^{edsn/+} x *Nisch*^{edsn/+} crosses

The observed genotypes from the *Nisch*^{edsn/+} x *Nisch*^{edsn/+} crosses were compared to the expected Mendelian ratios initially at weaning age, and when analysed there were a significantly reduced number of animals with the homozygous *Nisch*^{edsn/edsn} allele ($P = 0.0012$; Table 4.2).

Table 4.2| Numbers of live mice from *Nisch*^{edsn/+} x *Nisch*^{edsn/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	72	54	33.6%	0.0012
<i>Nisch</i> ^{edsn/+}	108	107	50.5%	
<i>Nisch</i> ^{edsn/edsn}	34	54	15.9%	
TOTAL	214			

In order to dissect this difference further, any mice found dead before weaning from these crosses were genotyped and combined with the numbers observed at weaning age to see if expected Mendelian ratios were restored (Table 4.3 and 4.4). It was

found that there was a significant difference between the expected genotypes in the dead pups from the *Nisch*^{edsn/+} x *Nisch*^{edsn/+} crosses ($P = 0.0008$). When the numbers of live mice from *Nisch*^{edsn/+} x *Nisch*^{edsn/+} crosses were combined with the numbers of dead pups, there was still a slightly significant difference between the expected genotypes ($P = 0.0171$) showing that Mendelian ratios were not restored.

Table 4.3| Numbers of dead pups from *Nisch*^{edsn/+} x *Nisch*^{edsn/+} crosses

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	3	9	8.6%	0.0008
<i>Nisch</i> ^{edsn/+}	14	18	40.0%	
<i>Nisch</i> ^{edsn/edsn}	18	9	51.4%	
TOTAL	35			

Table 4.4| Numbers of mice from *Nisch*^{edsn/+} x *Nisch*^{edsn/+} crosses including dead pups

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	83	64	32.3%	0.0171
<i>Nisch</i> ^{edsn/+}	121	129	47.5%	
<i>Nisch</i> ^{edsn/edsn}	52	64	20.2%	
TOTAL	257			

To help visualise and establish when exactly before weaning the lethality of *Nisch*^{edsn/edsn} mice was occurring, a survival plot was made using the Kaplan-Meier Survival Analysis Log-Rank method (Figure 4.2). *Nisch*^{edsn/edsn} mice had a significantly reduced survival rate of 0.63 at weaning ($P = 0.0004$), compared to wild-type and *Nisch*^{edsn/+} littermates with survival rates of 0.89 and 0.87 respectively. The majority of mice across all genotypes were found dead prior to one

week after birth; 11% of wild-type, 10% of *Nisch^{edsn/+}* and 33% of *Nisch^{edsn/edsn}* mice (Figure 4.2B).

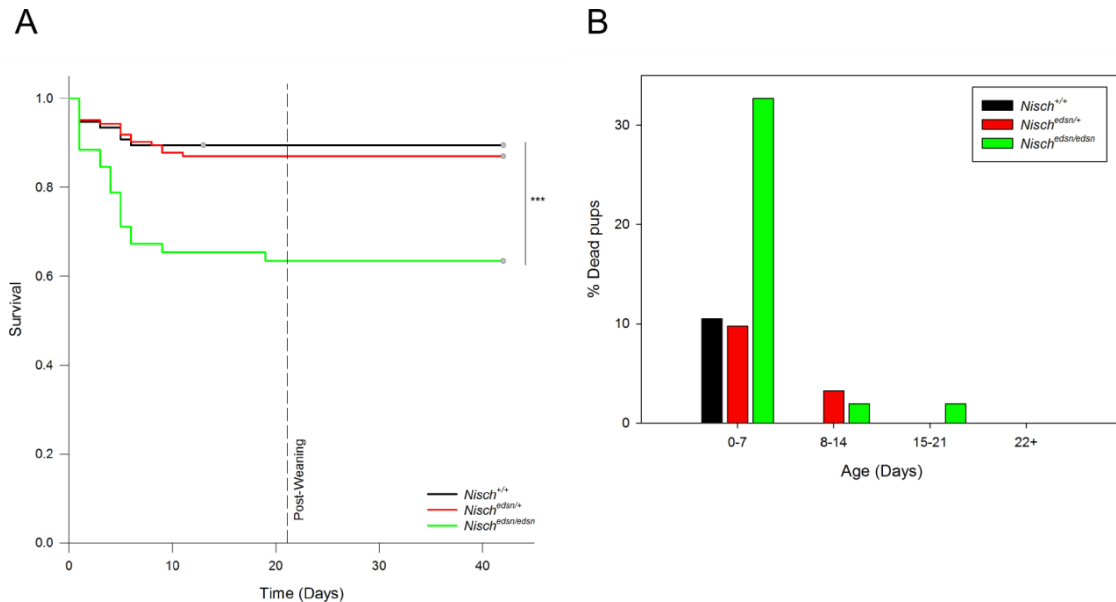


Figure 4.2| Survival plot analysis of mice from *Nisch^{edsn/+}* x *Nisch^{edsn/+}* crosses.

(A) *Nisch^{edsn/edsn}* mice have a reduced survival rate, compared to wild-type and *Nisch^{edsn/+}* littermates. **(B)** The majority of mice across all genotypes are found dead before 7 days of age. *** $P < 0.001$. A Kaplan-Meier Survival Analysis test was performed followed by Holm-Sidak's multiple comparison procedures for post-hoc testing.

Nisch^{+/+} $n = 83$; *Nisch^{edsn/+}* $n = 121$; *Nisch^{edsn/edsn}* $n = 52$.

4.2.1.2 *Nisch^{edsn/+}* x *Nisch^{edsn/edsn}* crosses

Similar observations were made when instead comparing genotypes from *Nisch^{edsn/+}* x *Nisch^{edsn/edsn}* crosses (Table 4.5 – 4.7). From the numbers of live mice observed at weaning age, there were a significantly reduced number of animals with the homozygous *Nisch^{edsn/edsn}* allele ($P < 0.0001$). Between the expected genotypes in the dead pups, from the *Nisch^{edsn/+}* x *Nisch^{edsn/edsn}* crosses, it was found that there was a significant excess of homozygotes ($P = 0.0002$). When the numbers of live mice from *Nisch^{edsn/+}* x *Nisch^{edsn/edsn}* crosses were combined with the numbers of

dead pups, there was still a significant difference between the expected genotypes ($P = 0.0045$) showing again that Mendelian ratios were not restored.

Table 4.5| Numbers of live mice from *Nisch*^{*edsn/+*} x *Nisch*^{*edsn/edsn*} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{<i>edsn/+</i>}	142	106	67.0%	<0.0001
<i>Nisch</i> ^{<i>edsn/edsn</i>}	69	106	33.0%	
TOTAL	212			

Table 4.6| Numbers of dead pups from *Nisch*^{*edsn/+*} x *Nisch*^{*edsn/edsn*} crosses

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{<i>edsn/+</i>}	12	25	24.0%	0.0002
<i>Nisch</i> ^{<i>edsn/edsn</i>}	38	25	76.0%	
TOTAL	50			

Table 4.7| Numbers of live mice from *Nisch*^{*edsn/+*} x *Nisch*^{*edsn/edsn*} crosses including dead pups

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{<i>edsn/+</i>}	154	131	58.8%	0.0045
<i>Nisch</i> ^{<i>edsn/edsn</i>}	108	131	41.2%	
TOTAL	262			

Again, to further investigate when the lethality in *Nisch*^{*edsn/edsn*} mice was occurring, a survival plot was constructed for the *Nisch*^{*edsn/+*} x *Nisch*^{*edsn/edsn*} crosses (Figure 4.3). *Nisch*^{*edsn/edsn*} mice were shown to have a significantly reduced survival rate of 0.64 at weaning ($P < 0.001$), compared to *Nisch*^{*edsn/+*} littermates with a 0.93 survival rate.

The majority of mice across both genotypes were again found dead by one week after birth, 4% of *Nisch^{edsn/+}* and 19% of *Nisch^{edsn/edsn}* mice (Figure 4.3B). Prior to two weeks after birth, 7% of *Nisch^{edsn/+}* mice and 34% of *Nisch^{edsn/edsn}* mice had been found dead.

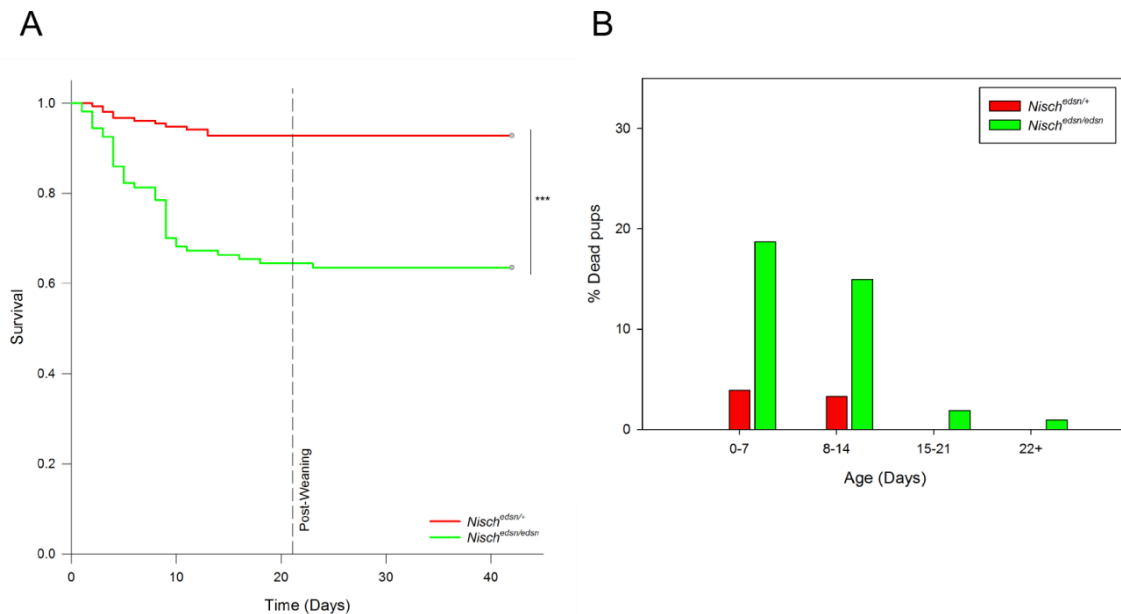


Figure 4.3| Survival plot analysis of mice from *Nisch^{edsn/+}* x *Nisch^{edsn/edsn}* crosses.

(A) *Nisch^{edsn/edsn}* mice have a reduced survival rate, compared to *Nisch^{edsn/+}* littermates. **(B)** The majority of mice across both genotypes are found dead before 7 days of age. *** $P < 0.001$. A Kaplan-Meier Survival Analysis test was performed followed by Holm-Sidak's multiple comparison procedures for post-hoc testing.

Nisch^{edsn/+} $n = 154$; *Nisch^{edsn/edsn}* $n = 108$.

I proceeded to cull litters from *Nisch^{edsn/+}* x *Nisch^{edsn/edsn}* mutant crosses (expected genotypes as described in Table 4.5 – 4.7) before birth at embryonic age 18.5 days post coitum (dpc) (E18.5) to ascertain if mice were found in more appropriate numbers in late gestation. Six litters with a total number of 34 embryos were analysed. 47% of embryos carried the *Nisch^{edsn/edsn}* allele, with 53% of embryos being *Nisch^{edsn/+}* (Table 4.8). This is in contrast to what we see by weaning age, where there are significantly fewer *Nisch^{edsn/edsn}* mice compared to *Nisch^{edsn/+}*.

Table 4.8| Numbers of embryos from *Nisch*^{edsn/+} x *Nisch*^{edsn/edsn} crosses at 18.5 dpc

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{edsn/+}	18	17	52.9%	0.7316
<i>Nisch</i> ^{edsn/edsn}	16	17	47.1%	
TOTAL	34			

4.2.2 *Nisch*^{edsn/edsn} animals display a perinatal lung phenotype

It was of interest to determine whether the observed decrease in *Nisch*^{edsn/edsn} animals at weaning was due to the presence of a cleft palate or any other physiological defect that may contribute to lethality. Cleft palate is observed in the *Fbxo11*^{Jeff} mouse model of OM (Hardisty-Hughes, Tateossian et al. 2006). In order to examine for any physiological defect six litters containing thirty mice were culled at P0 and H&E sections from palate, lung and heart examined. The whole body and brains of these animals were also sectioned and examined. While cleft palate was never observed in *edison* mice of any genotype (Figure 4.4), the lungs between wild-type and some *Nisch*^{edsn/edsn} animals had notable differences (Figure 4.5).

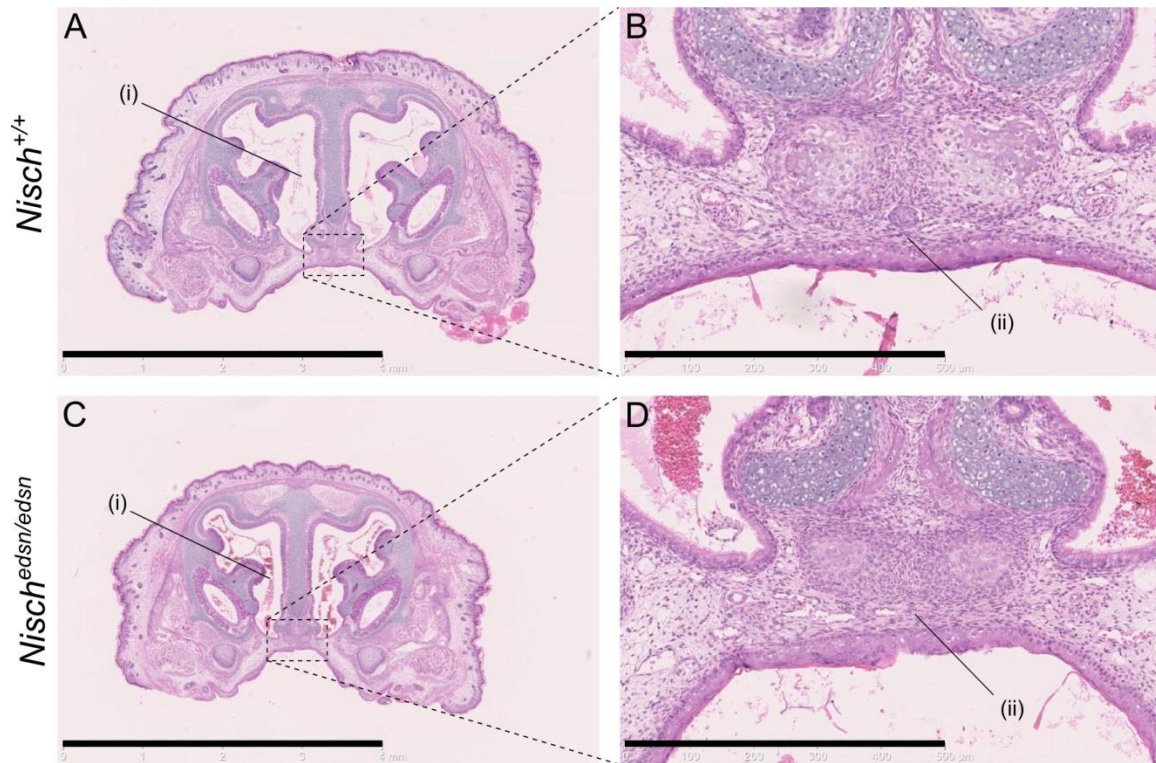


Figure 4.4| Assessment of neonate palatal shelves from *Nisch*^{edsn/edsn} and wild-type animals.

(A) Representative H&E stained coronal head section from a *Nisch*^{+/+} animal showing location of palatal shelves. **(B)** H&E stained coronal head section from a *Nisch*^{+/+} animal examining the fusion of the palatal shelves. **(C)** Representative H&E stained coronal head section from a *Nisch*^{edsn/edsn} animal. **(D)** H&E stained coronal head section from a *Nisch*^{edsn/edsn} animal examining the fusion of the palatal shelves. There are no genotypic differences observed in palate morphology. (i) indicates the nose; (ii) indicates the palatal shelves which fuse together. In cleft palate there is a gap between the palatal shelves. (A & C) Scale bar = 4 mm; (B & D) Scale bar = 500 μm.

Significant differences were seen in only a portion of P0 *Nisch*^{edsn/edsn} animals (Figure 4.5C), I was concerned that the lung inflation method required for neonates was confounding the analysis through its technical difficulty and differences between operators. Additionally, lungs from thirty three E16.5 embryos and twenty three E18.5 embryos were examined, as preparation of embryonic lungs requires no inflation. Again, the lungs between wild-type and some *Nisch*^{edsn/edsn} embryos differed significantly (Figure 4.5A and B). As there was a clear visual difference in the lung phenotype in some *Nisch*^{edsn/edsn} embryos, although an arbitrary decision, they were categorised into two groups; lungs that appeared abnormal compared to wild-type,

Nisch^{edsn/edsn} affected; and lungs indistinguishable from a wild-type phenotype, *Nisch*^{edsn/edsn} unaffected. At E16.5, 33% of *Nisch*^{edsn/edsn} lungs appeared affected (n = 6). This was replicated at E18.5 and P0, whereby 30% (n = 10) and 29% (n = 7) of *Nisch*^{edsn/edsn} lungs appeared affected respectively.

In order to quantify if there were genotypic differences in lung morphology, counts of the number of airways were conducted (Figure 4.6A) and the average airway widths were measured (Figure 4.6B). As only a few *Nisch*^{edsn/edsn} embryos appeared to have a difference in lung morphology, data was categorised into unaffected *Nisch*^{edsn/edsn} and affected *Nisch*^{edsn/edsn} for each time point. Counts of the number of airways showed no significant differences between littermates at any of the time points (E16.5 $P = 0.598$, E18.5 $P = 0.566$ and P0 $P = 0.533$).

At E16.5, measurements of the airway widths showed a significant difference ($P < 0.001$) in the widths between affected *Nisch*^{edsn/edsn} and wild-type mice. The average airway width in affected *Nisch*^{edsn/edsn} animals at E16.5 was 25.13 μm (± 0.901) and for *Nisch*^{+/+} animals was 36.86 μm (± 0.706). No significant difference was observed between unaffected *Nisch*^{edsn/edsn} animals and wild-type littermates ($P = 0.948$), with an average airway width in unaffected *Nisch*^{edsn/edsn} animals of 36.76 μm (± 1.615). These differences between *Nisch*^{edsn/edsn} and wild-type mice were mirrored at E18.5 and P0 ($P < 0.001$ for both time points).

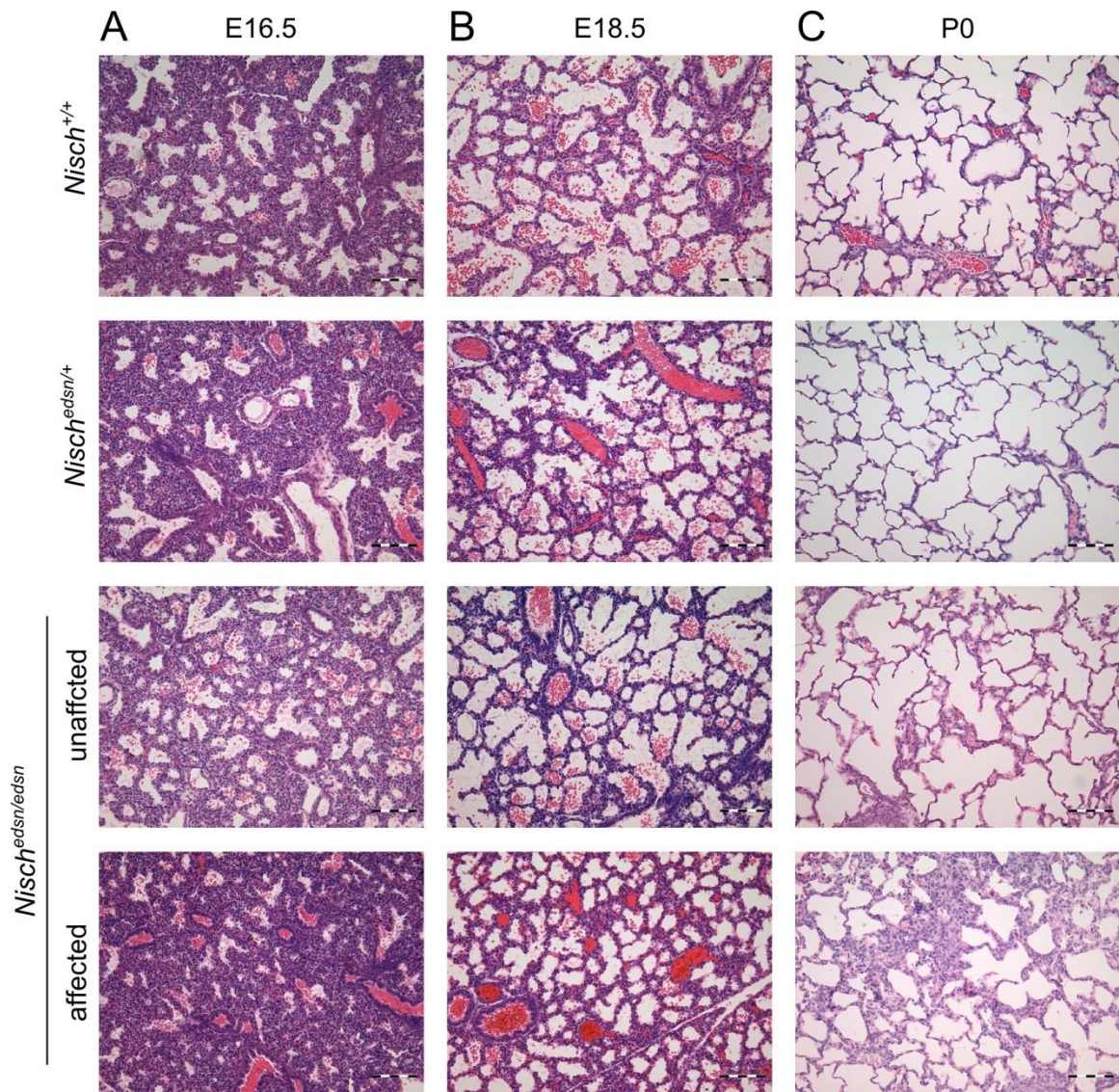


Figure 4.5| Embryo and neonate lung histology from *edison* animals.

Representative H&E stained lung sections at **(A)** E16.5, **(B)** E18.5, and **(C)** P0 time points from *Nisch*^{+/+}, *Nisch*^{edsn/+}, unaffected *Nisch*^{edsn/edsn}, and affected *Nisch*^{edsn/edsn} animals. Scale bars = 100 μ m.

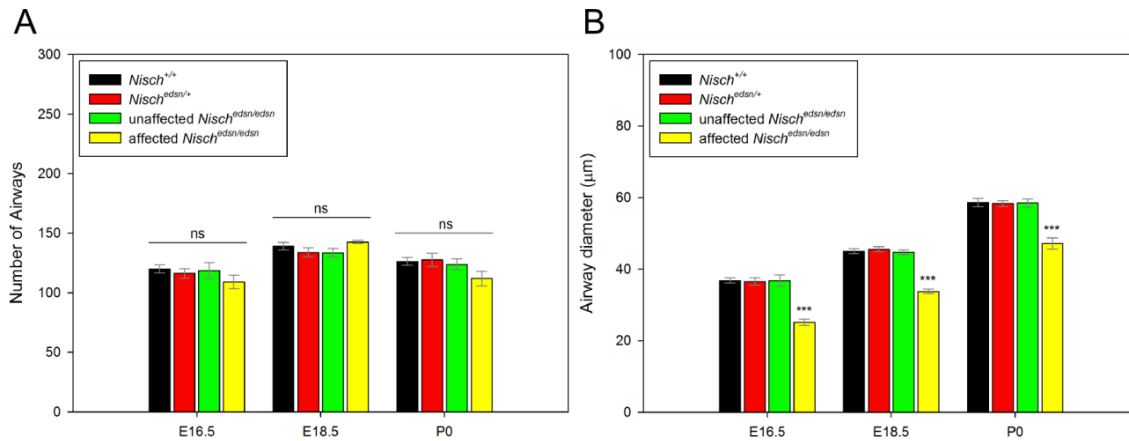


Figure 4.6| Analysis of lung from embryos and neonates from *edison* animals.

(A) Airways were counted in three different $6.5 \times 10^5 \mu\text{m}^2$ regions for all time points. There was no significant difference between genotypes. **(B)** Airway diameters were measured in three different regions for all embryos. The average airway width in affected *Nisch*^{edsn/edsn} animals was significantly smaller. ns $P > 0.05$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} E16.5 n = 6, E18.5 n = 9, P0 n = 8; *Nisch*^{edsn/+} E16.5 n = 11, E18.5 n = 14, P0 n = 15; unaffected *Nisch*^{edsn/edsn} E16.5 n = 4, E18.5 n = 7, P0 n = 5; affected *Nisch*^{edsn/edsn} E16.5 n = 2, E18.5 n = 3, P0 n = 2.

4.2.3 Morphological abnormalities displayed in *Nisch*^{edsn/edsn} animals

4.2.3.1 Weight measurements confirm mice with *edison* allele are undersized

Analysis of weights across a longitudinal time course, from four to twenty weeks of age, demonstrated that both male and female mice with the *Nisch*^{edsn/edsn} mutation were smaller than their wild-type littermates ($P < 0.001$; Figure 4.7). Male *Nisch*^{edsn/+} animals were also significantly smaller than *Nisch*^{+/+} animals from 13wk and older. Female *Nisch*^{edsn/+} animals showed some significant differences in weight compared to wild-types as well, but only in patches (between 5 to 11 and 14 to 18 weeks). *Nisch*^{edsn/edsn} mice were on average 36% smaller compared with their wild-type littermates, with *Nisch*^{edsn/+} mice 8% smaller.

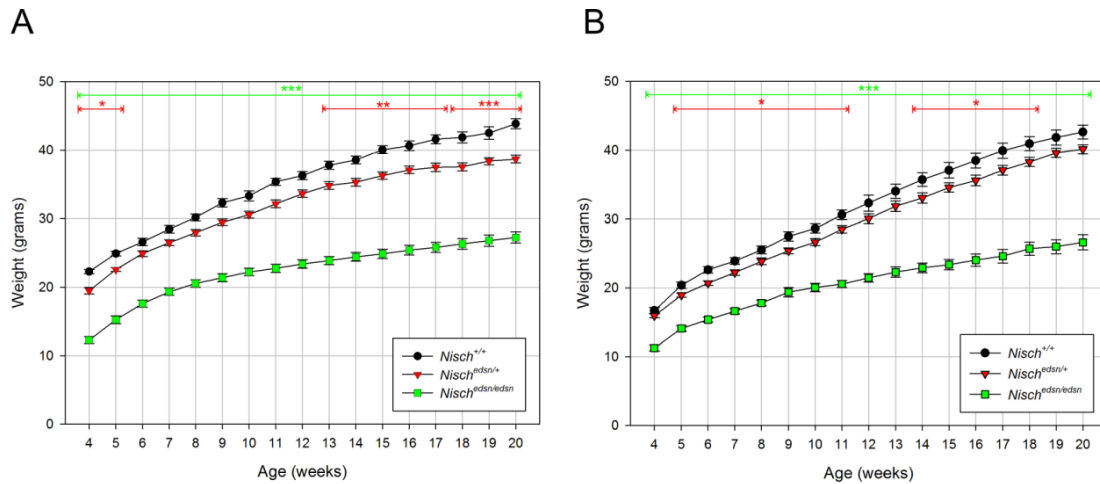


Figure 4.7| Weight data for *edison* mice over a 20wk longitudinal time course.

(A) Male *Nisch^{edsn/edsn}* mice are smaller than both wild-type and *Nisch^{edsn/+}* littermates throughout the time course. Male *Nisch^{edsn/+}* mice are also smaller than those wild-type for the allele, however there is no significant difference between 6 and 12 weeks. **(B)** Female *Nisch^{edsn/edsn}* animals are smaller than both wild-type and *Nisch^{edsn/+}* littermates throughout the time course. Female *Nisch^{edsn/+}* mice are also smaller than those wild-type for the allele, although there are only slight significant differences between 5 to 11 and 14 to 18 weeks. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} Male $n = 9$, Female $n = 12$; *Nisch^{edsn/+}* Male $n = 20$, Female $n = 17$; *Nisch^{edsn/edsn}* Male $n = 18$, Female $n = 13$.

4.2.3.2 Mild craniofacial defects result in a shortened snout

Nisch^{edsn/edsn} mice are smaller and appeared to have a shorter face when compared with their wild-type littermates (Figure 4.8A). To investigate the craniofacial phenotype of mice, I took dorsoventral X-ray images of the skulls and computed cranial measurements. The skull measurements were as follows: total skull length (tip of the nasal bone to back of the occipital bone), nasal bone length, frontal bone length, parietal bone length and skull width (measured at the widest points of the parietal bone) (Figure 4.8B). Most skull measurements proved to be significantly dependent on genotype (Figure 4.8C), which was to be expected considering the genotypic differences seen in body mass in the previous section. To determine whether any of the cranial bones showed disproportionate growth, allometric comparisons against skull length were used to normalise the data for differences in

body size. However, the data showed no genotypic effect between *Nisch*^{+/+} and *Nisch*^{edsn/+} mice on any of the following allometric comparisons; skull width/skull length, nasal bone length/skull length, frontal bone length/skull length and parietal bone length/skull length (Figure 4.8D). Comparison between *Nisch*^{edsn/edsn} and wild-type again showed no effect on skull width/skull length and parietal bone length/skull length. However, it did reveal a significant decrease in nasal bone length/skull length ($P < 0.001$), as well as a significant increase in frontal bone length/skull length ($P = 0.036$). These disproportions indicate the *Nisch*^{edsn/edsn} mutation affects growth at only the nasal and frontal bone. The magnitude of the difference between *Nisch*^{edsn/edsn} and wild-type mice was small (~3%). No other skeletal defects were evident.

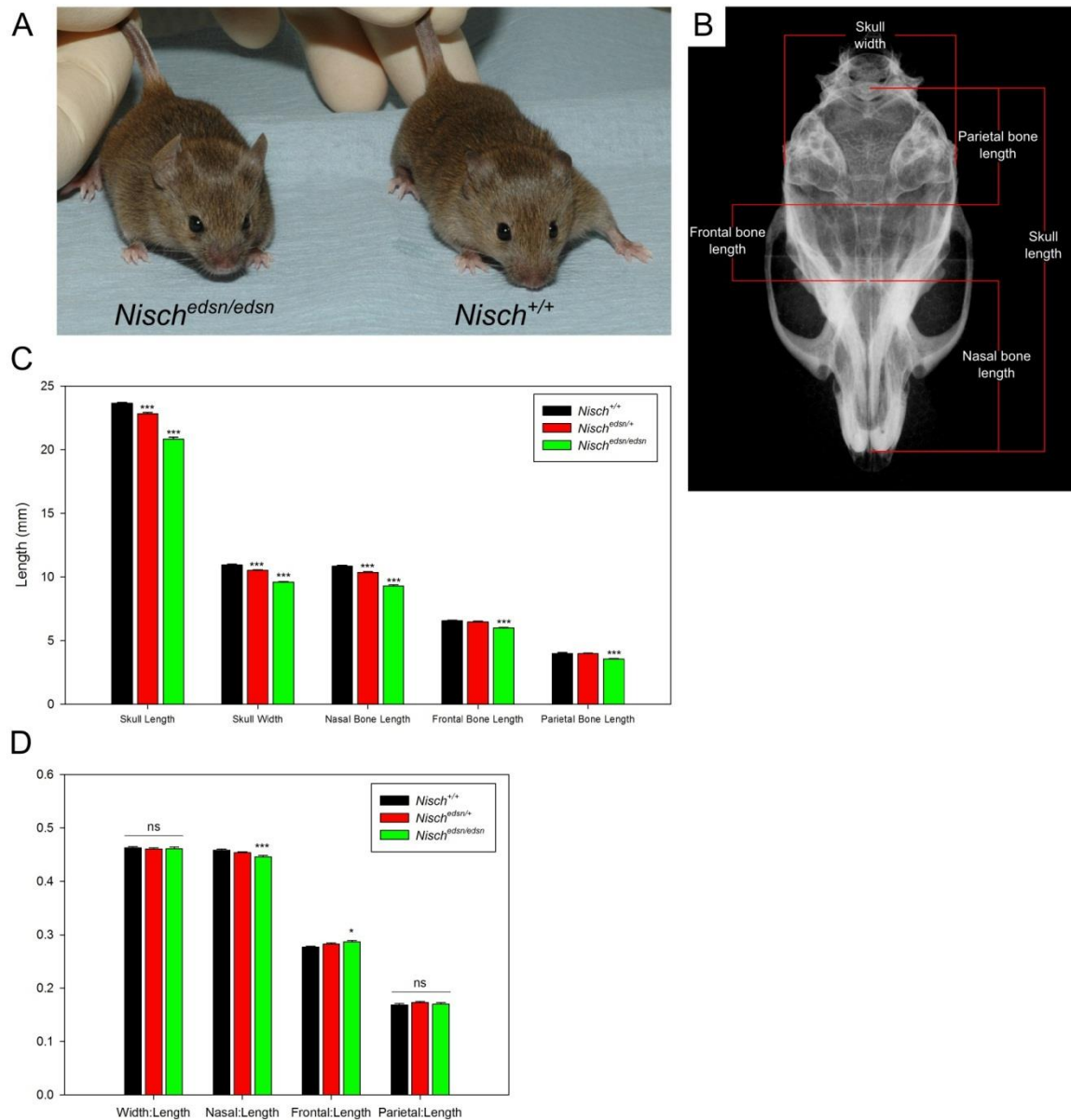


Figure 4.8| Craniofacial analysis of *edison* skulls at 20wk.

(A) Characteristic image of a *Nisch*^{edsn/edsn} mouse and a wild-type littermate. **(B)** Representative X-ray of a wild-type skull showing a dorsoventral view and the distances measured to analyse skull morphology of *edison* animals. **(C)** Specific bone measurements indicate that *Nisch*^{edsn/edsn} skulls are smaller than wild-type littermates for all measurements taken. *Nisch*^{edsn/+} skulls also differ significantly from wild-type for most measurements, apart from frontal bone length and parietal bone length. **(D)** Allometric comparisons against skull length show abnormal growth in *Nisch*^{edsn/edsn} skulls at the nasal bone and frontal bone. No effect was observed in *Nisch*^{edsn/+} skulls. ns $P > 0.05$; * $P < 0.05$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} $n = 13$; *Nisch*^{edsn/+} $n = 18$; *Nisch*^{edsn/edsn} $n = 12$.

4.2.3.3 Eustachian tube angle

To investigate the orientation of the Eustachian tube (ET) in *edison* mice, the skulls were stained and the angles between the midline of the skull and the bony part of the left and right ET's were measured (Figure 4.9). The mean angle in left wild-type ears was 43.11° (± 0.697), when compared with 42.66° (± 0.806) in *Nisch^{edsn/edsn}*, and the mean angle in right wild-type ears was 43.78° (± 0.455), when compared with 44.23° (± 0.553) in *Nisch^{edsn/edsn}*. These differences were not statistically significant (Left, $P = 0.848$; Right, $P = 0.324$).

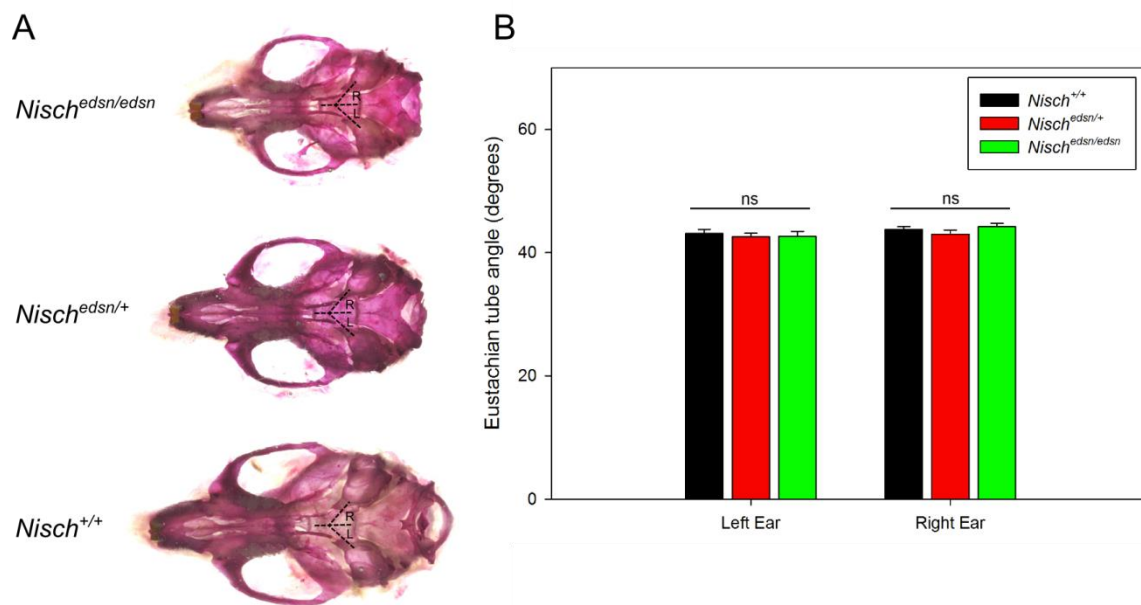


Figure 4.9| Eustachian tube (ET) angle of *edison* mice at 20wk.

(A) Representative alizarin stained skulls showing a dorsoventral view of all *edison* genotypes. ET angle measurements are indicated by dashed lines. R, right ear; L, left ear. **(B)** No genotypic effect is observed for ET angle. $n = 6$ for all genotypes. ns $P > 0.05$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

4.2.3.4 No abnormalities in auditory ossicles of *edison*

Ossicular malformations cause non-progressive conductive hearing loss (Stevenson and Hall 2005). To verify whether any such anomalies were present in *edison* mice, the ossicles were dissected from the ME of all genotypes (Figure 4.10). The ossicles dissected from *Nisch^{edsn/edsn}* mice were smaller in size; this was more than likely due to the mice being significantly smaller in size compared to wild-type littermates. There were no obvious morphological defects in the ossicles between *edison* genotypes.

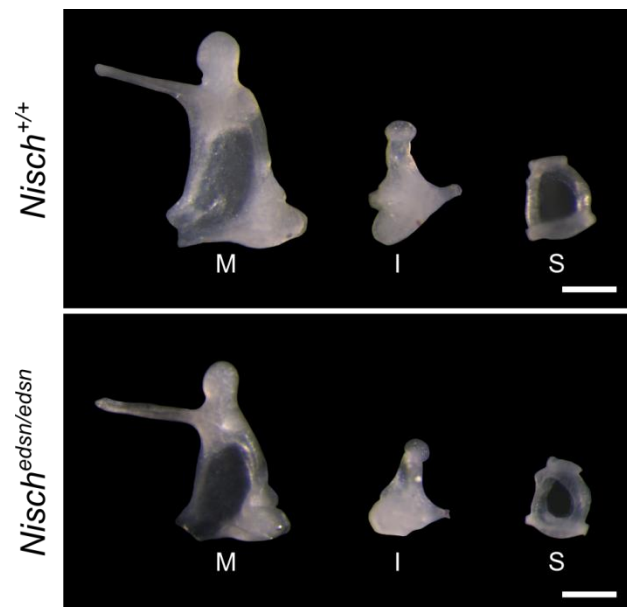


Figure 4.10| Auditory ossicles of *edison* mice at 8wk.

Representative images showing the three ossicles of the ME; malleus (M), incus (I) and stapes (S). No genotypic effect is observed. Photoshop was used to remove background artefacts, however ossicles themselves have not been altered. $n = 3$ for all genotypes. Scale bars = 100 μm .

4.2.4 Hearing deficits in *Nisch^{edsn/edsn}* animals

From preliminary data it was observed that affected *edison* animals had a hearing deficit at 12wk (see Figure 4.1). I proceeded to obtain auditory function data for each genotype across a time course to fully understand the development of the hearing phenotype.

4.2.4.1 Onset and progression of hearing loss in *Nisch^{edsn/edsn}* animals

In order to assess the onset and progression of the hearing phenotype in *edison* mice, ABR tests for auditory function were conducted across a time course with cohorts of age matched littermates (Figure 4.11). Hearing assessments were conducted with cohorts at 3, 4, 6, 8, 12, 16 and 20wk. Older mice were also assessed, but were not age matched (>20wk). Additionally, 3wk was used as the earliest time point at which ABR tests were conducted to get a baseline hearing threshold for all genotypes as previous mouse models display hearing phenotypes, due to OM, at 4wk. Mice were anesthetized and hearing thresholds determined using a click-evoked ABR ranging from 90 dB SPL to 5 dB SPL in decreasing 5 dB increments. The ABR threshold was measured for each ear.

Analysis using a non-parametric ANOVA found there was a significant difference in ABR thresholds between genotypes ($P < 0.001$) at each time point (Figure 4.11A). However, significant differences in ABR thresholds were only seen between *Nisch^{edsn/edsn}* and *Nisch^{+/+}* carrier mice. *Nisch^{edsn/+}* littermates displayed normal, wild-type hearing thresholds of between 15-30 dB SPL. At 3wk, mice with the *Nisch^{edsn/edsn}* mutation were found to have significantly elevated auditory thresholds compared to wild-types, with a mean ABR threshold of 30.28 dB SPL (± 1.143), just above the expected wild-type threshold range. Hearing progressively deteriorates with age in *Nisch^{edsn/edsn}* mice, displayed by increasingly elevated ABR thresholds. By

>20wk, mice with the *Nisch*^{edsn/edsn} mutation were recorded with a mean ABR threshold of 50.88 dB SPL ($\pm$ 2.499). It is also worth noting that there was no obvious gender or left-right preference observed in ABR threshold data from all genotypes (Appendix, Figure 10.3).

The ABR threshold range for each genotype was analysed using box and whisker plots. For both *Nisch*^{+/+} and *Nisch*^{edsn/+} mice, the boxes (25th to 75th percentile) and whiskers (10th to 90th percentiles) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for wild-type mice (Figure 4.11B and C). A small number of outliers were recorded in both genotypes outside of the expected wild-type ABR threshold range. The ABR threshold range in *Nisch*^{edsn/edsn} mice increased with age across the time course (Figure 4.11D). At 3wk, the threshold range was from 15 to 45 dB SPL and at >20wk, the threshold range was from 20 to 80 dB SPL. The median threshold also increased with age, from 30 dB SPL at 3wk to 55 dB SPL at >20wk. A number of ears still recorded an ABR threshold in the expected range for wild-type hearing, even in older mice at >20wk, indicating that in mice with the *Nisch*^{edsn/edsn} mutation the hearing phenotype is not fully penetrant.

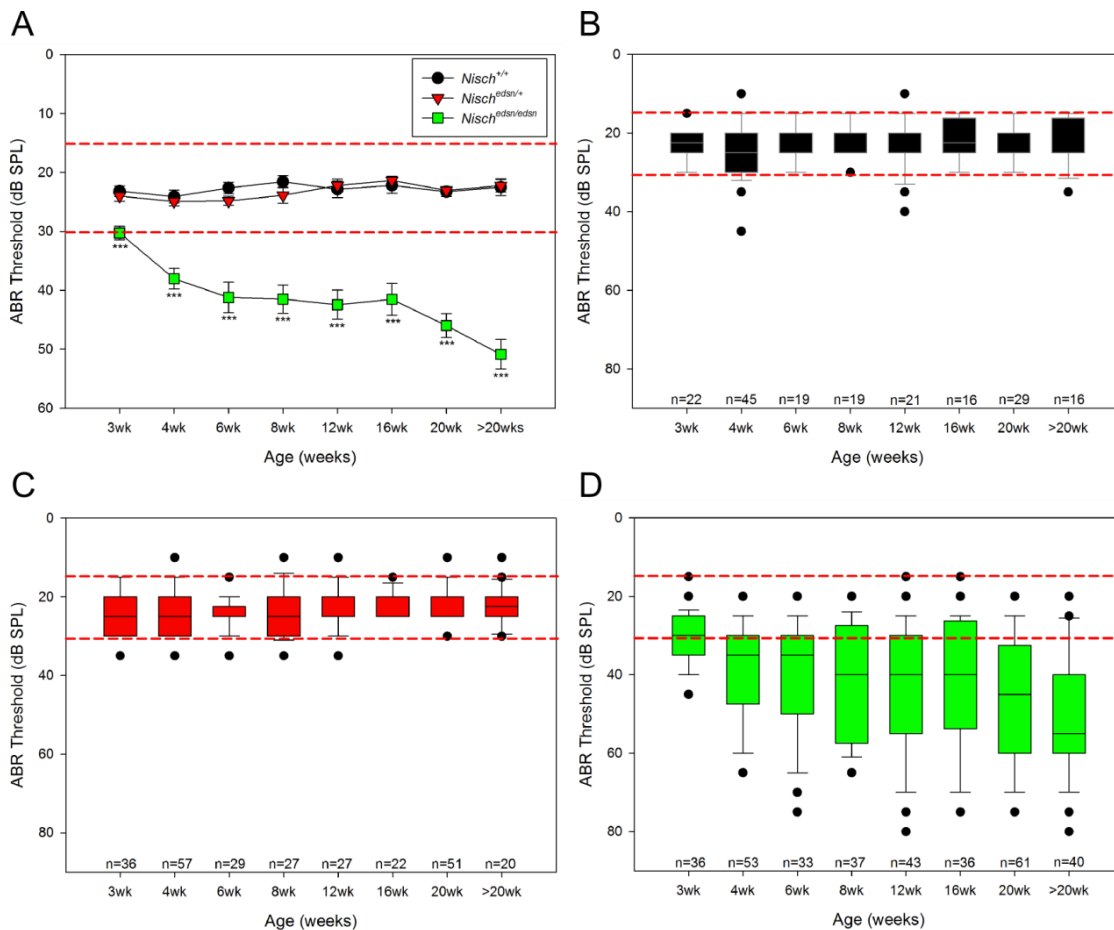


Figure 4.11 | Progression of click-evoked ABR thresholds across a cohort time course for *edison* mice.

(A) Mice with the *Nisch^{edsn/edsn}* mutation have elevated hearing thresholds. There is no difference in hearing thresholds between *Nisch^{edsn/+}* mice and wild-type littermates. (B-D) Box plots show the ABR threshold ranges of (B) *Nisch^{+/+}*, (C) *Nisch^{edsn/+}* and (D) *Nisch^{edsn/edsn}* mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (●) show any outliers. Sample number for each genotype and time point indicated on box plots (B-D). *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc testing.

4.2.4.2 Variable penetrance of OM in *Nisch^{edsn/edsn}* mice

To investigate the hearing phenotype in *Nisch^{edsn/edsn}* mice, the incidence of OM was determined from visual inspection of the tympanic membrane and the presence or absence of a cloudy eardrum (Figure 4.12). The cloudy appearance of an eardrum is a semi-quantitative measure of bulla fluid accumulation and hence OM. There was a

small incidence of unilateral OM seen in both *Nisch*^{+/+} and *Nisch*^{edsn/+} animals, 1% and 2% respectively, and there was no significant difference between genotypes ($P = 0.546$). In mice with *Nisch*^{edsn/edsn} allele there was a significant increase in the incidence of OM compared to littermates ($P < 0.001$), with 42% of mice with bilateral OM, 38% with unilateral OM and 20% with no OM phenotype.

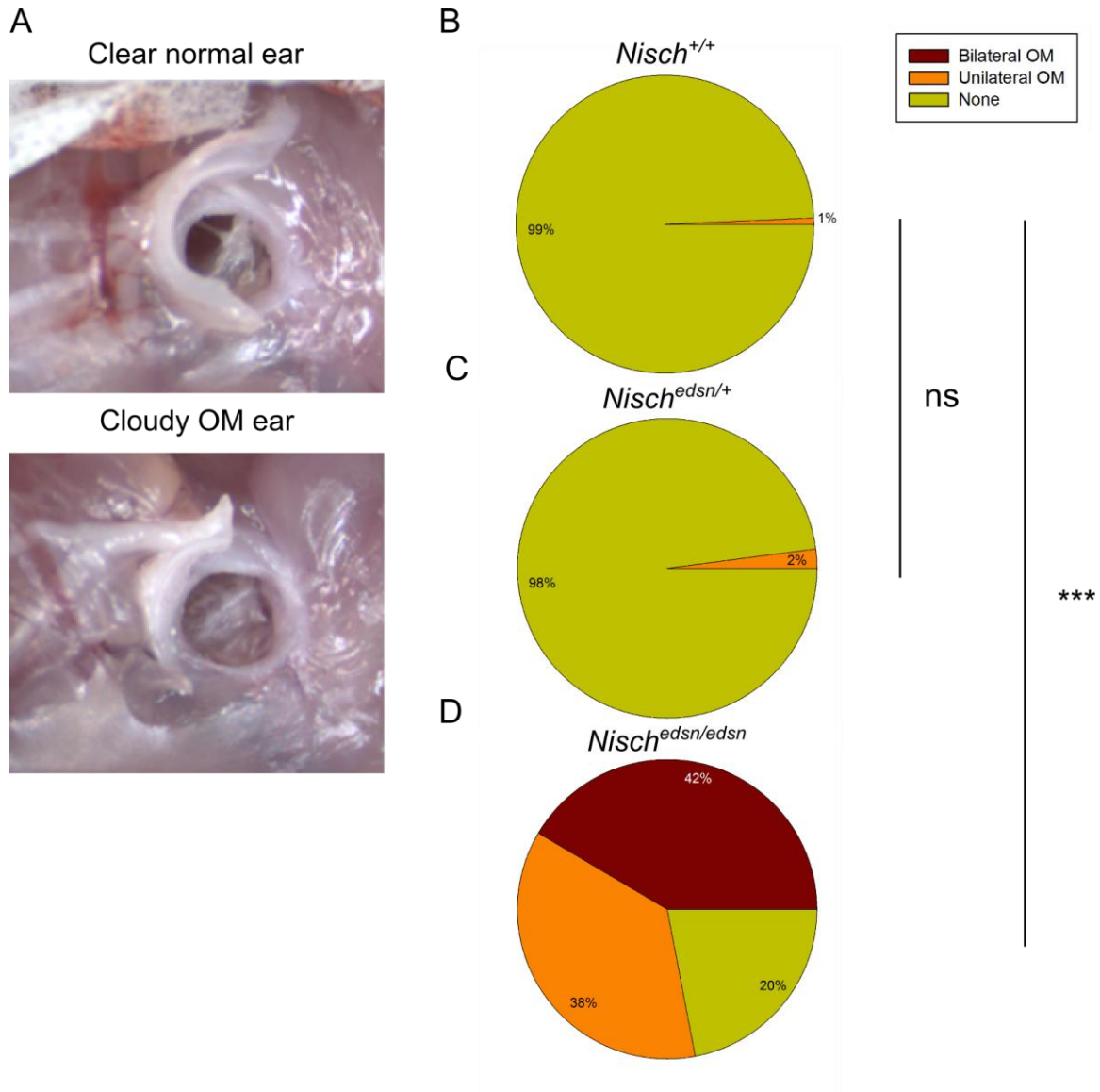


Figure 4.12| Incidence of OM in adult *edison* mice.

(A) Mice were sacrificed, heads skinned and ears examined using a dissecting microscope. The presence or absence of a cloudy eardrum was recorded from visual inspection. (Top) Clear eardrums have no visible fluid behind the tympanic membrane and it is possible to see the malleus. (Bottom) Cloudy eardrums have fluid behind the tympanic membrane and the malleus is obscured. **(B-D)** The incidence of OM was recorded in (B) *Nisch*^{+/+}, (C) *Nisch*^{edsn/+} and (D) *Nisch*^{edsn/edsn} mice. OM in *Nisch*^{edsn/edsn} animals is significantly more prevalent compared to littermates. ns $P > 0.05$; *** $P < 0.001$. Statistics were conducted using z-tests. *Nisch*^{+/+} n = 148; *Nisch*^{edsn/+} n = 194; *Nisch*^{edsn/edsn} n = 200.

The incidence of OM was also assessed across a time course for each genotype (Figure 4.13 A-C). Again, both *Nisch*^{+/+} and *Nisch*^{edsn/+} animals showed a small incidence of unilateral OM. Only one wild-type and four *Nisch*^{edsn/+} mice had an OM

phenotype throughout the time course, and they occurred at no particular time point. In *Nisch^{edsn/edsn}* mice there was a clear trend in increasing prevalence of OM from 3wk onwards. At 3wk, 60% of *Nisch^{edsn/edsn}* mice had bilateral clear ears with no OM phenotype present and 40% had unilateral OM, which was a significant difference compared to wild-type (z-test, $P = 0.045$). The prevalence of both unilateral and bilateral OM increases throughout the time course, where at >20wk 60% had bilateral OM, 37% unilateral OM and only 3% had no OM phenotype (z-test, $P < 0.001$).

As 40% of *Nisch^{edsn/edsn}* mice had unilateral OM at the 3wk time point, mice were also analysed at 2wk to see whether there was any incidence of OM prior to 3wk. Interestingly, all *Nisch^{edsn/edsn}* animals had at least one cloudy ear at this earlier time point, where 75% had bilateral cloudy ears and 25% unilateral (z-test, $P < 0.001$).

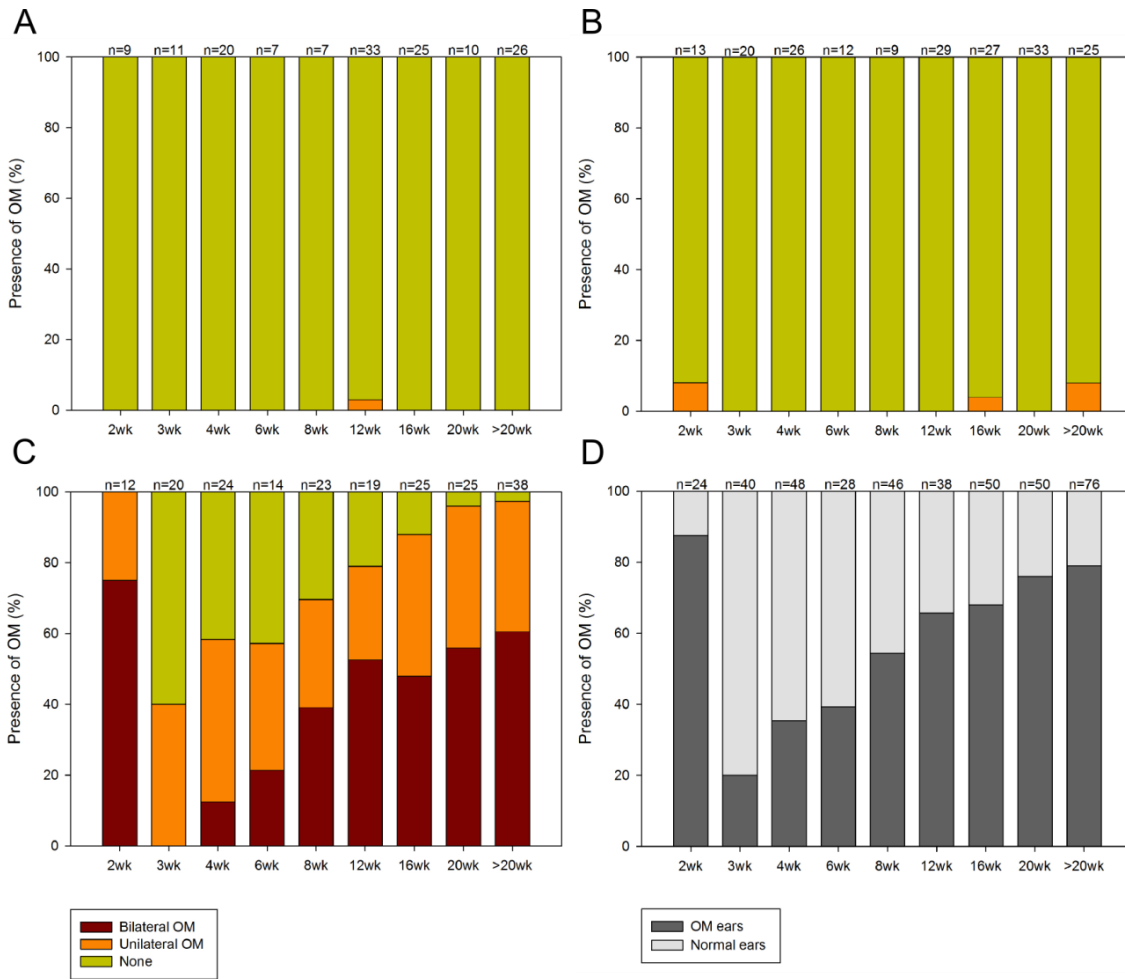


Figure 4.13| Incidence of OM in *edison* mice across a time course.

(A-C) The presence or absence of OM for each mouse was recorded from visual inspection in (A) *Nisch*^{+/+}, (B) *Nisch*^{edsn/+} and (C) *Nisch*^{edsn/edsn} mice. OM in *Nisch*^{edsn/edsn} animals increases in prevalence with age compared to littermates. From 8wk the majority of *Nisch*^{edsn/edsn} mice have bilateral OM. **(D)** The incidence of OM when looking at individual ears in *Nisch*^{edsn/edsn} mice.

4.2.4.3 Comparison between normal and OM ears in *Nisch*^{edsn/edsn} mice

As there is variation in the penetrance of OM in *Nisch*^{edsn/edsn} mice, with OM becoming more prevalent with age, the ABR data recorded from individual ears was categorised into two groups; ears that had an OM phenotype, *Nisch*^{edsn/edsn} OM, and ears with a normal wild-type phenotype, *Nisch*^{edsn/edsn} Normal (Figure 4.14). The criteria used to impartially categorise the ears into *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal was from the subsequent visualisation of tympanic inflammation after

dissection. *Nisch^{edsn/edsn}* Normal ears displayed normal, wild-type hearing thresholds of between 15-30 dB SPL, with no significant differences compared to wild-type littermates. ABR thresholds from *Nisch^{edsn/edsn}* OM ears still showed a hearing loss that progressively deteriorates over the time course (Figure 4.14A). At 3wk, *Nisch^{edsn/edsn}* OM ears had a mean ABR threshold of 37.00 dB SPL ($\pm$ 0.951) and by >20wk, the mean ABR threshold had increased to 54.86 dB SPL ($\pm$ 2.095). *Nisch^{edsn/edsn}* OM ears showed significantly increased ABR thresholds at every time point, when compared to *Nisch^{+/+}* ears ($P < 0.001$).

The ABR threshold range in *Nisch^{edsn/edsn}* OM ears again increased with age across the time course (Figure 4.14B). At 3wk, the threshold range was from 35 to 45 dB SPL and at >20wk, the threshold range was from 35 to 80 dB SPL, indicating that there was variation in the severity of the OM phenotype in *Nisch^{edsn/edsn}* OM ears.

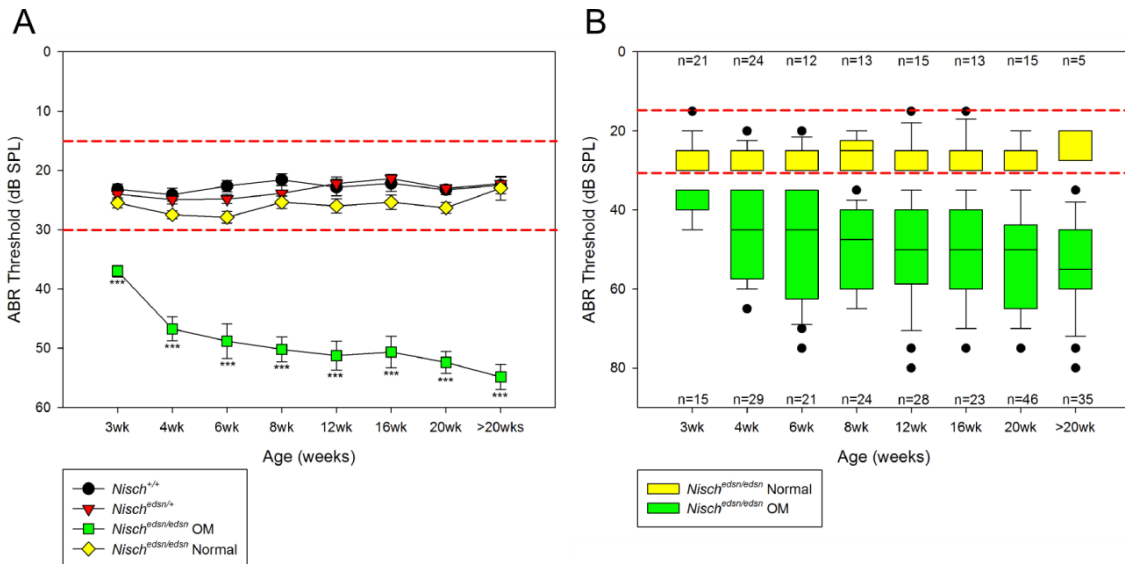


Figure 4.14 | Progression of click-evoked ABR thresholds across a cohort time course comparing normal and OM ears of *Nisch*^{*edsn/edsn*} mice.

Nisch^{*edsn/edsn*} data used in Figure 4.11 was separated into two groups, *Nisch*^{*edsn/edsn*} OM and *Nisch*^{*edsn/edsn*} Normal. **(A)** *Nisch*^{*edsn/edsn*} ears with OM have elevated hearing thresholds due to OM. There is no difference in hearing thresholds between *Nisch*^{*edsn/edsn*} Normal ears and ears of wild-type littermates. **(B)** Box plots show the ABR threshold ranges of *Nisch*^{*edsn/edsn*} Normal ears and *Nisch*^{*edsn/edsn*} OM ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (●) show any outliers. Sample number for each time point indicated on box plots (B-D). *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc testing.

4.2.4.4 Resolution of hearing deficit is observed in some *Nisch*^{*edsn/edsn*} animals

Due to the large range in ABR thresholds and variation in incidence of OM seen across the cohort study, I was interested in assessing how the OM phenotype and associated hearing loss developed in individual mice over a longitudinal time course. Hearing assessments were conducted at 4, 6, 8, 12, 16 and 20wk, with thirty *Nisch*^{*edsn/edsn*} mice tested at each time point for both ears.

Animals were sacrificed at the end of the longitudinal time course to confirm presence or absence of an OM phenotype in each ear. At the 20wk end point, forty

nine *Nisch*^{*edsn/edsn*} ears had an OM phenotype and eleven ears had a wild-type phenotype with the ME clear of exudate (Table 4.9). The majority of ears tested showed a progressive hearing loss ($n = 46$, 76.7%), which resulted in an OM phenotype at 20wk. As seen in Figure 4.15, the hearing loss observed in these ears mostly replicated the progression of hearing loss observed in the cohort study, with ABR thresholds of approximately 40 dB SPL that progressed to around 60 dB SPL (Figure 4.15B and D). Some ears had a persistently high ABR threshold throughout the time course, at around 60 dB SPL (Figure 4.15A, EDSN/90.2b R). A number of ears had normal ABR thresholds of around 25 dB SPL at 4wk and only recorded a hearing loss due to OM at a later time point, some as late as 20wk (Figure 4.15C, EDSN/94.7a L).

Table 4.9| Numbers of ears showing improvement in ABR thresholds over a 20wk longitudinal time course.

Changes in ABR thresholds	Observed	Percent	OM at 20wk	Clear at 20wk
Progressive hearing loss	46	76.7%	46	0
Improvement in hearing	5	8.3%	3	2
Normal hearing (no change)	9	15.0%	0	9
TOTAL	60		49	11

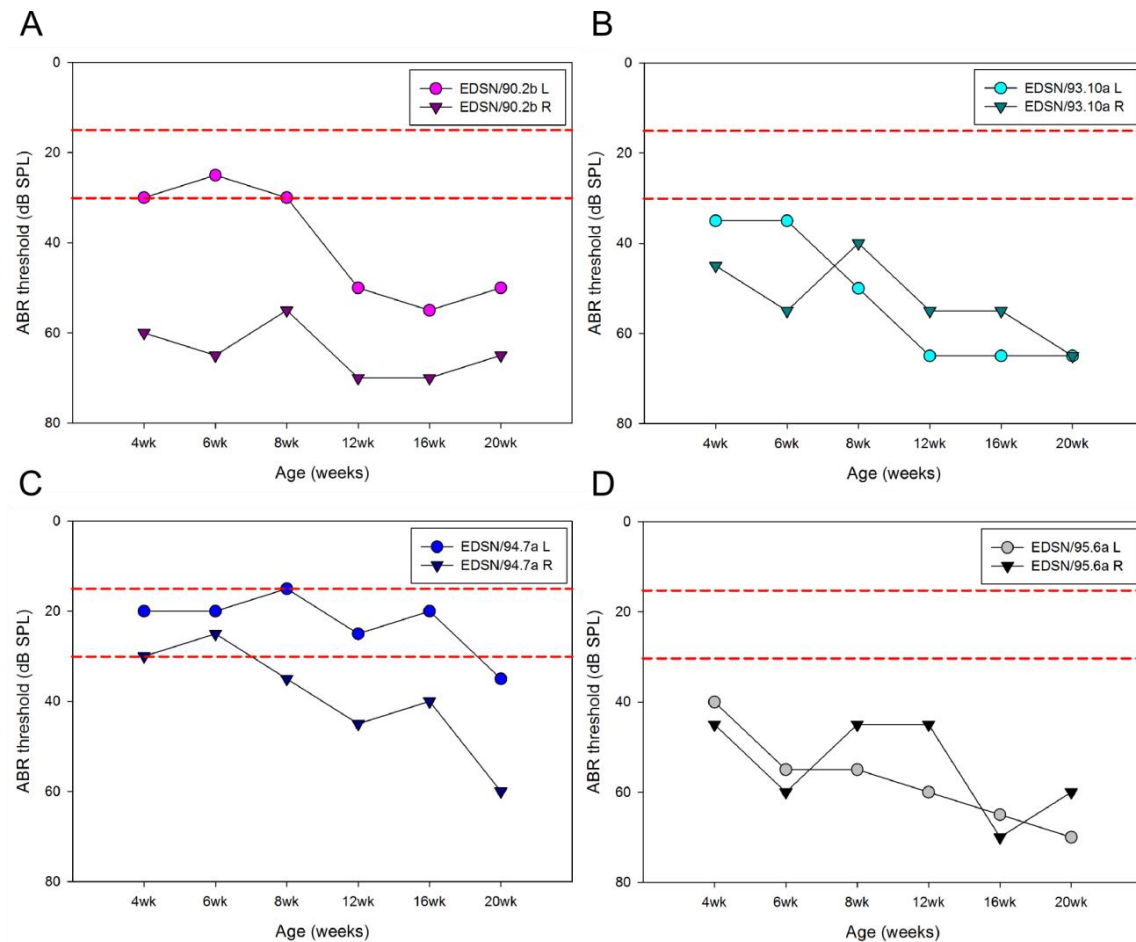


Figure 4.15| Progression of abnormal click-evoked ABR thresholds observed in *Nisch^{edsn/edsn}* mice.

Line and scatter plots showing longitudinal ABR thresholds for individual *Nisch^{edsn/edsn}* animals **(A)** EDSN/90.2b, **(B)** EDSN/93.10a, **(C)** EDSN/94.7a and **(D)** EDSN/95.6a. Majority of *Nisch^{edsn/edsn}* mice show a progressive hearing deficit due to OM. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). L, left ear; R, right ear.

In nine ears (15.0%), normal hearing thresholds between 15-30 dB SPL were observed at each time point throughout the longitudinal study (Figure 4.16A, B and D). Interestingly, five *Nisch^{edsn/edsn}* ears (8.3%) showed an improvement in their ABR thresholds throughout the time course compared to their 4wk ABR thresholds (Figure 4.16). Three of these ears still showed an OM phenotype at 20wk even though there was a reduction in their ABR thresholds from 4wk (Figure 4.16A, EDSN/90.2c R; C, EDSN/93.9g R; and D, EDSN/94.7d R). The other two ears that showed an

improvement in their hearing had a wild-type ME phenotype with no fluid present at 20wk (Figure 4.16B, EDSN/93.9b L and C, EDSN/93.9g L). The left ear in EDSN/93.9b had an ABR threshold of 45 dB SPL at 4wk that by 20wk had reduced to a normal hearing threshold at 25 dB SPL. This was also observed in the left ear of EDSN/93.9g, at 4wk an ABR threshold of 60 dB SPL was recorded compared to 30 dB SPL at 20wk.

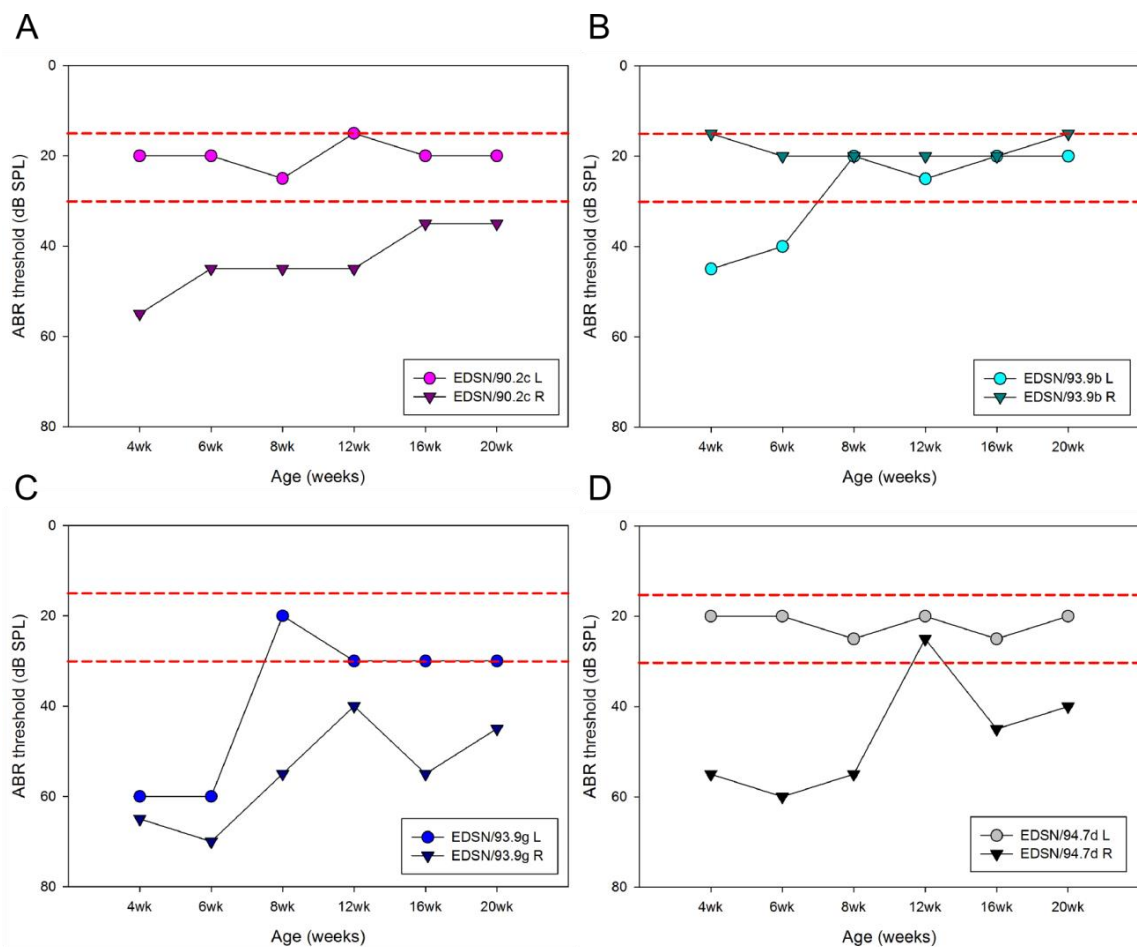


Figure 4.16| Resolution of abnormal click-evoked ABR thresholds observed in some *Nisch^{edsn/edsn}* mice.

Line and scatter plots showing longitudinal ABR thresholds for individual *Nisch^{edsn/edsn}* animals **(A)** EDSN/90.2c, **(B)** EDSN/93.9b, **(C)** EDSN/93.9g and **(D)** EDSN/94.7d. Four out of thirty *Nisch^{edsn/edsn}* animals showed improvement and/or resolution of an initial hearing deficit due to OM. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). L, left ear; R, right ear.

4.2.5 Histological analysis of ME

H&E stained sections can be used to examine the morphology of the ME. Histopathological examination of *edison* mice at 20wk demonstrated that *Nisch^{edsn/edsn}* mice develop a chronic otitis media (Figure 4.17B). The mucosal inflammation was diffuse and of moderate severity. There were differing types of ME effusion: a dense granulocyte-rich effusion, a thin watery effusion or a combination of both (Figure 4.18A-D). Papillary to polypoid exophytic growths were observed projecting into the ME cavity (MEC) (Figure 4.17D). The polypoid projections were most likely due to fibroblast stimulation as a consequence of chronic inflammation. There were small accumulations of inflammatory cells and dilated lymphatic and blood vessels in the mucoperiosteum (Figure 4.18E). In ME with the most severe phenotype, there were instances showing inflammation of the tympanic membrane (Figure 4.18F). Wild-type mice did not develop OM and had a ME free of effusion, with no thickening of the mucosal lining (Figure 4.17A and C). The histology of *Nisch^{edsn/+}* mice was indistinguishable from wild-type littermates.

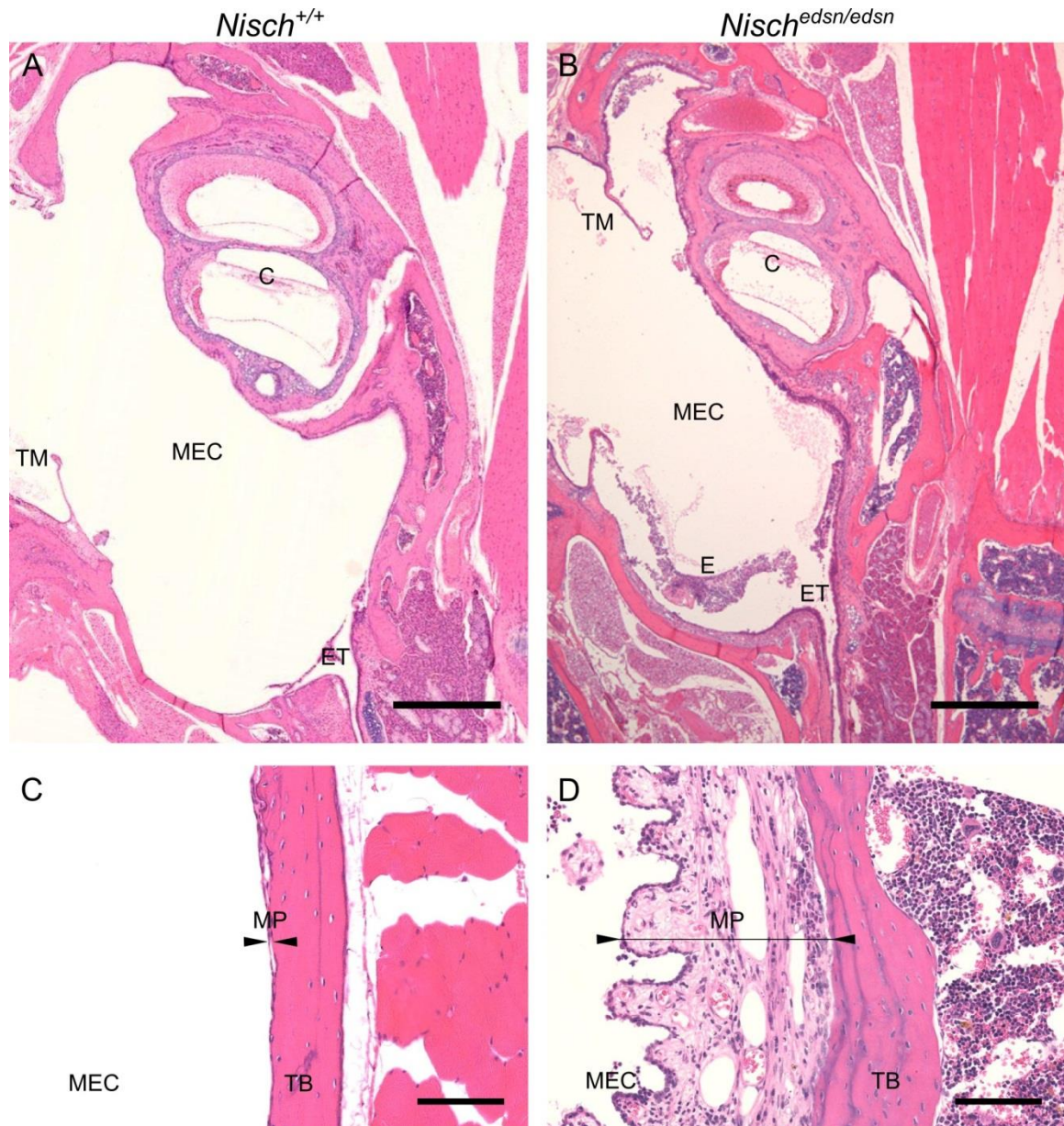


Figure 4.17| Characteristic ME histology in wild-type and *Nisch*^{edsn/edsn} animals at 20wk.

H&E stained transverse sections of the MEC and mucoperiosteum, in **(A & C)** *Nisch*^{+/+} and **(B & D)** *Nisch*^{edsn/edsn}, at 20wk. Histopathology of the MEC from *Nisch*^{edsn/edsn} mice demonstrates chronic inflammation with an exudate. Thickened mucoperiosteum found in *Nisch*^{edsn/edsn} animals has fibrous polyps that project into the MEC. Wild-type animals have no inflammation or exudate in the MEC and a thin mucoperiosteum covers the temporal bone. C, cochlea; ET, eustachian tube; E, exudate; MEC, middle ear cavity; MP, mucoperiosteum (arrowheads); TB, temporal bone; TM, tympanic membrane. (A & B) Scale bar = 500 μ m; (C & D) Scale bar = 100 μ m.

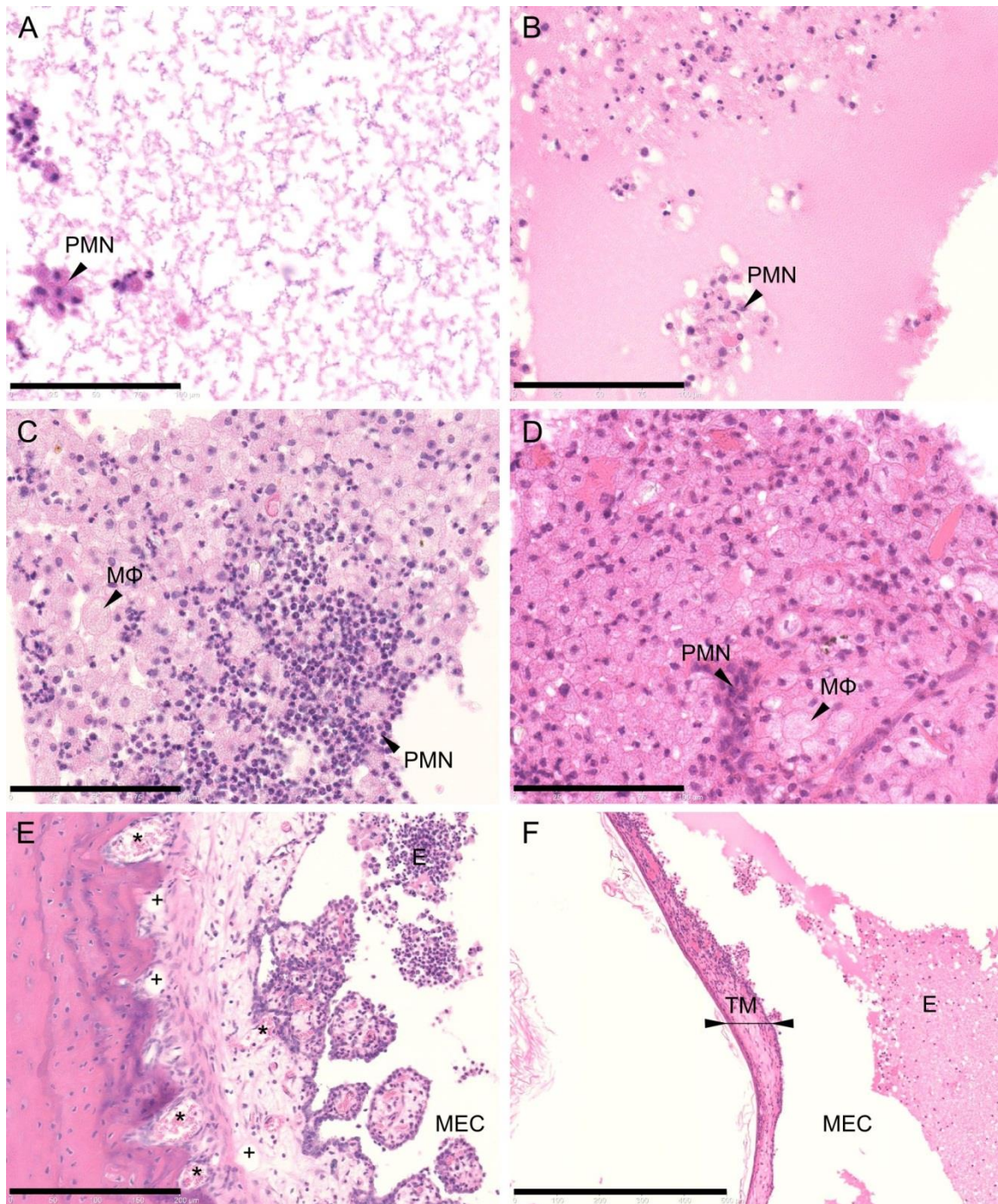


Figure 4.18| Additional characteristics observed in *Nisch^{edsn/edsn}* ME histology at 20wk.

(A-D) H&E stained transverse section of the MEC showing the differing types of ME effusion, from a thin watery effusion (A & B), to a dense granulocyte-rich effusion (C & D). **(E)** H&E stained transverse section of the mucoperiosteum showing dilated lymphatic and blood vessels. **(F)** H&E stained transverse section of the MEC showing an inflamed tympanic membrane. E, exudate; MΦ, foamy macrophage; MEC, middle ear cavity; PMN, polymorphonuclear cells; TM, tympanic membrane; *, blood vessels; +, lymphatic vessels. (A-D) Scale bar = 100 μm; (E) Scale bar = 200 μm; (F) Scale bar = 500 μm.

4.2.5.1 Histological time course of OM in *edison*

To further investigate the ME phenotype in *edison*, heads were sectioned at 2, 3, 4, 6, 8 and 20wk (Figure 4.19 and 4.20). An 'OM phenotype' was detected in the ME of *Nisch^{edsn/edsn}* mice as early as 2wk. At that time point, most of the *Nisch^{edsn/edsn}* mice (67%, n = 24 ears) had fluid in the MEC and a thickened epithelial lining, some of them only had a thickened epithelial lining with no fluid present (21%, n = 24 ears). In all the ears that contained fluid, it was a thin watery effusion. Wild-type littermates showed no fluid in the ear and no thickening of the epithelial lining (n = 18 ears). By comparison, one *Nisch^{edsn/+}* animal demonstrated a thickened epithelial lining in one ear (4%, n = 26 ears), all other ears showed a clear phenotype equivalent to wild-type.

Surprisingly at 3wk, most *Nisch^{edsn/edsn}* animals displayed a normal wild-type phenotype with clear ears (71%, n = 14 ears), and only three mice had a thin watery effusion (21%, n = 14 ears). None of the wild-type or *Nisch^{edsn/+}* mice displayed signs of an OM phenotype (n = 10 and n = 16 respectively). At 4wk, there were five *Nisch^{edsn/edsn}* ears that contained fluid in the MEC with a thickened epithelial lining (50%, n = 10 ears). Two ears had a thin watery effusion, and three ears displayed a cellular effusion with the presence of polymorphonuclear cells (PMNs) that are the first responding inflammatory cells that migrate towards the site of inflammation. Again no phenotype was demonstrated in *Nisch^{+/+}* and *Nisch^{edsn/+}* mice (n = 10 ears for both genotypes). In 6wk *Nisch^{edsn/edsn}* animals, four ears showed inflammation in the MEC with a thickened epithelial lining (33%, n = 12 ears), with three of these ears also containing a cellular effusion.

By the 8wk time point, the inflammation in *Nisch^{edsn/edsn}* animals had progressed to a chronic inflammation with effusion, as evidenced by a thick cellular fluid in the MEC along with a greatly thickened ME mucosa (67%, n = 12 ears). There was variability in the type of effusion found, where two ears exhibited a serous effusion and in six

ears the ME fluid was thick with the presence of macrophages and PMNs. *Nisch*^{*edsn/edsn*} mice studied at 20wk displayed a chronic inflammation with effusion, similar to what was observed at 8wk. Seventeen ears presented a chronic inflammation (77%, n = 22 ears), eleven of which contained fluid. Ten ears exhibited a thick effusion with the presence of macrophages and PMNs and in only one ear there was a serous exudate. Cellular debris-like aggregates were present in the thick effusions and developing polyps were observed in the ME mucosa. At 20wk, no OM was displayed in *Nisch*^{*edsn/+*} and wild-type littermates (n = 22 and n = 14 ears respectively).

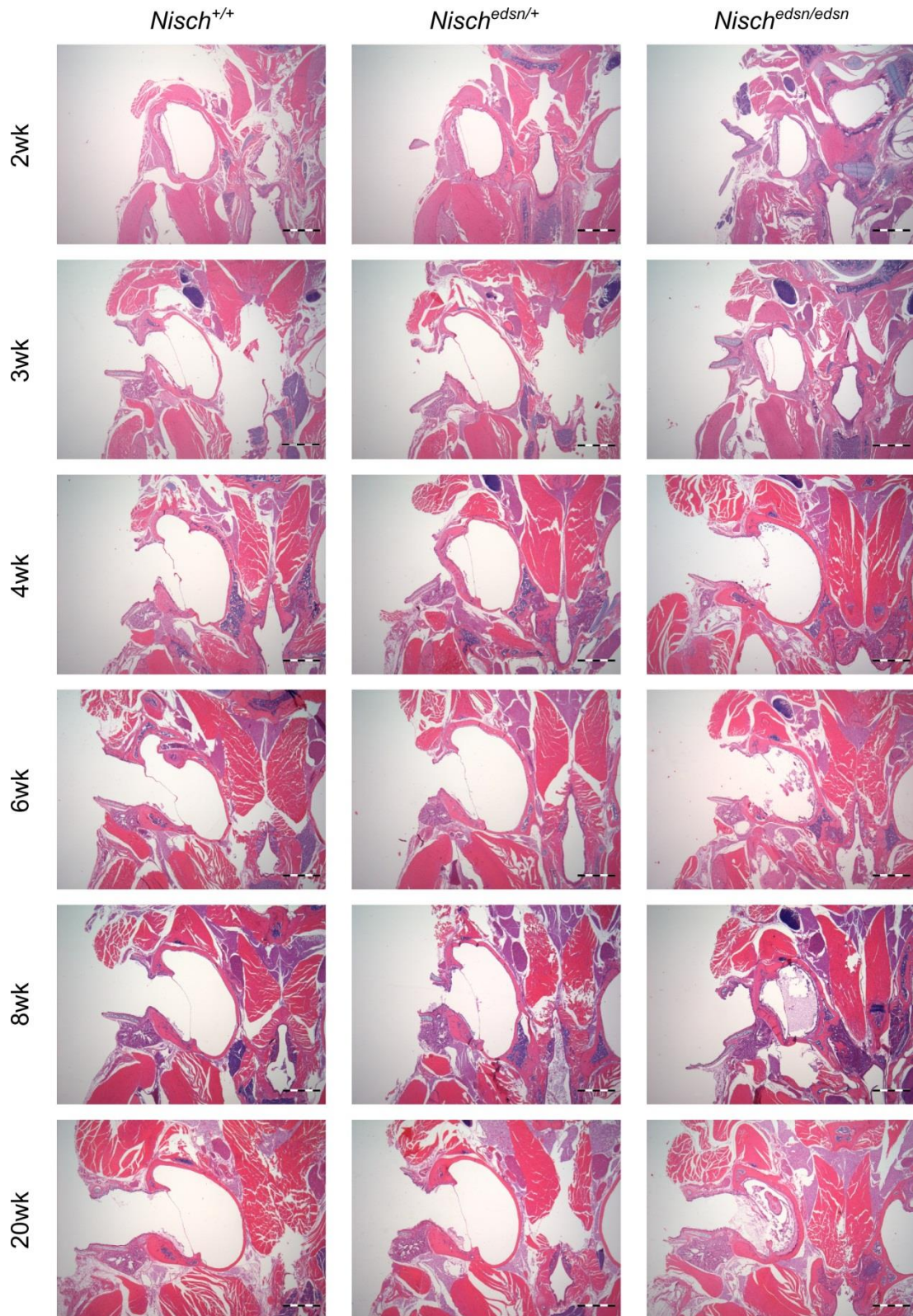


Figure 4.19| Progression of ME histology in *edison* mice.

Each group of panels consists of representative H&E stained transverse sections of the ME of *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{edsn/edsn} mice at different time points. Scale bars= 1 mm.

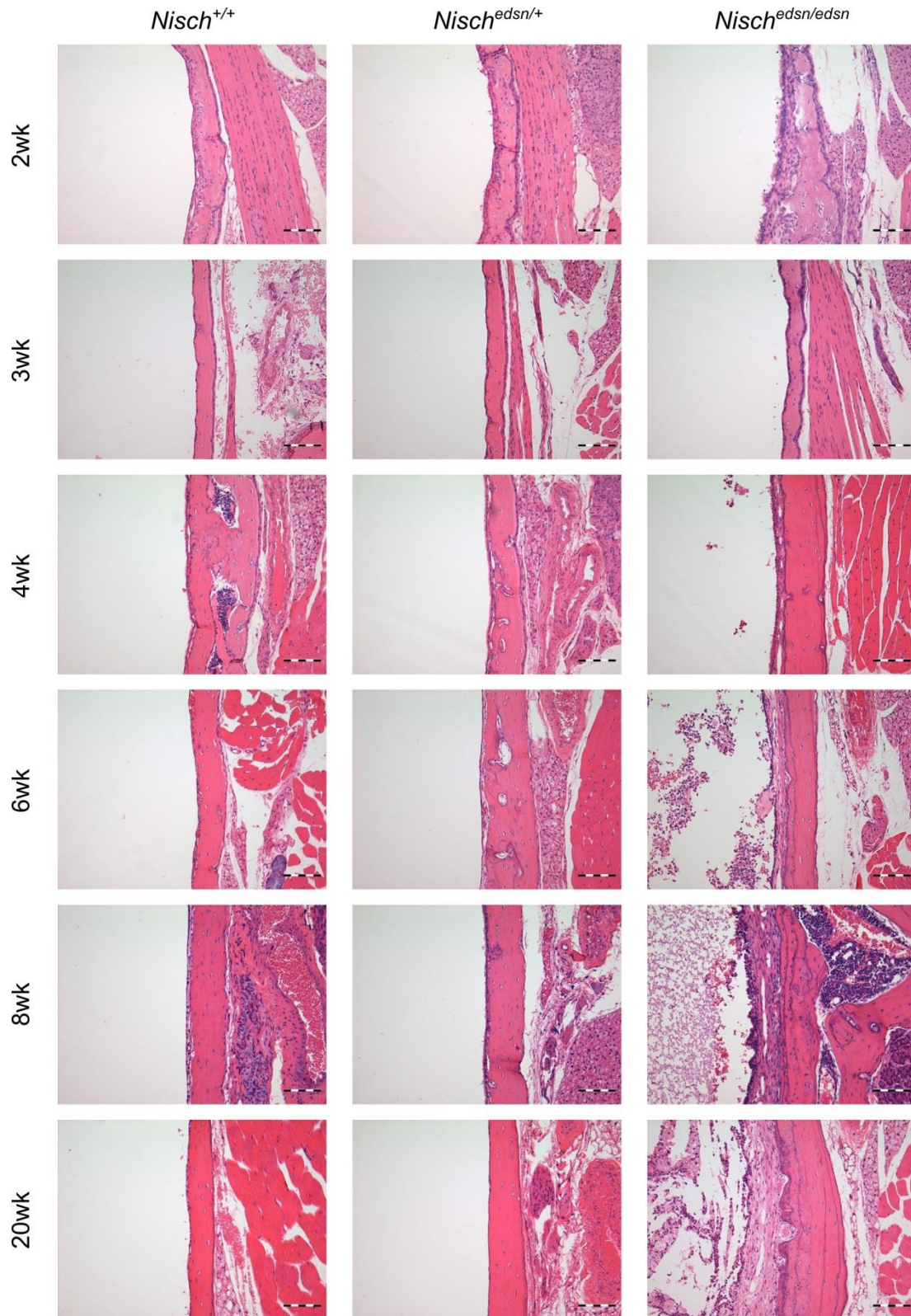


Figure 4.20| Progression of ME mucoperiosteum histology in *edison* mice.

Each group of panels consists of magnified views from Figure 4.19 showing representative H&E stained transverse sections of ME mucoperiosteum in *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{edsn/edsn} mice at different time points. Scale bar = 100 μm

4.2.5.2 Thickening of *edison* ME mucosa due to chronic inflammation

To quantify the chronic inflammation observed in *Nisch^{edsn/edsn}* mice and assess any subtle changes in the OM phenotype, blinded assessment of the mucoperiosteum thickness was carried out in all *edison* genotypes across the time course (Figure 4.21). Comparable regions of ME were measured for each ear assessed. Consistent with inflammation, *Nisch^{edsn/edsn}* animals have a thicker ME mucosa than wild-types, which gets progressively thicker across the time course.

Analysis of ME thickness at 2wk, revealed *Nisch^{edsn/edsn}* mice had a significantly thickened ME mucosa in comparison to littermates ($P < 0.001$). The mean mucosal thickness was 36.32 μm (± 3.403) in *Nisch^{edsn/edsn}* animals and 16.52 μm (± 0.741) for wild-types (Figure 4.21B). Interestingly, analysis of mucosal thickness in *Nisch^{edsn/+}* animals showed that the thickness of the mucosa ranged from between 11.15 μm to 42.50 μm (Figure 4.21A), with a mean of 18.23 μm (± 1.307). When the mucosal thickness is compared between all the time points for *Nisch^{edsn/+}* animals, the thickness at 2wk is significantly increased compared to 4, 8, 20 and >20wk. This is replicated in the wild-type data as well, where the mucosal thickness at 2wk is significantly increased compared to 4, 6, 20 and >20wk time points. The differences seen at 2wk are most likely part of the anatomically normal process of mesenchyme remodelling to form the bulla cavity, rather than inflammation.

There was no significant difference in the mucosal thickness between mice of any genotype at 3wk ($P = 0.321$), with a mean thickness of 11.36 μm (± 1.506) for wild-types and 16.86 μm (± 3.240) for *Nisch^{edsn/edsn}*. As there are no significant differences between genotypes observed at the 3wk time point, it suggests again that the differences seen at 2wk can be attributed to mesenchymal remodelling.

From 4wk onwards, the ME mucosa in *Nisch^{edsn/edsn}* mice gets progressively thicker and there is a significant difference compared to wild-type littermates at 4, 8, 20 and

>20wk ($P = 0.003$, $P = 0.006$, $P < 0.001$ and $P < 0.001$ respectively). By >20wk, the mean mucosal thickness was $10.38 \mu\text{m}$ (± 0.761) for wild-types and $86.04 \mu\text{m}$ (± 10.541) for *Nisch^{edsn/edsn}* animals. The range in mucosal thicknesses observed in *Nisch^{edsn/edsn}* animals at >20wk is very large, from between $5.12 \mu\text{m}$ to $186.80 \mu\text{m}$ (Figure 4.21A), confirming the variation in penetrance and severity of the OM phenotype in mice with the *Nisch^{edsn/edsn}* mutation.

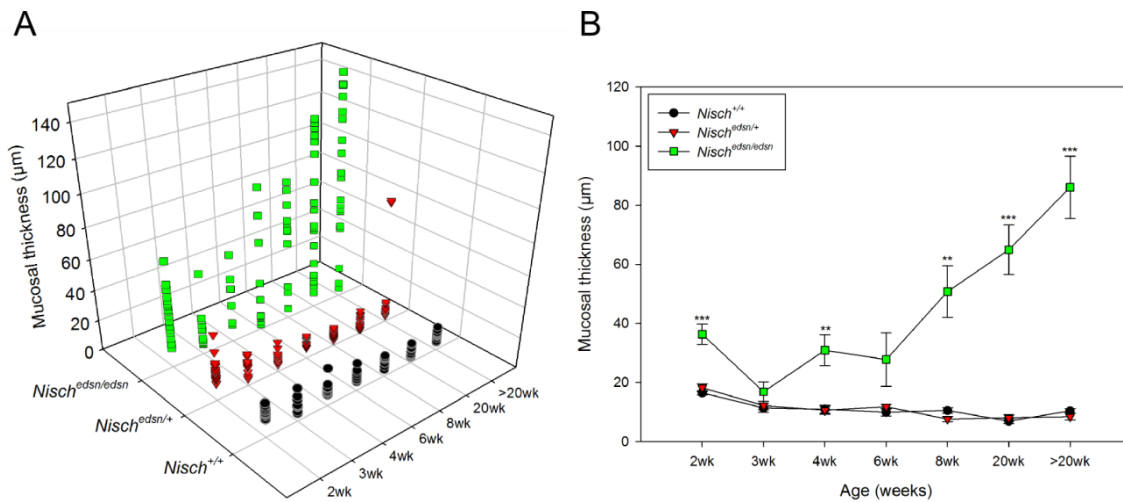


Figure 4.21 | Analysis of mucoperiosteum thickness in the MEC of *edison* mice.

H&E sections of *edison* ME were scanned into the Hamamatsu Pathology Imager and analysed. The mucosal thickness was averaged from 5 measurements of a standard $1000 \mu\text{m}$ length of ME mucosa (avoiding the cochlea and the region close to the ET). **(A)** Scatter plot showing the variation in mucosal thickness of the ME for indicated genotypes. Each point represents a single ear. **(B)** Line graph showing the average mucosal thickness of the ME for each genotype. Mice with the *Nisch^{edsn/edsn}* mutation show a progressive increased thickening of the mucosa due to OM. There is no histological difference seen in mucosal thickness between *Nisch^{edsn/+}* mice and wild-type littermates. ** $P < 0.01$; *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc testing.

Nisch^{+/+} 2wk n = 18, 3wk n = 10, 4wk n = 12, 6wk n = 10, 8wk n = 10, 20wk n = 14, >20wk n = 14; *Nisch^{edsn/+}* 2wk n = 26, 3wk n = 16, 4wk n = 10, 6wk n = 14, 8wk n = 10, 20wk n = 22, >20wk n = 12; *Nisch^{edsn/edsn}* 2wk n = 18, 3wk n = 14, 4wk n = 9, 6wk n = 12, 8wk n = 12, 20wk n = 21, >20wk n = 21.

As there was variation in the penetrance of OM in *Nisch^{edsn/edsn}* mice throughout the time course, the ears used in the blinded mucosal assessment were categorised into

those that had an OM phenotype and those that did not, *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal respectively (Figure 4.22). The criteria used to impartially categorise the ears into *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal was from the subsequent visualisation of tympanic inflammation after dissection. *Nisch*^{edsn/edsn} Normal ears displayed normal, wild-type ME mucosal morphology of between 8.95 μm to 16.95 μm (Figure 4.22B), with no significant differences compared to wild-type littermates. The mucosal thickness observed in *Nisch*^{edsn/edsn} OM ears still showed a mucosal morphology that progressively thickens over the time course. At 3wk, *Nisch*^{edsn/edsn} OM ears had a mean mucosal thickness of 28.41 μm ($\pm$ 8.490) and by >20wk, the mean mucosal thickness had increased to 98.50 μm ($\pm$ 9.391). *Nisch*^{edsn/edsn} OM ears showed significantly increased thickening of the ME mucosa at every time point, when compared to *Nisch*^{+/+} ears (3wk, $P = 0.013$; >20wk, $P < 0.001$).

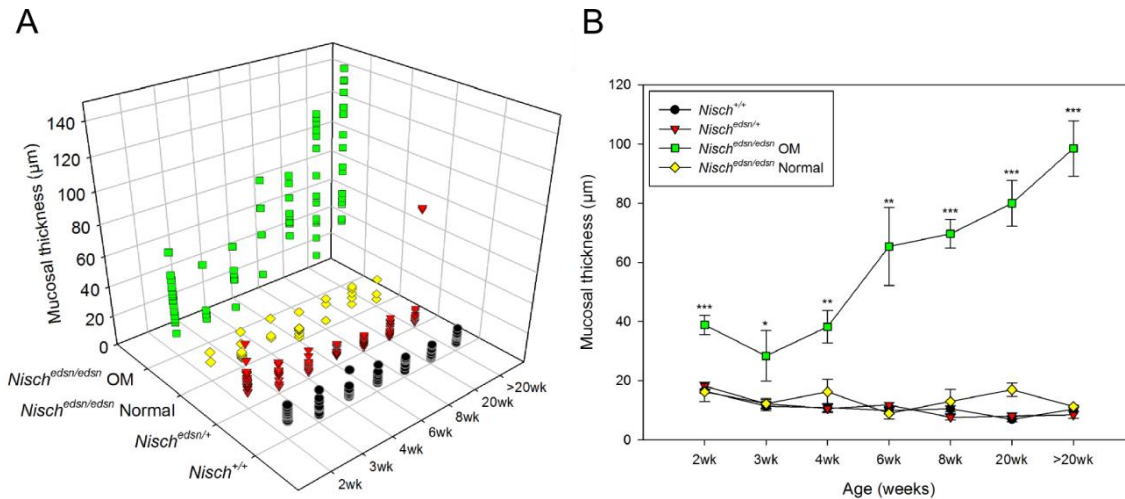


Figure 4.22| Analysis of mucoperiosteum thickness in the MEC comparing normal and OM ears of *Nisch*^{edsn/edsn} mice.

Nisch^{edsn/edsn} data used in Figure 4.21 was separated in to two groups, *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal. **(A)** Scatter plot showing the variation in mucosal thickness of the ME for indicated genotypes. Each point represents a single ear. **(B)** Line graph showing the average mucosal thickness of the ME. OM ears from mice with the *Nisch*^{edsn/edsn} mutation show a significantly increased thickening of the mucosa at each time point. There is no histological difference seen in mucosal thickness between *Nisch*^{edsn/edsn} Normal ears and wild-type littermates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc testing.

Nisch^{+/+} 2wk n = 18, 3wk n = 10, 4wk n = 12, 6wk n = 10, 8wk n = 10, 20wk n = 14, >20wk n = 14; *Nisch*^{edsn/+} 2wk n = 26, 3wk n = 16, 4wk n = 10, 6wk n = 14, 8wk n = 10, 20wk n = 22, >20wk n = 12; *Nisch*^{edsn/edsn} OM 2wk n = 16, 3wk n = 4, 4wk n = 6, 6wk n = 4, 8wk n = 8, 20wk n = 16, >20wk n = 18; *Nisch*^{edsn/edsn} Normal 2wk n = 2, 3wk n = 10, 4wk n = 3, 6wk n = 8, 8wk n = 4, 20wk n = 5, >20wk n = 3.

4.2.5.3 Smaller MEC in *Nisch*^{edsn/edsn} ears with OM

The MEC appeared to be reduced in size in *Nisch*^{edsn/edsn} mice compared to wild-type littermates. The area of the MEC in *edison* mice was measured from transverse sections, using the temporal bone and tympanic membrane as an outline for the MEC.

Analysis of the MEC area at 20wk revealed *Nisch*^{edsn/edsn} mice had a significantly smaller MEC in comparison to littermates ($P < 0.001$). The mean MEC area was $2.30 \mu\text{m}^2 (\pm 0.206)$ in *Nisch*^{edsn/edsn} animals and $3.55 \mu\text{m}^2 (\pm 0.225)$ for wild-types (Figure 4.23A). The measurements taken for MEC area in *Nisch*^{edsn/edsn} animals, ranged from

0.83 to 4.29 μm^2 . Whereas in *Nisch*^{+/+} animals, the MEC area ranged from between 1.88 and 5.05 μm^2 .

As there was a large amount of overlap between genotypes I thought it would be interesting to see if the MEC area is affected by the presence of an OM phenotype in the *Nisch*^{edsn/edsn} ME. The sections used to measure the MEC area were divided into those that had an OM phenotype and those that did not, *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal respectively (Figure 4.23B). The criteria used to impartially categorise the ears into *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal was from the subsequent visualisation of tympanic inflammation after dissection. *Nisch*^{edsn/edsn} Normal ears still displayed a slightly smaller MEC area of between 1.59 and 4.29 μm^2 , however no significant difference was observed compared to wild-type littermates ($P = 0.246$). The MEC area observed in *Nisch*^{edsn/edsn} OM ears was significantly smaller than both wild-type littermates and *Nisch*^{edsn/edsn} Normal ears ($P < 0.001$ and $P = 0.024$ respectively). The MEC area of *Nisch*^{edsn/edsn} OM ears ranged from 0.83 to 3.47 μm^2 .

The analysis of the MEC area was also assessed across a time course, from 2wk to 20wk for each genotype (Figure 4.23C). Again, analysis revealed *Nisch*^{edsn/edsn} mice had a significantly smaller MEC in comparison to littermates from 3wk to 20wk ($P < 0.05$). There was no significant difference in the MEC area between mice of any genotype at 2wk ($P = 0.121$), with a mean area of 1.68 μm^2 (± 0.167) for wild-types and 1.33 μm^2 (± 0.195) for *Nisch*^{edsn/edsn}. As mentioned from 3wk onwards the MEC area is significantly smaller compared to wild-type littermates, where at 3wk the mean MEC area was 2.10 μm^2 (± 0.239) in *Nisch*^{edsn/edsn} animals and 2.83 μm^2 (± 0.305) for wild-types, increasing to what was observed above at 20wk.

For the time course assessment of MEC area the sections used to measure the area were again divided into those that had an OM phenotype and those that did not, *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal respectively (Figure 4.23D). The criteria

used to impartially categorise the ears into *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal was the same as above. *Nisch*^{edsn/edsn} Normal ears still displayed a slightly smaller MEC area, however no significant difference was observed compared to wild-type littermates across the time course ($P > 0.05$). The MEC area observed in *Nisch*^{edsn/edsn} OM ears were significantly smaller than wild-type littermates from 4wk onwards ($P < 0.05$), and only showed a significant difference between *Nisch*^{edsn/edsn} Normal ears at 20wk as seen above. The mean MEC area of *Nisch*^{edsn/edsn} OM ears ranged from $1.35 \mu\text{m}^2$ at 2wk to $2.03 \mu\text{m}^2$ at 20wk, whereby the mean MEC area of *Nisch*^{edsn/edsn} Normal ears ranged from $1.15 \mu\text{m}^2$ at 2wk to $3.05 \mu\text{m}^2$ at 20wk.

H&E stained sections were used to examine the bone pathology of the bulla wall at 20wk (Figure 4.24). Histopathological examination of *edison* mice demonstrated that in the chronically inflamed ME of *Nisch*^{edsn/edsn} mice osteoneogenesis of the bulla wall occurs, where new bone deposits are seen on the original bone of the bulla wall in *Nisch*^{edsn/edsn} OM ears. In *Nisch*^{edsn/edsn} Normal ears no osteoneogenesis was observed at the bulla wall. The histology of *Nisch*^{edsn/edsn} Normal and *Nisch*^{edsn/+} ears was indistinguishable from wild-type littermates.

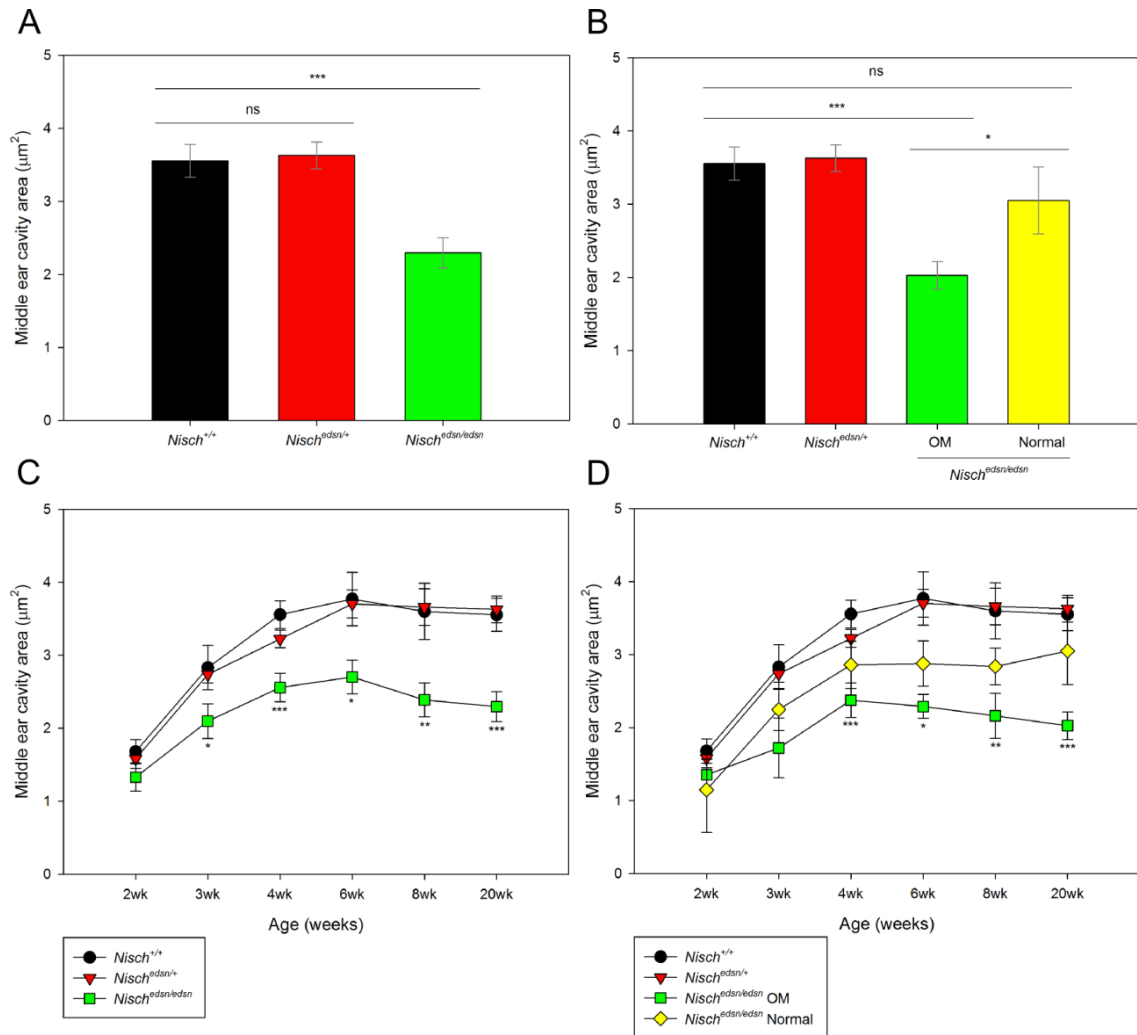


Figure 4.23 | Analysis of MEC area of *edison* mice.

H&E sections of *edison* ME were scanned into the Hamamatsu Pathology Imager and analysed. The area of the MEC was recorded using the temporal bone and tympanic membrane as the framework for the measurement. **(A)** Bar chart showing the distribution of MEC areas for *edison* animals at 20wk. Mice with the *Nisch*^{edsn/edsn} mutation have a smaller MEC. **(B)** Bar chart showing *Nisch*^{edsn/edsn} data used in (A) after being separated into two groups, *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal. OM ears from *Nisch*^{edsn/edsn} mice have a significantly smaller MEC compared to *Nisch*^{edsn/edsn} Normal ears and wild-type littermates. There is no difference between *Nisch*^{edsn/edsn} Normal ears and wild-type littermates. **(C)** Time course showing the mean MEC areas for *edison* animals from 2 to 20wk. **(D)** Time course showing *Nisch*^{edsn/edsn} data used in (C) after being separated into two groups, *Nisch*^{edsn/edsn} OM and *Nisch*^{edsn/edsn} Normal. Observations seen at 20wk are duplicated at earlier time points, as early as 4wk. ns $P > 0.05$; * $P < 0.05$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} n = 14; *Nisch*^{edsn/+} n = 21; *Nisch*^{edsn/edsn} n = 18; *Nisch*^{edsn/edsn} OM n = 13; *Nisch*^{edsn/edsn} Normal n = 5.

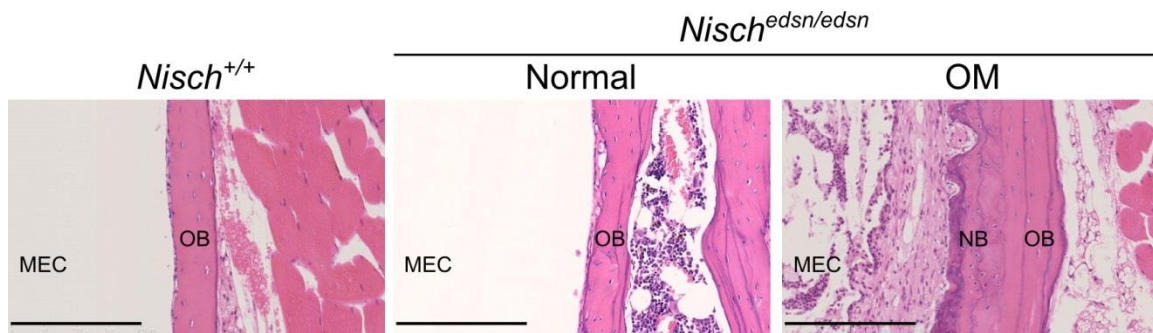


Figure 4.24 | Osteoneogenesis of the bulla wall in *edison* mice.

H&E stained transverse sections of the bulla wall in *Nisch*^{+/+}, *Nisch*^{edns/edns} Normal and *Nisch*^{edns/edns} OM ears at 20wk. New bone (NB) deposits are seen on the original bone (OB) of the bulla wall in *Nisch*^{edns/edns} OM ears. Wild-type and *Nisch*^{edns/edns} Normal ears display no osteoneogenesis at the bulla wall. *Nisch*^{edns/+} mice show the same phenotype as wild-type littermates (data not shown). MEC, middle ear cavity. Scale bar = 200 μ m.

4.2.5.4 Increased goblet cell population in *Nisch*^{edns/edns} Eustachian tube

Periodic acid–Schiff (PAS) staining was used to identify the presence of goblet cells in *edison* mice at 20wk (Figure 4.25). Goblet cells are glandular simple epithelial cells whose function is to secrete mucin, which dissolves in water to form mucus. Throughout the NP and ET, goblet cells were identified in all *edison* genotypes. In the MEC, no goblet cells were found in any *edison* animals, even in the thickened ME mucosa found in *Nisch*^{edns/edns} mice.

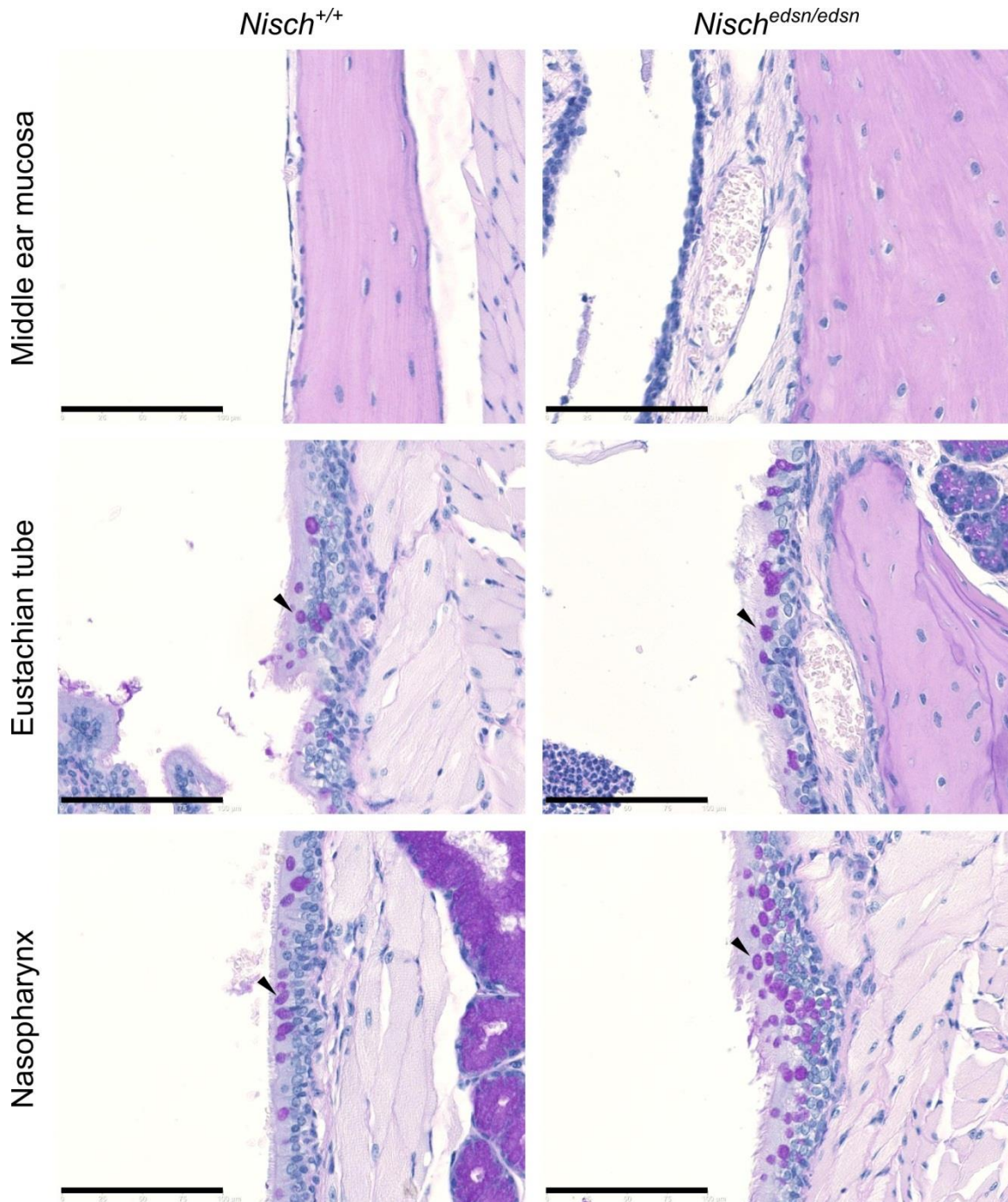


Figure 4.25| Mucin secreting goblet cells are found in the nasopharynx and Eustachian tube of *edison* mice at 20wk.

Periodic acid–Schiff (PAS) stained sections from the ME mucosa (top), Eustachian tube (middle) and nasopharynx (bottom). PAS stains glycoproteins, including mucins, bright purple. Goblet cells (arrowheads) are clearly present in the nasopharynx and Eustachian tube of both *Nisch*^{+/+} and *Nisch*^{edsn/edsn} mice. In the ME mucosa, goblet cells are not found in either *Nisch*^{+/+} or *Nisch*^{edsn/edsn} sections. *Nisch*^{edsn/+} mice show the same phenotype as wild-type littermates (data not shown). Scale bars = 100 μm.

In order to quantify if there were significant genotypic differences in the density of goblet cells in *edison* mice, counts of the number of goblet cells were conducted in the NP and ET (Figure 4.26A and B respectively). Goblet cell density was measured by counting the number of goblet cells per 500 μ m of epithelium.

Counts of the number of goblet cells showed no significant difference between *Nisch^{edsn/edsn}* and wild-type mice in the NP ($P = 0.480$). The mean number of goblet cells in *Nisch^{edsn/edsn}* animals in the NP was 21.3 (± 5.45) and for *Nisch^{+/+}* animals was 20.5 (± 2.37). However, a significant increase in the number of goblet cells was shown in the *Nisch^{edsn/edsn}* ET, when compared to wild-type littermates ($P = 0.006$). The average number of goblet cells in *Nisch^{edsn/edsn}* animals in the ET was 36.2 (± 4.29) and for *Nisch^{+/+}* animals was 19.0 (± 2.65).

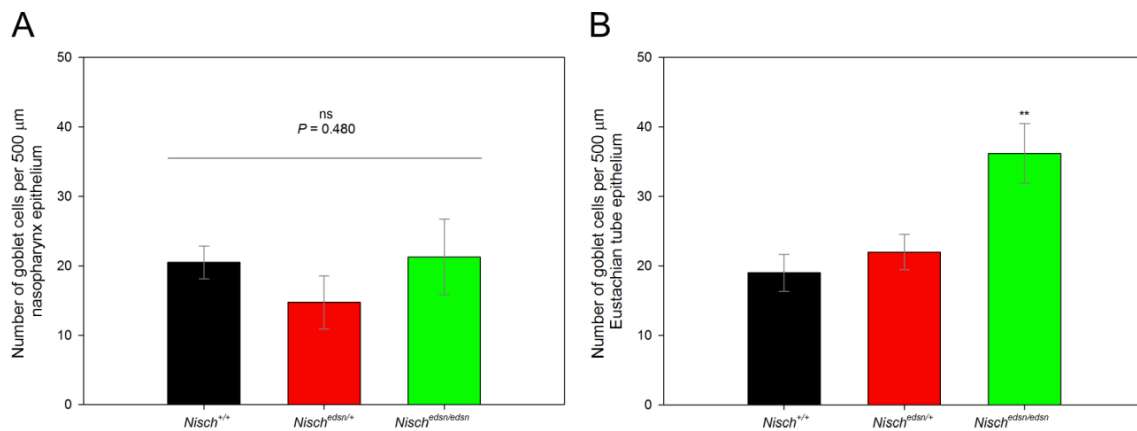


Figure 4.26 | Analysis of goblet cell histology from *edison* animals.

(A) Histogram showing counts of goblet cells per 500 μ m in nasopharynx (NP) epithelium for each genotype. **(B)** Histogram showing counts of goblet cells per 500 μ m in Eustachian tube (ET) epithelium. ns $P > 0.05$; ** $P < 0.01$. Data in panel A was analysed by one-way ANOVA and Holm-Sidak's multiple comparison test for post-hoc analysis. WT data (panel B) was not normally distributed and a Kruskal-Wallis test was performed followed by Dunn's multiple comparison tests for post-hoc testing.

Nisch^{+/+} NP n = 8, ET n = 5; *Nisch^{edsn/+}* NP n = 8, ET n = 6; *Nisch^{edsn/edsn}* NP n = 8, ET n = 10.

4.2.5.1 Cellular exudate in edison MEC rich in macrophages

Chronic inflammation of the ME mucosa in *Nisch^{edsn/edsn}* mice is marked by increased macrophage infiltration in contrast to the uninflamed wild-type ME (Figure 4.27A and B). Sections were stained with the monocyte lineage marker F4/80 antibody showing macrophages in brown. The ME effusions of *Nisch^{edsn/edsn}* mice predominantly consisted of neutrophils with some macrophages, similar to that depicted (Figure 4.27C). In a number of instances, enlarged foamy macrophages containing coarsely granular material were observed within the *Nisch^{edsn/edsn}* exudate as well (Figure 4.27D).

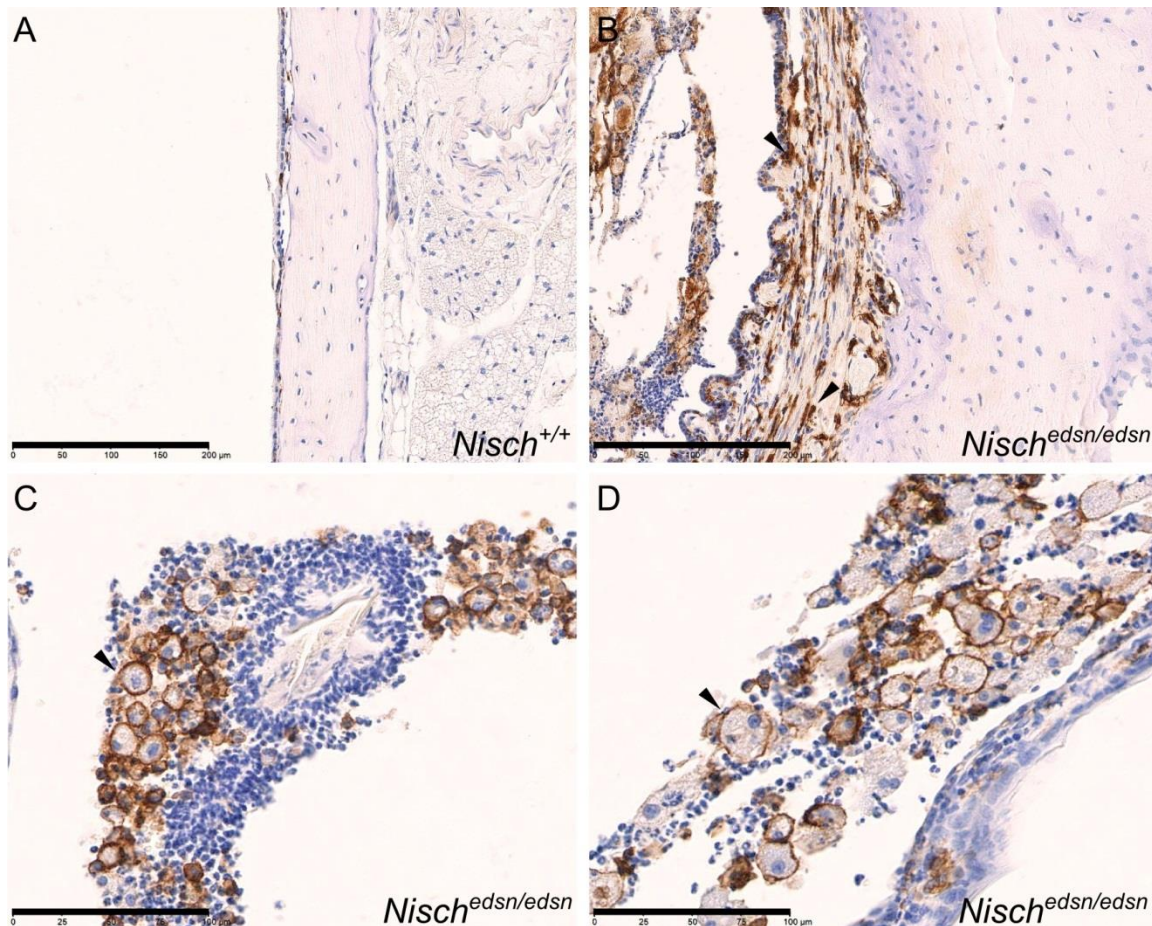


Figure 4.27| Foamy macrophages are found in the mucoperiosteum and exudate in the MEC of *Nisch^{edsn/edsn}* animals.

(A and B) Representative F4/80 stained sections of the MEC showing the mucosal lining in (A) *Nisch^{+/+}* and (B) *Nisch^{edsn/edsn}* mice. In the chronically inflamed ME of *Nisch^{edsn/edsn}* mice, macrophages (arrowheads) infiltrate the thickened mucoperiosteum. **(C and D)** ME exudates rich in inflammatory cells in *Nisch^{edsn/edsn}* mice. (C) Most exudates are rich in both neutrophils and macrophages. (D) In some exudates enlarged macrophages containing coarsely granular material are seen. The sections were stained with the monocyte lineage marker F4/80 antibody and counterstained; showing neutrophils (purple) and foamy macrophages (brown). (A and B) Scale bar = 200 µm; (C and D) Scale bar = 100 µm.

4.2.6 Gene expression of ME fluids in *Nisch^{edsn/edsn}* mice shows upregulation in genes associated with OM

ME exudates were collected from 20wk *Nisch^{edsn/edsn}* mice for gene expression analysis using RT-qPCR. *Nisch^{edsn/edsn}* venous blood white blood cells (WBC) were used as a baseline control to calculate the fold-change (the normalised gene expression in the bulla fluid sample divided by the normalised gene expression in the

control blood sample). TaqMan assays for five genes associated with inflammation and hypoxia (*Hif1-a*, *Il-1 β* , *Tnfa*, *Vegfa* and *Src*) were selected for analysis, along with *Evi1* and *Fbxo11*, which are the causative genes in the OM mouse models *Junbo* and *Jeff*, respectively. Three biological replicates (pools of 4 exudate/blood samples) and three technical replicates were run for each assay. Wild-type and *Nisch^{edsn/+}* animals were not assayed as they do not develop OM and have no obtainable ear fluid.

Analysis of ear fluids from *Nisch^{edsn/edsn}* mice at 20wk shows that significant elevation of *Hif-1a* and *Vegfa* expression occurs in OM when compared to the normoxic control of blood serum (Figure 4.28). Ear fluid aspirated from *Nisch^{edsn/edsn}* mice demonstrates *Hif-1a* expression at 30.48 fold above blood and the HIF responsive gene *Vegfa* was elevated 87.12 fold ($P < 0.001$ for both). *Il-1 β* and *Tnfa* are known modulators of *Hif-1a* translation and expression data indicated that they were significantly elevated in *Nisch^{edsn/edsn}* ($P = 0.003$ and $P < 0.001$ respectively). The fold change for *Il-1 β* was 15.10 and for *Tnfa* it was 66.13. *Src* has been implicated in the regulation of the macrophage inflammatory response and expression in *Nisch^{edsn/edsn}* exudates is also significantly elevated 23.87 fold ($P = 0.005$). *Evi1* and *Fbxo11* were expressed in the bulla fluid of *Nisch^{edsn/edsn}* mice, but only *Evi1* (256.23 fold; $P = 0.006$) was expressed at higher levels relative to a normoxic baseline control of *Nisch^{edsn/edsn}* venous blood WBC.

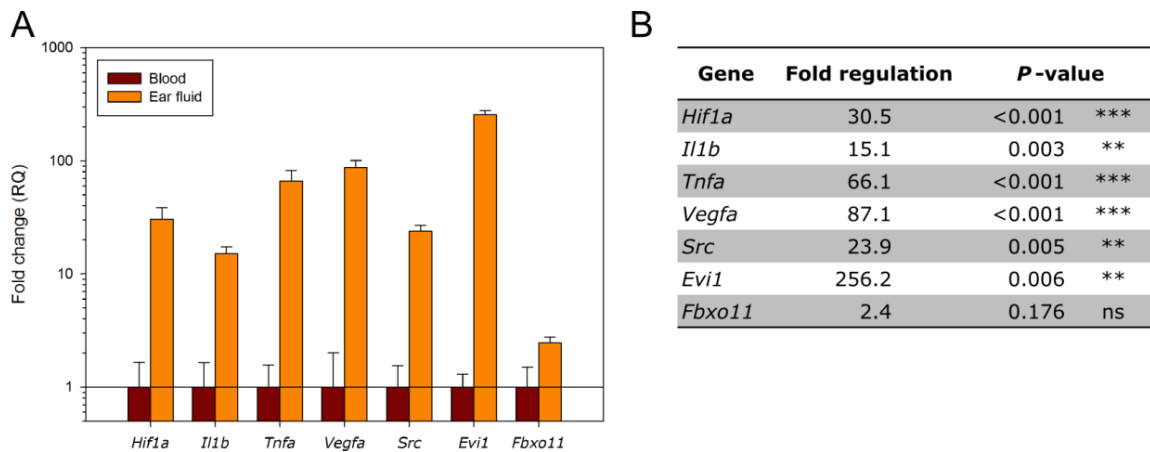


Figure 4.28 | Relative gene expression of ME fluids from *Nisch^{edsn/edsn}* mice at 20wk.

ME fluids from *Nisch^{edsn/edsn}* mice were collected and pools created for RT-qPCR to analyse relative expression of key genes associated with OM (*Hif-1a*, *Il1-β*, *Tnfa*, *Vegfa*, *Src*, *Evi1*, *Fbxo11*). **(A)** Relative Quantification (RQ) of gene expression in 20wk *Nisch^{edsn/edsn}* bulla fluid compared with blood WBC. **(B)** Table showing fold change differences and statistical significance of gene expression data. *P*-values are based on a Student's *t*-test of the replicate $2^{(-\Delta Ct)}$ values. Data represents mean RQ $\pm$ min/max 95% CL. Blood, *n* = 3 pools; Ear fluid, *n* = 3 pools.

At 2wk, 67% of *Nisch^{edsn/edsn}* ears contained a thin watery effusion (as mentioned in section 4.2.5). Ear fluids were collected at 2wk and analysed for expression of the same genes as investigated with 20wk ear fluid (Figure 4.29). All the genes analysed were expressed in the bulla fluid of 2wk *Nisch^{edsn/edsn}* mice, but only *Il-1β* (4.06 fold; *P* = 0.028), *Vegfa* (11.16 fold; *P* = 0.028) and *Src* (8.09 fold; *P* = 0.029) were expressed at higher levels relative to the *Nisch^{edsn/edsn}* venous blood WBC baseline. When compared to elevated levels in ME fluid from 20wk *Nisch^{edsn/edsn}* mice they were much less elevated.

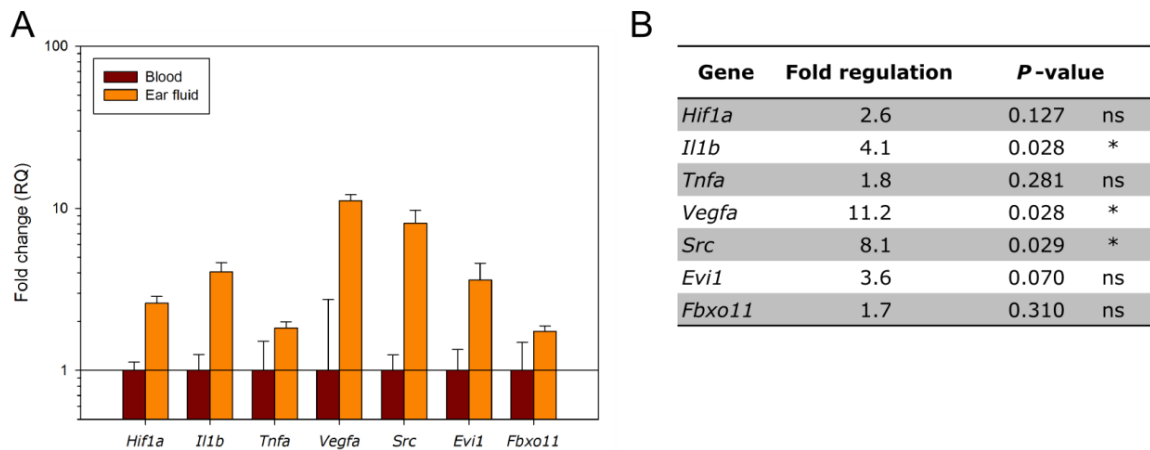


Figure 4.29| Relative gene expression of ME fluids from *Nisch*^{edsn/edsn} mice at 2wk.

ME fluids from *Nisch*^{edsn/edsn} mice were collected and pools created for RT-qPCR to analyse relative expression of key genes associated with OM (*Hif-1a*, *Il1-β*, *Tnfa*, *Vegfa*, *Src*, *Evi1*, *Fbxo11*). **(A)** Relative Quantification (RQ) of gene expression in 2wk *Nisch*^{edsn/edsn} bulla fluid compared with blood WBC. **(B)** Table showing fold change differences and statistical significance of gene expression data. *P*-values are based on a Student's t-test of the replicate $2^{(-\Delta\Delta Ct)}$ values. Data represents mean RQ $\pm$ min/max 95% CL. Blood, *n* = 3 pools; Ear fluid, *n* = 3 pools.

4.2.7 Inner ear morphology assessed

As mentioned in the section 4.1.1 (Preliminary characterisation of *edison*), one mouse from the *edison* founder pedigree litters circled during the swim test from the preliminary SHIRPA tests undertaken (Table 4.1; PEDV/7.2h). Mice with vestibular anomalies normally display deficits in swim tests. PEDV/7.2h showed a normal wild-type phenotype in the trunk curl test, which is also a test to discover vestibular anomalies. Also, a number of *Nisch*^{edsn/edsn} mice displayed a profound elevation in hearing thresholds, as high as 80 dB SPL, which is greater than expected for a conductive hearing loss. COM can be associated with sensorineural hearing loss (Cureoglu, Schachern et al. 2004). To investigate whether the hearing loss seen in *Nisch*^{edsn/edsn} mice was only a conductive hearing loss, with no additional inner ear defects confounding the hearing deficits observed, the multi-frequency auditory function and the inner ear morphology was evaluated in *edison* mice.

4.2.7.1 No vestibular anomalies observed in congenic *edison* mice

To evaluate whether there was any vestibular component to the hearing phenotype observed in *edison* mice, a number of tests were utilised to assess vestibular dysfunction with congenic *edison* animals. Firstly, a trunk curl test was undertaken to examine the ability of mice when held by the tail to reach out to a presented horizontal landing surface. Control mice reach towards the landing surface, whereas mice with a severe vestibular anomaly curl towards their abdomen (Trunk curl: 0 = Absent; 1 = Present). Subsequently, contact righting reflex was analysed to assess if animals will respond to pressure applied to the feet, where mice are placed in a perspex tube and inverted. Control littermates will ignore this surface and rotate back to their upright position, while animals with vestibular impairment would remain inverted (Contact righting reflex: 0 = Present; 1 = Absent). Finally, a swim test was carried out where mice have to rely on vestibular information for orientation and irregular swimming reveals mutants with vestibular abnormalities (Swim: 0 = swims (mouse body is elongated and the tail propels in a flagella-like motion); 1 = Irregular swimming (Includes: vertical swimming, swimming in a circle, swimming on side)). All *edison* genotypes recorded normal scores for all of the vestibular tests undertaken (Table 4.10), hence there appears to be no vestibular component contributing to the hearing loss in *Nisch^{edsn/edsn}* mice.

Table 4.10| Vestibular phenotyping in *edison* mice.

Six mice of each *edison* genotype (*Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{edsn/edsn}) were tested for trunk curl, contact righting reflex and swim response. No differences were observed between genotypes.

Trunk curl: 0 = Absent; 1 = Present.

Contact righting reflex: 0 = Present; 1 = Absent.

Swim: 0 = swims (mouse body is elongated and the tail propels in a flagella-like motion); 1 = Irregular swimming (Includes: vertical swimming, swimming in a circle, swimming on side).

Mouse ID	Genotype	Trunk curl	Contact righting reflex	Swim
EDSN/119.2b	<i>Nisch</i> ^{+/+}	0	0	0
EDSN/119.2e	<i>Nisch</i> ^{+/+}	0	0	0
EDSN/119.2e	<i>Nisch</i> ^{+/+}	0	0	0
EDSN/120.1b	<i>Nisch</i> ^{+/+}	0	0	0
EDSN/120.2a	<i>Nisch</i> ^{+/+}	0	0	0
EDSN/120.2b	<i>Nisch</i> ^{+/+}	0	0	0
EDSN/111.2a	<i>Nisch</i> ^{edsn/+}	0	0	0
EDSN/111.2b	<i>Nisch</i> ^{edsn/+}	0	0	0
EDSN/111.2b	<i>Nisch</i> ^{edsn/+}	0	0	0
EDSN/116.2b	<i>Nisch</i> ^{edsn/+}	0	0	0
EDSN/117.6c	<i>Nisch</i> ^{edsn/+}	0	0	0
EDSN/120.3a	<i>Nisch</i> ^{edsn/+}	0	0	0
EDSN/103.6b	<i>Nisch</i> ^{edsn/edsn}	0	0	0
EDSN/103.6b	<i>Nisch</i> ^{edsn/edsn}	0	0	0
EDSN/110.13d	<i>Nisch</i> ^{edsn/edsn}	0	0	0
EDSN/110.13d	<i>Nisch</i> ^{edsn/edsn}	0	0	0
EDSN/118.3c	<i>Nisch</i> ^{edsn/edsn}	0	0	0
EDSN/122.2d	<i>Nisch</i> ^{edsn/edsn}	0	0	0

4.2.7.2 Frequency specific auditory function consistent with conductive hearing loss

In order to assess whether there was any sensorineural element to the hearing phenotype observed in *edison* mice, frequency specific toneburst-evoked ABR tests for auditory function were conducted across a time course with cohorts of age matched littermates (Figure 4.30). Hearing assessments were conducted with cohorts at 4, 6, 8, 12 and 20wk. Mice were anesthetised and hearing thresholds determined using specific frequencies of 8, 16, and 32 kHz, ranging from 90 dB SPL to 5 dB SPL in decreasing 5 dB increments. Click-evoked ABR thresholds were also

measured as a baseline for the OM phenotype. The ABR threshold was measured for the right ear only.

Across all frequencies *Nisch*^{edsn/edsn} mice displayed elevated ABR thresholds at each time point (Figure 4.30). Hearing loss progressively deteriorated with age, displayed by increasingly elevated ABR thresholds. *Nisch*^{edsn/+} littermates displayed normal, wild-type hearing thresholds of between 15-30 dB SPL and there was no significant difference compared to *Nisch*^{+/+}. At 4wk, mice with the *Nisch*^{edsn/edsn} mutation were found to have significantly elevated auditory thresholds for both 8 kHz ($P = 0.011$) and click-evoked ($P = 0.004$) ABR data, with a mean ABR threshold of 37.0 dB SPL (± 3.74) and 47.0 dB SPL (± 7.00) respectively. By 12wk, *Nisch*^{edsn/edsn} mice displayed significantly elevated ABR thresholds in all frequencies tested (8 kHz, $P = 0.013$; 16 kHz, $P = 0.005$; 32 kHz, $P = 0.022$; click, $P = 0.003$). This significant increase continued at 20wk, where mice with the *Nisch*^{edsn/edsn} mutation were recorded with a mean ABR threshold of 49.0 dB SPL (± 6.40) at 8 kHz, 43.0 dB SPL (± 4.64) at 16 kHz, 47.0 dB SPL (± 5.61) at 32 kHz and 53.0 dB SPL (± 6.82) with click-evoked ABR (8 kHz, $P = 0.002$; 16 kHz, $P < 0.001$; 32 kHz, $P = 0.001$; click, $P = 0.008$). The ABR data from all three frequencies analysed duplicated the progressive hearing loss observed in the click-evoked ABR data with an increase in ABR threshold of ~ 30 dB SPL by 20wk (Figure 4.30E). This indicates that it is probable there is no additional sensorineural hearing loss confounding the conductive hearing loss caused by the OM phenotype.

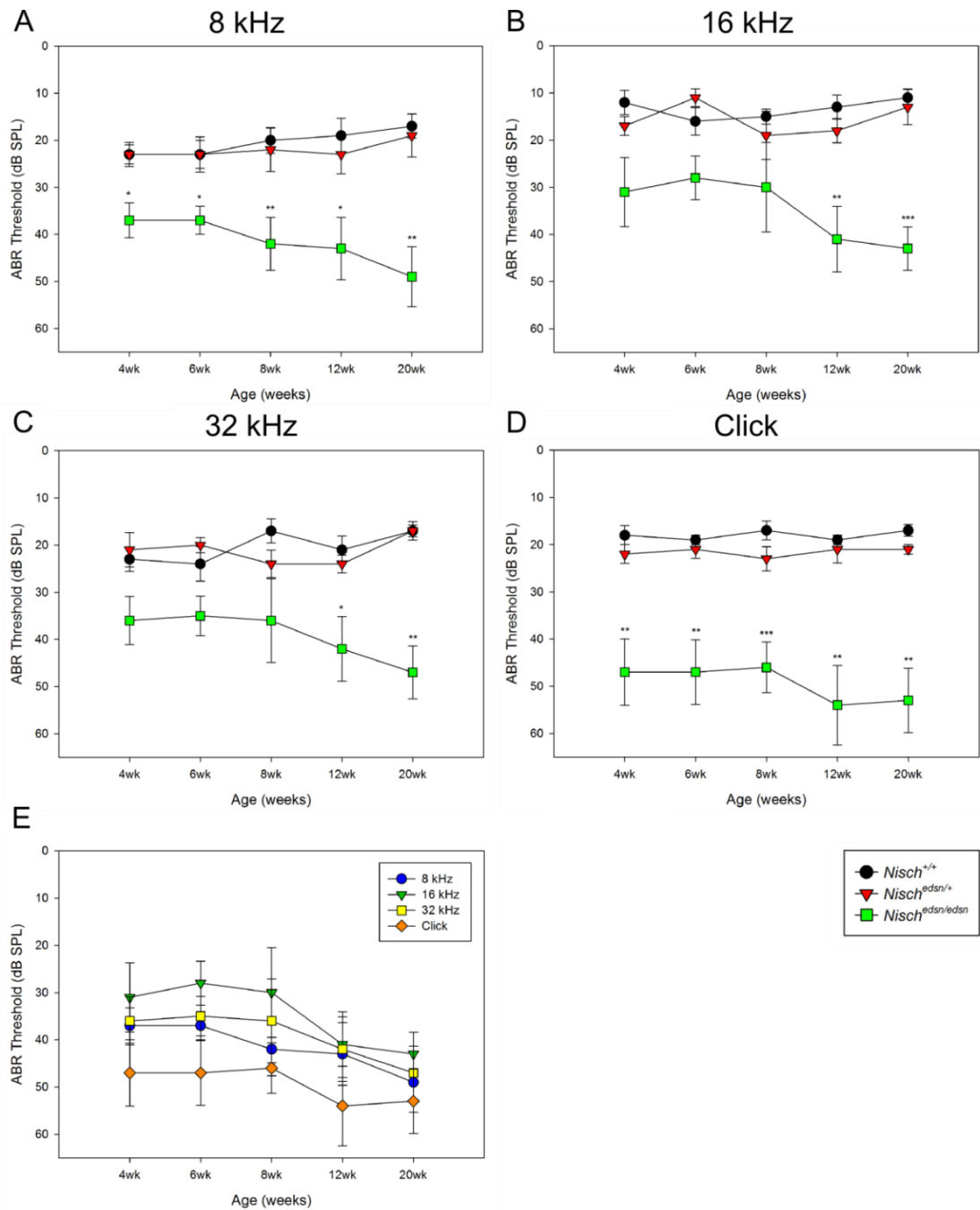


Figure 4.30| Multi-frequency ABR thresholds throughout a longitudinal time course for *edison* mice.

Line and scatter plots show the ABR thresholds of all *edison* genotypes tested at **(A)** 8 kHz, **(B)** 16 kHz, **(C)** 32 kHz and **(D)** click-evoked. At each specific frequency tested, mice with the *Nisch*^{edsn/edsn} mutation had a progressive hearing loss similar to that observed with the click-evoked ABR. There are no significant differences in hearing thresholds between *Nisch*^{edsn/+} mice and wild-type littermates. **(E)** Parallel shifts in audiometric profiles across frequencies at the different ages are consistent with an underlying conductive hearing loss. ABR thresholds were measured for right ear only. $n = 5$ for all genotypes at each time point. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

4.2.7.3 Organ of Corti displays no abnormal morphology

H&E stained sections can be used to examine the inner ear morphology, specifically the cochlea and the organ of Corti. Histopathological examination of *edison* mice at 20wk demonstrated that there were no genotypic differences in inner ear morphology (Figure 4.31). The cochlea morphology appears normal, with the organ of Corti, spiral ganglion, spiral ligament and Reissner's membrane all present throughout the basal, mid and apical cochlear turns (Figure 4.31A and B). There was no loss of spiral ganglion neurons. At closer observation the organ of Corti also appears normal, with three rows of outer hair cells and one row of inner hair cells (Figure 4.31C and D). This is again observed throughout the cochlear turn. The histology of *Nisch^{edsn/edsn}* mice was indistinguishable from *Nisch^{edsn/+}* and wild-type littermates.

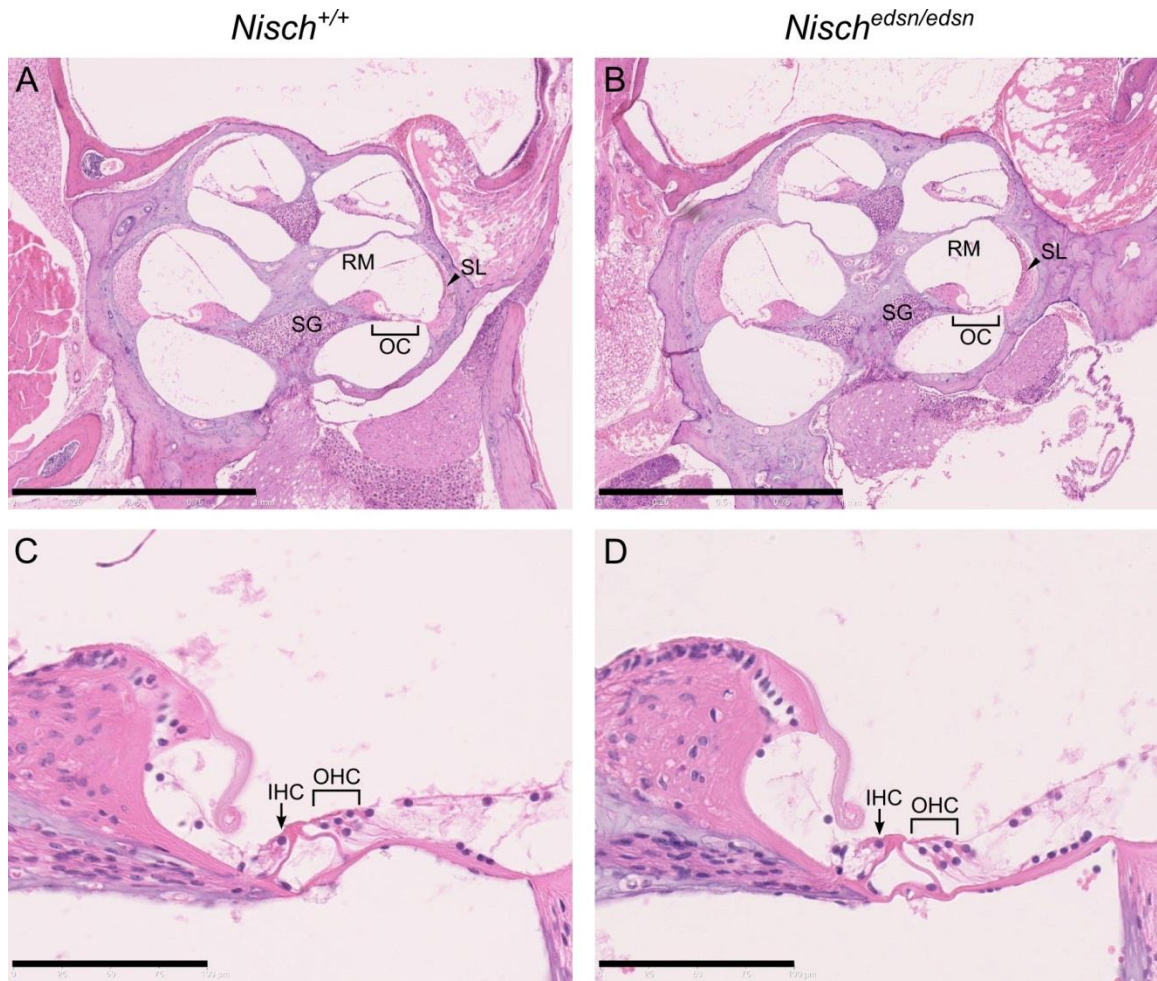


Figure 4.31| Histological analysis of the inner ear morphology in *edison* mice at 20wk.

(A & B) Representative H&E stained mid-modiolar sections of the cochlea from (A) *Nisch*^{+/+} and (B) *Nisch*^{edn/edn} mice. Cross-sections of the basal, mid and apical turns are visible as they spiral around the modiolus. **(C & D)** Representative H&E stained sections of the organ of Corti from (C) *Nisch*^{+/+} and (D) *Nisch*^{edn/edn} mice. There are no differences observed between genotypes in the morphology of the organ of Corti throughout the turns of the cochlea (basal, mid and apical). OC, organ of Corti; SG, spiral ganglion; SL, spiral ligament; RM, Reissner's membrane; OHC, outer hair cell; IHC, inner hair cell. *n* = 5 for each genotype. (A & B) Scale bar = 1 mm; (C & D) Scale bar = 100 μm.

To further establish whether there was a sensorineural element to the hearing loss seen in *Nisch*^{edn/edn} mice, scanning electron microscopy (SEM) was used for ultra-structural analysis of the inner ear. Both outer and inner ear hair cells of the inner ear were examined and there were no obvious abnormalities, such as hair cell damage or hair cell loss. All through the cochlear turn (basal, mid and apical),

normal cell and bundle morphology was observed (Figure 4.32A and B). No defects were observed in stereocilia bundles in *Nisch^{edsn/edsn}* mice compared to wild-type littermates, with well organised rows of outer hair cells and normal sized inner hair cells in the organ of Corti (Figure 4.32C and D). Two of the *Nisch^{edsn/edsn}* ears that were used for SEM had a higher than average ABR threshold of 70 dB SPL and still they both showed no inner ear abnormalities.

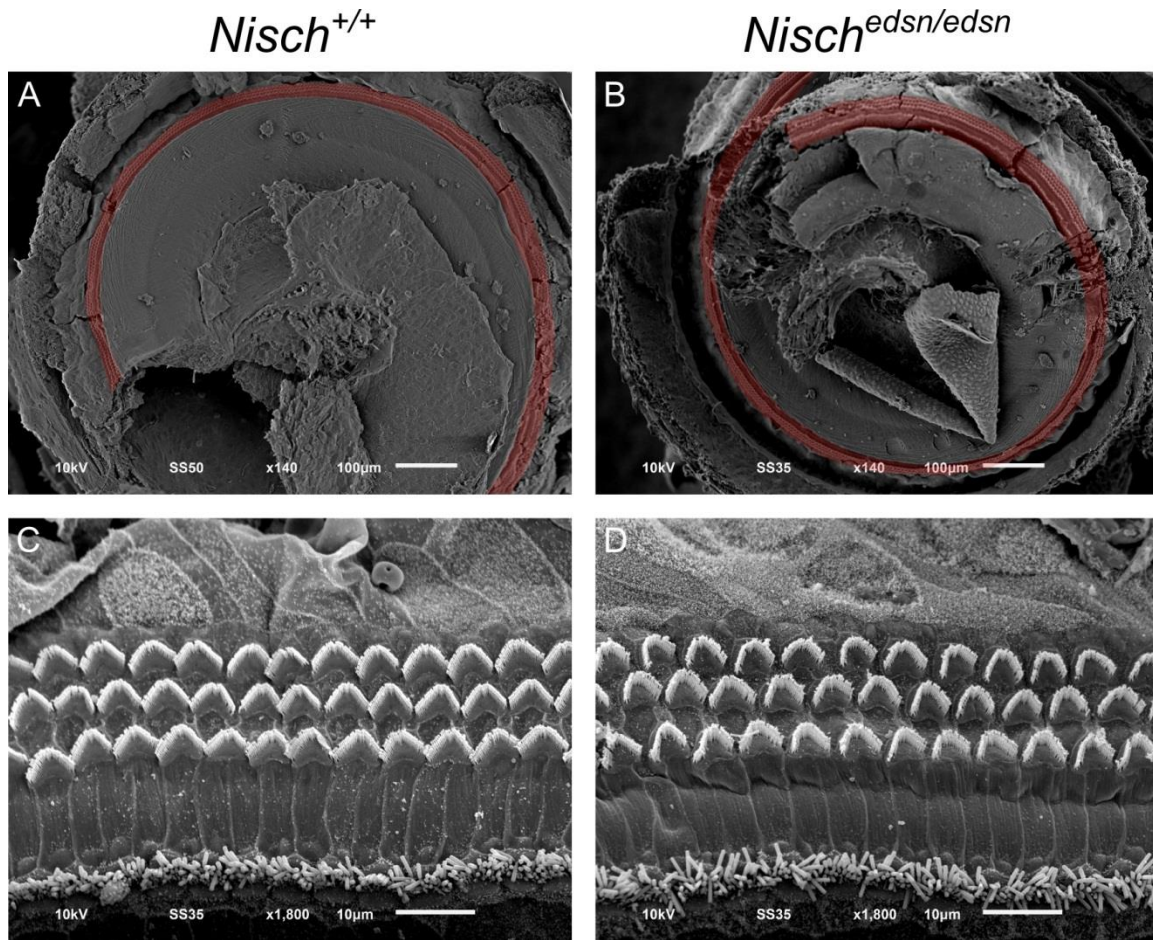


Figure 4.32| Scanning electron microscopy (SEM) images of inner ear from *edison* mice.

(A & B) Representative SEM images of the cochlear spiral from (A) *Nisch*^{+/+} and (B) *Nisch*^{edsn/edsn} mice. The bony capsule, stria and tectorial membrane have been removed. The organ of Corti is highlighted in red. **(C & D)** Representative SEM images of the organ of Corti, showing hair cell morphology from (C) *Nisch*^{+/+} and (D) *Nisch*^{edsn/edsn} mice. Outer hair cells are the three upper rows with inner hair cells the single lower row. There are no differences between genotypes observed in the morphology of the organ of Corti and the hair cell bundles for both OHCs and IHCs. This is observed throughout the entire cochlea spiral (basal, mid and apical). SEM acceleration voltage was 10 kV. SS, spot size. n = 5 for all genotypes.

4.2.8 Analysis of lungs in adult *edison* mice

Nisch^{edsn/edsn} animals display a perinatal lung phenotype, as described in section 4.2.2. A comprehensive investigation into inflammatory response, using widespread pathology phenotyping, was undertaken to investigate if there was an inflammatory response observed outside of the ME in adult *Nisch*^{edsn/edsn} mice, which might be a sign of an immune deficiency. Widespread inflammation was not observed in *edison*

mice of any genotype (see Appendix, Figure 10.4). However, histologic evaluation of lungs revealed enlargement of the air spaces accompanied by destruction of the normal alveolar architecture in *Nisch^{edsn/edsn}* mice, characteristic of emphysema (Figure 4.33A).

4.2.8.1 Histological analysis of edison lungs

In order to quantify if there were significant genotypic differences in the lung morphology of adult *edison* mice, counts of the number of airways were conducted (Figure 4.33B) and the average airway widths were measured (Figure 4.33C).

Counts of the number of airways showed a significant decrease in the number of airways between *Nisch^{edsn/edsn}* and wild-type mice ($P < 0.001$). The average number of airways in *Nisch^{edsn/edsn}* animals was 128 (± 10.5) and for *Nisch^{+/+}* animals was 250 (± 6.4). No significant difference was observed between *Nisch^{edsn/+}* animals and wild-type littermates, with the average number of airways in *Nisch^{edsn/+}* animals as 255 (± 4.2).

Nisch^{edsn/edsn} mice also showed a difference in airway width, however it was a significant increase in the measurements compared to wild-type ($P < 0.001$). The average airway width in *Nisch^{edsn/edsn}* animals was 71.16 μm (± 2.883) and for *Nisch^{+/+}* animals was 36.42 μm (± 0.658). Again no significant difference was observed between *Nisch^{edsn/+}* animals and wild-type littermates, with the average airway width in *Nisch^{edsn/+}* animals as 35.97 μm (± 0.524).

Similar to what was observed in the ME of *Nisch^{edsn/edsn}* mice, the lung phenotype was not fully penetrant and some *Nisch^{edsn/edsn}* lungs had a wild-type appearance. The measurements recorded from *Nisch^{edsn/edsn}* lungs were categorised into two groups;

lungs that had an emphysema-like phenotype, affected *Nisch*^{edsn/edsn}; and lungs with a normal wild-type phenotype, unaffected *Nisch*^{edsn/edsn} (Figure 4.33D and E).

Counts of the number of airways again showed a significant decrease in the number of airways in affected *Nisch*^{edsn/edsn} animals compared to wild-type littermates ($P < 0.001$). The mean number of airways in affected *Nisch*^{edsn/edsn} animals was 112 (± 7.1). Interestingly, a significant difference was still observed between unaffected *Nisch*^{edsn/edsn} animals and wild-type littermates ($P < 0.001$), but the mean number of airways was almost double that seen in affected lungs at 206 (± 8.3).

Affected *Nisch*^{edsn/edsn} mice still showed a significant increase in airway width compared to wild-type ($P < 0.001$). The average airway width in affected *Nisch*^{edsn/edsn} animals was slightly higher at 78.22 μm (± 2.266). No significant difference was observed between unaffected *Nisch*^{edsn/edsn} animals and wild-type littermates, the average airway width was 35.89 μm (± 1.616).

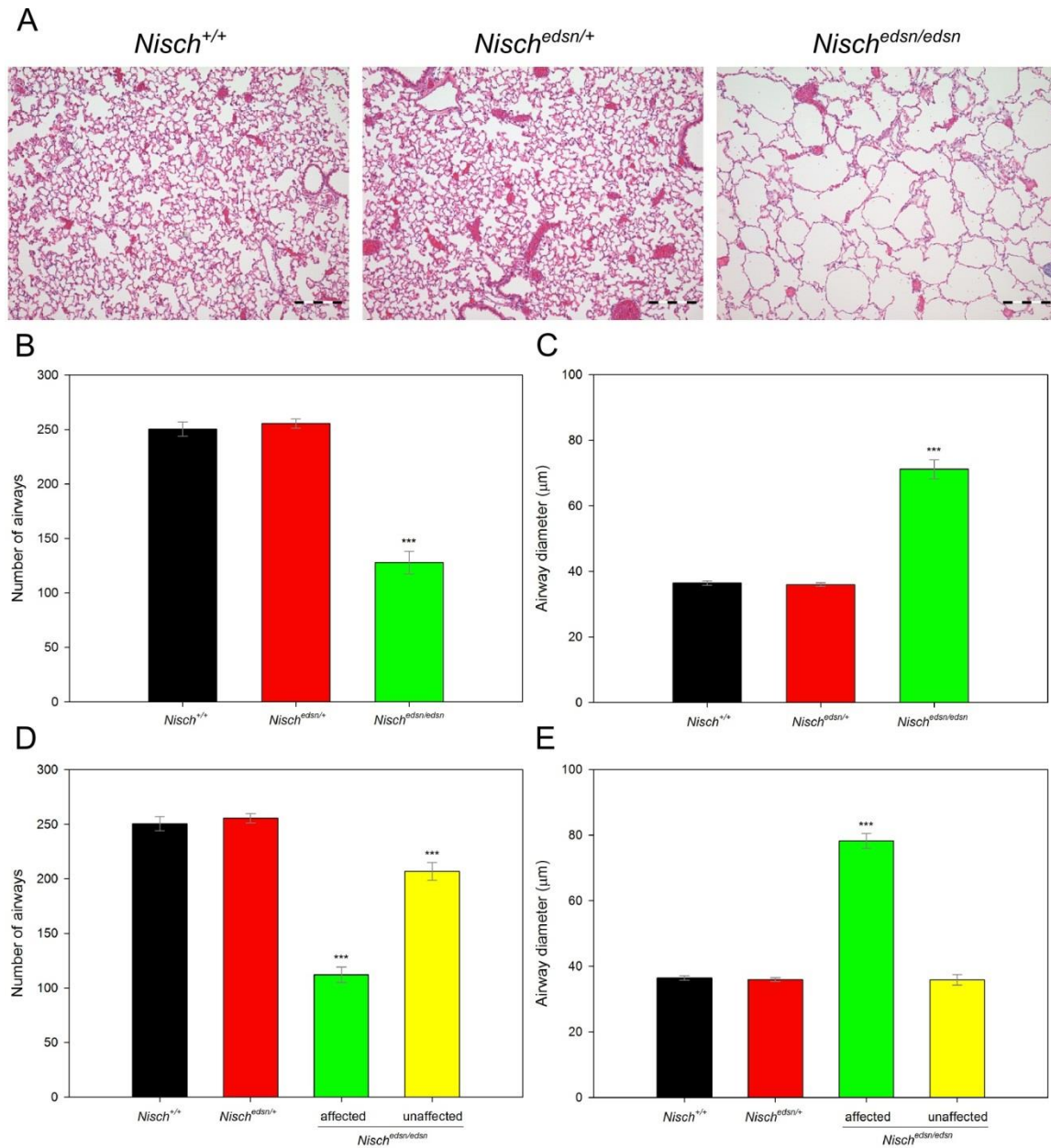


Figure 4.33 | Analysis of adult lung histology from *edison* animals.

(A) Representative H&E stained lung sections from *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{edsn/edsn} animals at 20wk. Scale bars = 100 μ m. **(B)** Bar chart showing counts of lung airways for each genotype. Airways were counted in three different $6.5 \times 10^5 \mu\text{m}^2$ regions for all lungs. **(C)** Bar chart showing airway diameters for each genotype. Airway diameters were measured in three different regions for all lungs. **(D)** *Nisch*^{edsn/edsn} number of airways data used in (B) was separated in to two groups, affected *Nisch*^{edsn/edsn} and unaffected *Nisch*^{edsn/edsn}. **(E)** *Nisch*^{edsn/edsn} airway diameter data used in (C) was separated in to two groups, affected *Nisch*^{edsn/edsn} and unaffected *Nisch*^{edsn/edsn}. *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis. *Nisch*^{+/+} $n = 30$; *Nisch*^{edsn/+} $n = 30$; *Nisch*^{edsn/edsn} $n = 54$; affected *Nisch*^{edsn/edsn} $n = 45$; unaffected *Nisch*^{edsn/edsn} $n = 9$.

When categorising the lungs from *Nisch^{edsn/edsn}* mice into affected and unaffected groups, I noticed that the lungs that appeared unaffected also had no OM phenotype and the most affected lungs displayed a bilateral OM phenotype. I decided to group the measurements recorded from *Nisch^{edsn/edsn}* lungs into three categories corresponding to the severity of OM phenotype; bilateral OM, unilateral OM and clear. Interestingly, the severity of the emphysema-like lung phenotype observed in *Nisch^{edsn/edsn}* replicates the severity of the OM phenotype in the ME (Figure 4.34A).

In the number of airways counted from *Nisch^{edsn/edsn}* lungs a significant difference was implicated when comparing clear and bilateral OM mice ($P < 0.001$; Figure 4.34B). The mean number of airways in clear *Nisch^{edsn/edsn}* animals was 200 (± 6.8) and for *Nisch^{edsn/edsn}* animals with bilateral OM the mean was 104 (± 6.8). *Nisch^{edsn/edsn}* mice with unilateral OM also showed a significant difference to bilateral OM *Nisch^{edsn/edsn}* animals ($P = 0.002$). Unilateral OM animals had a mean of 144 (± 19.5). No significant difference was observed in lungs between *Nisch^{edsn/edsn}* animals with no OM phenotype and those with unilateral OM.

Similar associations were observed in airway width data from *Nisch^{edsn/edsn}* lungs (Figure 4.34C). Mice with no OM phenotype showed a significant difference in airway width compared to both unilateral OM and bilateral OM mice ($P < 0.001$ for both). The average airway width in clear *Nisch^{edsn/edsn}* animals was 36.38 μm (± 1.313). In animals with unilateral OM the mean airway width was 67.31 μm (± 4.413), and in bilateral animals the mean was 80.43 μm (± 2.971). A significant difference was also observed between mice with bilateral OM and those with unilateral OM ($P = 0.010$).

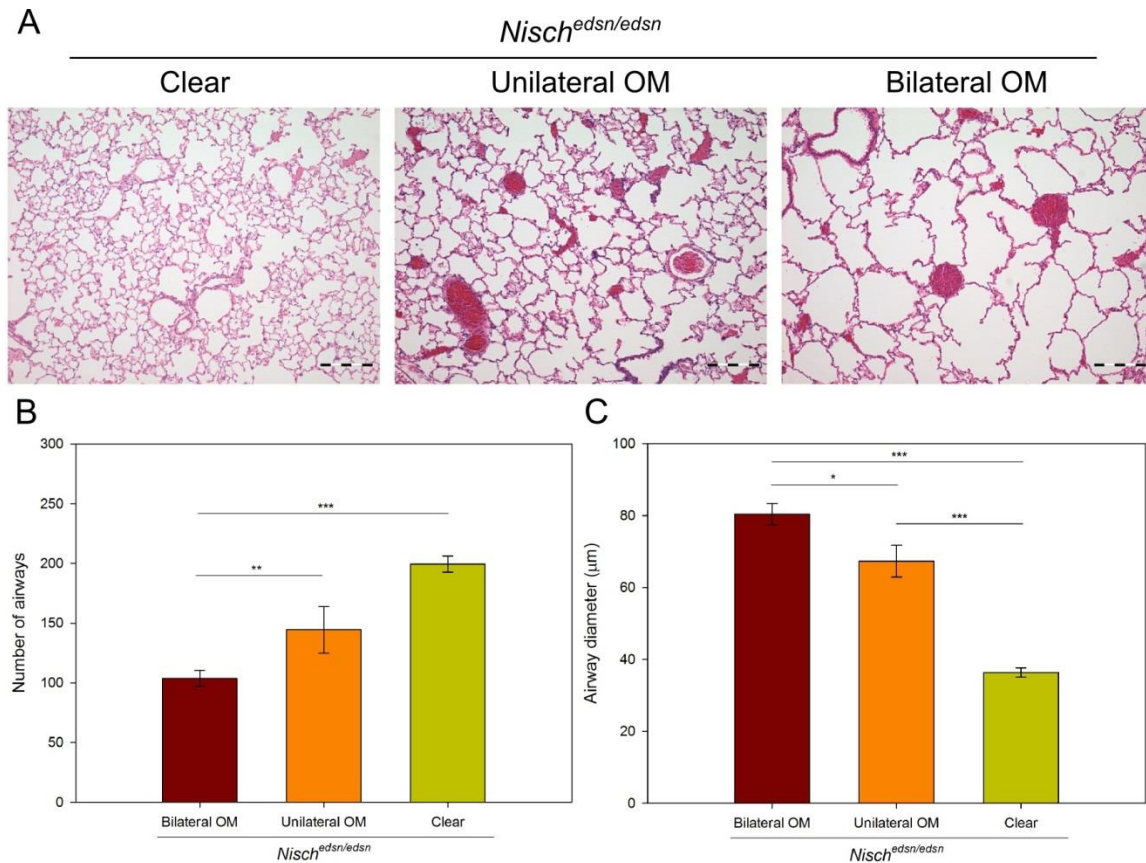


Figure 4.34 | Association between severity of OM phenotype and adult lung histology of *Nisch^{edsn/edsn}* animals.

Nisch^{edsn/edsn} data used in Figure 4.33 was separated into three groups; mice with bilateral OM, mice with unilateral OM and mice with no OM phenotype. **(A)** Representative H&E stained lung sections from *Nisch^{edsn/edsn}* animals at 20wk with bilateral OM, unilateral OM and no OM phenotype. Scale bars = 100 μm. **(B)** Bar chart showing counts of lung airways for *Nisch^{edsn/edsn}* animals with varying incidence of OM. Airways were counted in three different $6.5 \times 10^5 \mu\text{m}^2$ regions for all lungs. **(C)** Bar chart showing airway diameters for *Nisch^{edsn/edsn}* animals with varying incidence of OM. Airway diameters were measured in three different regions for all lungs. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Bilateral OM n = 30; Unilateral OM n = 18; Clear n = 6.

Alveolar macrophages are the primary phagocytic cells in the uninflamed lower airways. Lung sections were stained with the monocyte lineage marker F4/80 antibody showing macrophages in brown. In wild-type lungs, F4/80-positive cells are generally relatively small and rounded, and are found within the lung parenchyma or attached to the airway wall (Figure 4.35A). In contrast, abnormal accumulations of

enlarged alveolar macrophages containing coarsely granular material were observed within the emphysematous lungs of *Nisch^{edsn/edsn}* mice (Figure 4.35B).

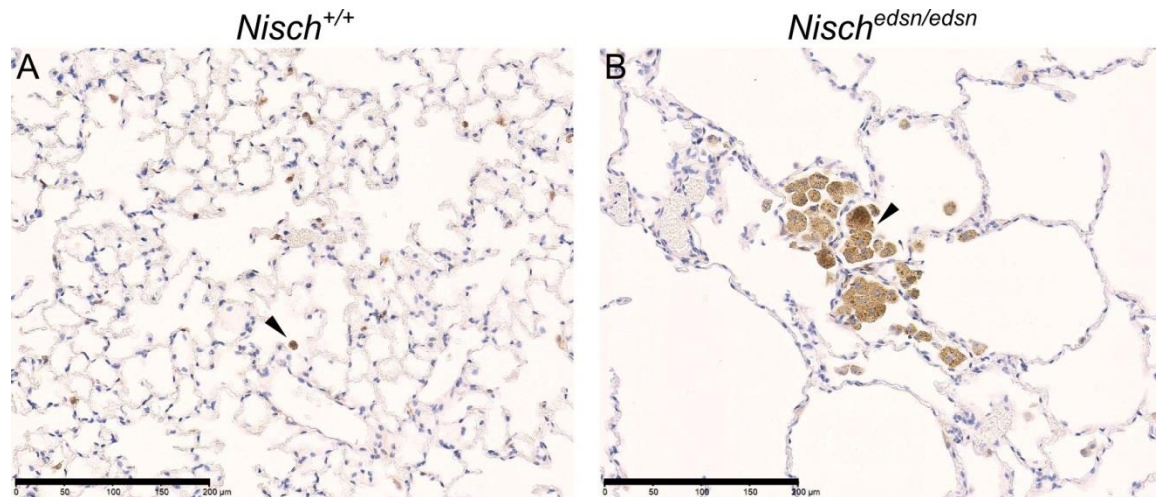


Figure 4.35| Clusters of enlarged alveolar macrophages containing coarsely granular material are found in emphysematous *Nisch^{edsn/edsn}* lungs.

Representative F4/80 stained lung sections from (A) *Nisch^{+/+}* and (B) *Nisch^{edsn/edsn}* animals at 20wk. Collections of enlarged alveolar macrophages containing coarsely granular material are seen in *Nisch^{edsn/edsn}* lungs compared to wild-type littermates. The sections were stained with the monocyte lineage marker F4/80 antibody and counterstained; showing alveolar macrophages (brown). Scale bars = 200 μ m.

4.2.8.2 Whole body plethysmography indicates impaired ventilatory response from *Nisch^{edsn/edsn}* lungs

To analyse whether lung function was affected by the emphysema-like phenotype observed in *Nisch^{edsn/edsn}* animals, whole body plethysmography was undertaken. A dual challenge approach was used; firstly the mice underwent a hypoxia-hypercapnia challenge, followed secondly by a methacholine challenge. The hypoxia-hypercapnia challenge consisted of a 5 minute exposure to 10% O₂ and 3% CO₂ in the inspired gas mixture, which was preceded and followed by a baseline and recovery period of 21% O₂. The methacholine challenge involved inhalation of increasing concentrations of nebulised methacholine (0 mg/ml (PBS only), 12.5 mg/ml, 25 mg/ml and 50

mg/ml). Methacholine causes bronchoconstriction by acting as a non-selective muscarinic receptor agonist. Four parameters were used to analyse the ventilatory response in *edison* mice; breathing frequency (f [breaths/min]), tidal volume (TVb [ml]; the inspired volume), minute volume (MVb [ml/min]; product of the tidal volume and respiratory rate) and enhanced pause (Penh; an indicator of bronchoconstriction).

4.2.8.2.1 Hypoxia-hypercapnia response

In resting conditions (normoxia-normocapnia), breathing rate (f) was similar in *Nisch^{edsn/edsn}* mice (454.9 ± 4.15 breaths/min) and their wild-type counterparts (444.8 ± 13.35 breaths/min). When exposed to hypoxic-hypercapnic conditions, both *Nisch^{edsn/edsn}* and wild-type mice showed a reduced breathing rate (Figure 4.36A). However, the reduced breathing rate in response to hypoxia-hypercapnia exposure was significantly greater in *Nisch^{edsn/edsn}* mice compared to wild-type littermates ($P = 0.047$). In 10% O₂; 3% CO₂, *Nisch^{edsn/edsn}* animals had a breathing rate of 366.8 ± 3.22 breaths/min, whereas wild-type animals displayed a breathing rate of 381.0 ± 6.27 breaths/min. During the recovery phase (return to normoxia-normocapnia), the breathing rate in all littermates returned to a similar level as previously observed under resting conditions.

Corresponding breathing rates were normalised against the baseline responsiveness to analyse the fold change in breathing rate for each genotype (Figure 4.36E). Under hypoxic-hypercapnic conditions, *Nisch^{edsn/edsn}* animals had a fold change of 0.81 ± 0.007 compared to baseline responsiveness, whereas wild-type animals displayed a fold change of 0.86 ± 0.027 . The normalised breathing rate data amplified the result seen in Figure 4.36A, again showing a significantly reduced breathing rate in response to hypoxia-hypercapnia exposure in *Nisch^{edsn/edsn}* mice ($P = 0.038$).

Interestingly, during resting conditions enhanced pause (Penh) was significantly increased in *Nisch^{edsn/edsn}* mice compared to wild-type littermates ($P < 0.001$). In 21% O₂, *Nisch^{edsn/edsn}* animals had a Penh of 1.10 ± 0.048 , whereas wild-type animals displayed a Penh of 0.87 ± 0.056 (Figure 4.36D). When exposed to hypoxic-hypercapnic conditions, both *Nisch^{edsn/edsn}* and wild-type mice showed an increased Penh. In 10% O₂; 3% CO₂, *Nisch^{edsn/edsn}* animals had a Penh of 1.48 ± 0.067 , whereas wild-type animals displayed a Penh of 1.33 ± 0.091 . However, even though *Nisch^{edsn/edsn}* mice still observed an increased Penh compared to wild-type mice, this was no longer significant ($P = 0.125$). During the recovery phase (return to normoxia-normocapnia), the Penh in all littermates returned to a similar level as previously observed under resting conditions and again was significantly increased in *Nisch^{edsn/edsn}* mice compared to wild-type littermates ($P < 0.001$).

Tidal volume (TVb) and minute volume (MVb) were also measured throughout the challenge (Figure 4.36B and C respectively), however no significant genotypic differences were observed during both normoxia-normocapnia and hypoxia-hypercapnia. No significant difference was observed between *Nisch^{+/+}* and *Nisch^{edsn/+}* animals at any point during the challenge.

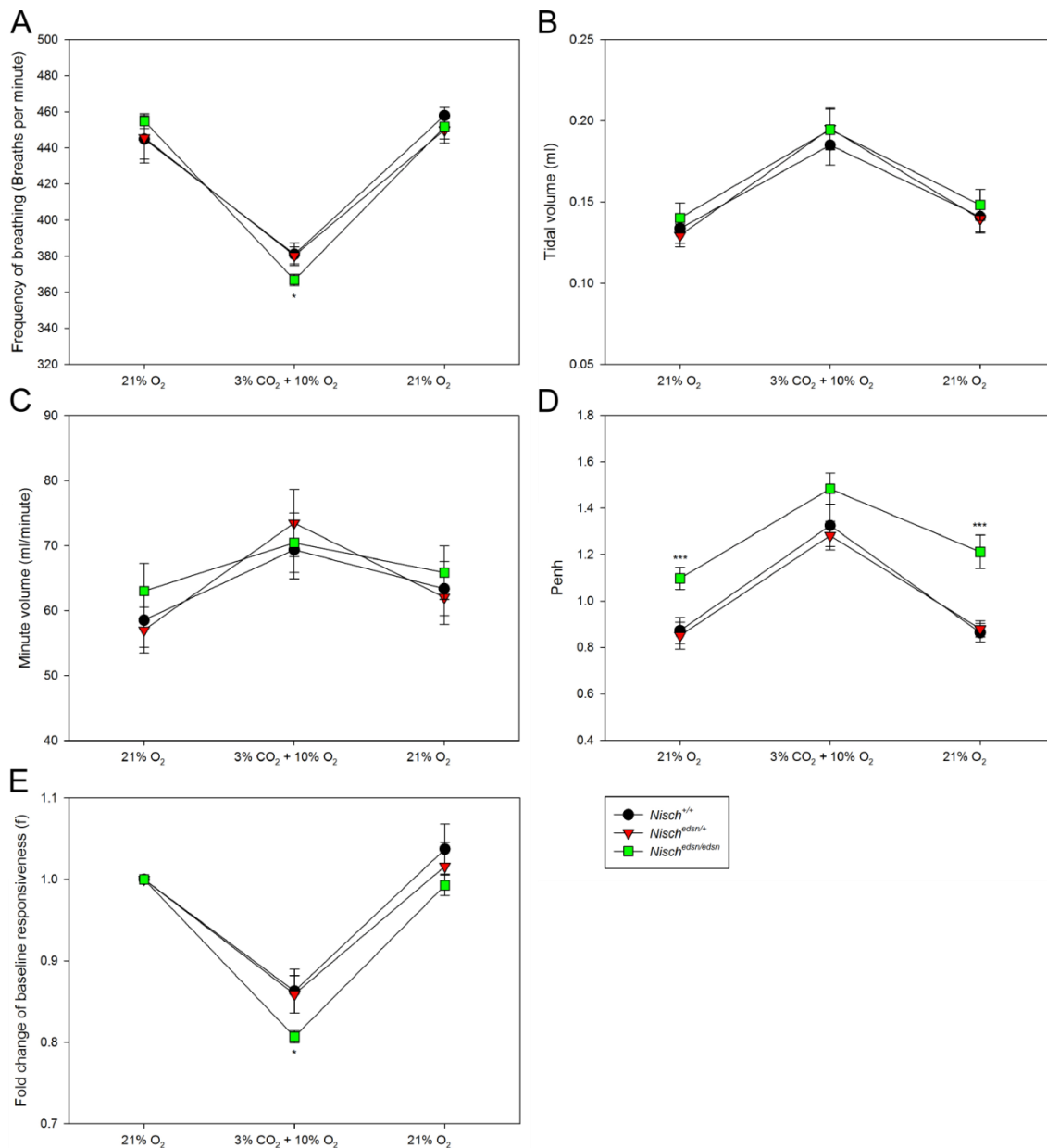


Figure 4.36| Ventilatory responses to 10% oxygen with 3% carbon dioxide.

(A-D) Lung function was analysed, using whole body plethysmography, in response to a hypoxia-hypercapnia challenge (10% O_2 and 3% CO_2 for 5 minutes). Mean plethysmogram data collected from *edison* mice was plotted for (A) breathing frequency (f), (B) tidal volume (TVb), (C) minute volume (MVb) and (D) enhanced pause (Penh). **(E)** Corresponding f values were normalised to the baseline responsiveness to analyse fold change. * $P < 0.05$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

$Nisch^{+/+}$ n = 10; $Nisch^{edsn/+}$ n = 11; $Nisch^{edsn/edsn}$ n = 12.

4.2.8.2.2 Methacholine response

During baseline conditions (nebulised PBS only), breathing rate (f), tidal volume (TVb) and minute volume (MVb) were similar in *Nisch^{edsn/edsn}* mice and their wild-type counterparts (Figure 4.37A-C respectively). With exposure to increasing methacholine (MCh) concentrations, both *Nisch^{edsn/edsn}* and wild-type mice showed a decreased breathing rate and increased TVb and MVb, however no significant genotypic differences were observed during the challenge. Also, no significant difference was observed between *Nisch^{+/+}* and *Nisch^{edsn/+}* animals throughout the challenge.

As seen previously in the hypoxia-hypercapnia challenge, during baseline conditions enhanced pause (Penh) was significantly increased in *Nisch^{edsn/edsn}* mice compared to wild-type littermates ($P = 0.005$). Before exposure to MCh, *Nisch^{edsn/edsn}* animals had a Penh of 1.58 ± 0.161 , whereas wild-type animals displayed a Penh of 0.71 ± 0.059 (Figure 4.37D). When exposed to increasing concentrations of MCh, both *Nisch^{edsn/edsn}* and wild-type mice displayed an incremental increase in Penh. At 50mg/ml MCh, *Nisch^{edsn/edsn}* animals had a Penh of 4.62 ± 0.559 , whereas wild-type animals displayed a Penh of 5.12 ± 0.489 . No significant genotypic differences were observed in Penh during MCh exposure.

Corresponding Penh values were normalised for each genotype against the baseline responsiveness to analyse the fold change in Penh with increasing MCh concentrations (Figure 4.37E). At higher concentrations, *Nisch^{edsn/edsn}* mice appear hyporesponsive to MCh. At 12.5 mg/ml MCh, *Nisch^{edsn/edsn}* animals had a fold change of 2.00 ± 0.307 compared to baseline responsiveness, whereas wild-type animals displayed a fold change of 2.60 ± 0.194 ($P = 0.310$). With both further increases in MCh concentration to 25 then 50 mg/ml, the fold change in Penh compared to baseline responsiveness was significantly decreased in *Nisch^{edsn/edsn}* mice compared to wild-type littermates ($P = 0.005$). At 50 mg/ml MCh, *Nisch^{edsn/edsn}* animals had a

fold change of 3.20 ± 0.394 , whereas wild-type animals displayed a fold change of 7.29 ± 0.732 compared to baseline responsiveness.

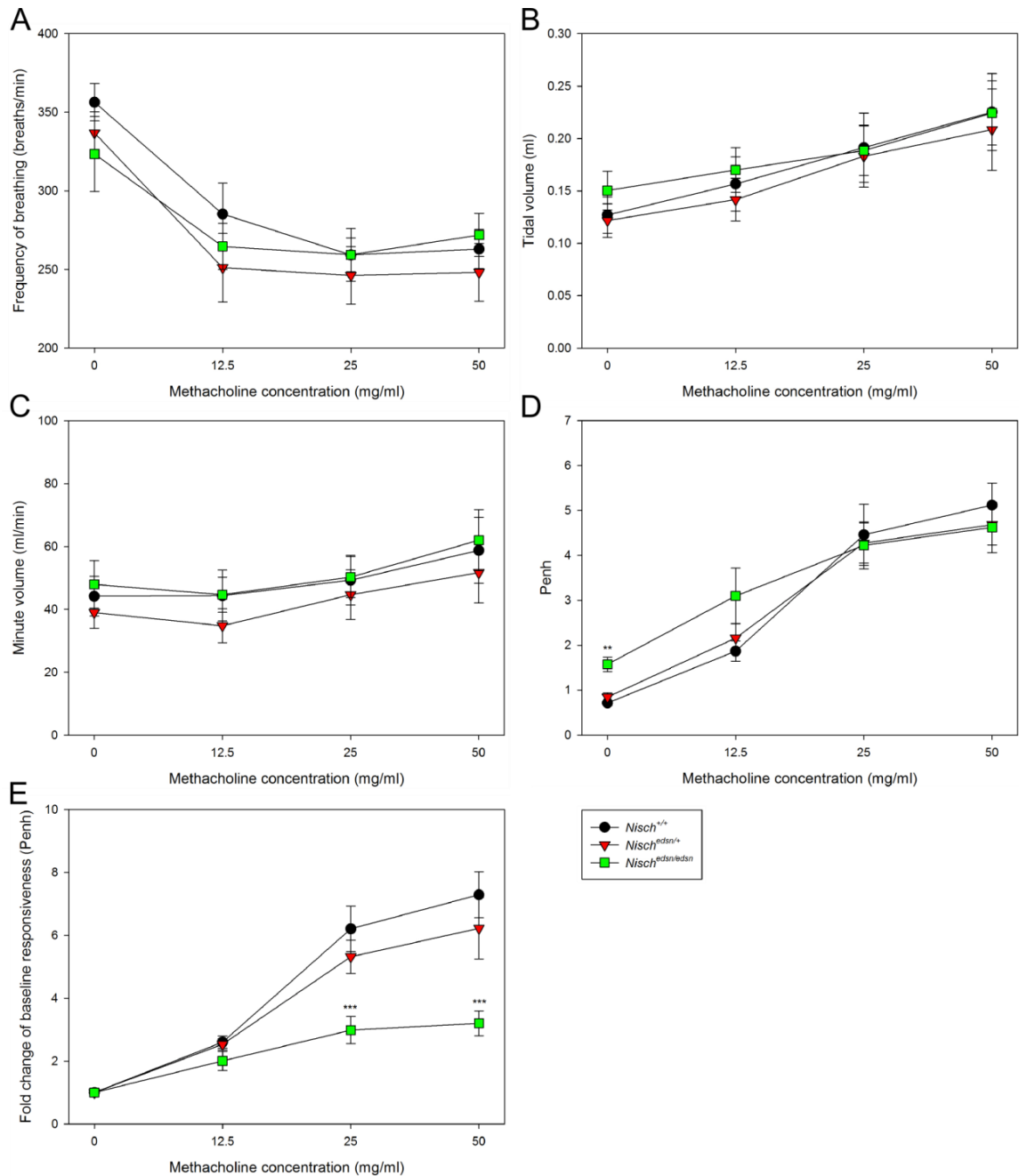


Figure 4.37| Ventilatory responses to increasing methacholine concentrations.

Lung function responses were measured, using whole body plethysmography, under increasing concentrations of methacholine (0, 12.5, 25 and 30 mg/ml). Mean plethysmogram data was normalised by the mean baseline responsiveness to show ventilatory responses for **(A)** breathing frequency (f), **(B)** tidal volume (TVb), **(C)** minute volume (MVb) and **(D)** enhanced pause (Penh) in *edison* mice. **(E)** Corresponding Penh values were normalised to the baseline responsiveness to analyse fold change. ** $P < 0.01$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} n = 10; *Nisch*^{edsn/+} n = 11; *Nisch*^{edsn/edsn} n = 12.

4.3 Discussion

4.3.1 Summary

The *edison* mouse exhibits a complex phenotype with an abnormal cranial morphology, reduced body size, emphysematous lungs and a hearing deficit caused by COM. Data indicates that *edison* is an additional non-syndromic OM mutant to be characterised at MRC Harwell and represents another powerful model for understanding chronic ME disease in humans.

4.3.2 *edison* exhibits spontaneous COM with onset at 3wk

In humans, the histopathological features of CSOM firstly present as an early acute phase, with mucosal and bony pathological changes. This continues to a late chronic phase with the presence of inflammatory granulation tissue, newly formed capillaries and ME metaplasia, sometimes with an active suppuration and polyps (Meyerhoff, Kim et al. 1978; Salvinelli, Trivelli et al. 1999). The OM histology displayed in *edison* mice shows a good resemblance to human disease. In SPF conditions, spontaneous COM develops in mice with the *Nisch*^{*edsn/edsn*} allele, which in turn is associated with the elevated hearing thresholds recorded by ABR. Onset of the reduced hearing deficit is observed as early as 3wk and is progressive. The moderate hearing impairment seen in *Nisch*^{*edsn/edsn*} mice is robust and reproducible across different cohorts of mice, tested at ages from 3 to >20wk. The incidence of OM in *Nisch*^{*edsn/edsn*} mice is also progressive, becoming more prevalent in older mice and is comparable to other non-syndromic mouse models of COM (Table 4.11). As the *edison* mutation is hypomorphic, *Nisch*^{*edsn/+*} mice did not develop OM and had a ME free of effusion, with no thickening of the mucosal lining. Interestingly, resolution of hearing deficit was observed in some *Nisch*^{*edsn/edsn*} ears (8.3%), which could be a result of

abatement of the ME inflammation or reduction in exudate, most likely associated with the hypomorphic nature of the *edison* allele.

Table 4.11| Penetrance of OM phenotype in *Jeff*, *Junbo*, *Tgif* and *edison* mutant mice at 8wk.

Table showing the penetrance of OM phenotype for *Fbxo11*^{Jf/+}, *Evi1*^{Jbo/+}, *Tgif*^{-/-} and *Nisch*^{edsn/edsn} mice. *Fbxo11*^{Jf/+}, *Evi1*^{Jbo/+} and *Tgif*^{-/-} data adapted from Cheeseman *et al.*, 2011 and Tateossian *et al.*, 2013. *Fbxo11*^{Jf/+}, n = 54; *Evi1*^{Jbo/+}, n = 50; *Tgif*^{-/-}, n = 12; *Nisch*^{edsn/edsn}, n = 23.

OM mouse mutant	Bilateral OM	Unilateral OM	None
<i>Fbxo11</i> ^{Jf/+}	46%	29%	25%
<i>Evi1</i> ^{Jbo/+}	78%	13%	9%
<i>Tgif</i> ^{-/-}	42%	41%	17%
<i>Nisch</i> ^{edsn/edsn}	39%	30%	30%

Typical changes due to COM occur in the MEC mucoperiosteum including edematous polyps, stromal edema, and increased lymphatics with variable numbers of infiltrating neutrophil leukocytes. At 3wk the mucosal thickness in the MEC of *Nisch*^{edsn/edsn} mice appears normal, but from 4wk onwards it gets progressively thicker as the COM develops. Interestingly, at 2wk there is a significant increase in the thickness of mucosa compared to both wild-type and *Nisch*^{edsn/+} littermates. The ME fluid observed at 2wk was serous, compared to the more purulent exudates found in adult *Nisch*^{edsn/edsn} mice. Mammalian ME development and modelling of the ossicles takes place in combination with cavitation, the process by which the ME is cleared of the mesenchymal tissue that the ossicles initially develop in to form the bulla cavity (Jaskoll and Maderson 1978; Palva and Ramsay 2002; Palva, Pääkkö *et al.* 2003). In the *Tcof1* mutant mouse, defects in ME cavitation lead to conductive hearing loss (Richter, Amin *et al.* 2010). Cavitation in the ME of CBy/RF mice was suggested to be complete at P10 (Roberts and Miller 1998), and by P14 in DBA/1

mice (Richter, Amin et al. 2010). In *edison*, as there are no significant differences between genotypes observed in mucosal thickness at the 3wk time point, the difference seen at 2wk is most likely due to delayed cavitation rather than ME inflammatory disease.

The TGF- β pathway has been postulated as being important in OM development (Hardisty, Erven et al. 2003) and *in vivo* studies have highlighted the potential importance of TGF- β pathways in the control of mucin production (Jono, Shuto et al. 2002; Jono, Xu et al. 2003). This is of particular interest as a feature of OM is mucus hyper-secretion, with studies finding that MUC5B is the predominant mucin glycoprotein in OME and COM (Elsheikh and Mahfouz 2006; Preciado, Goyal et al. 2010). COM in *Nisch^{edsn/edsn}* mice is associated with increased mucin-secreting goblet cell populations in the ET, similar to what has been observed in *Tgif* KO mice (Tateossian, Morse et al. 2013). However, this is probably a secondary effect of the ME inflammation, as in mice with induced AOM increased goblet cell density was observed in the ET and remained high for at least six months after the disease had resolved (Cayé-Thomasen and Tos 2003).

4.3.3 Mild morphological abnormalities in *edison* mice

The cranial deformity observed in *edison* affects the nasal and frontal bones of *Nisch^{edsn/edsn}* mice and they therefore exhibit a shortened snout. Similar shortening of the snout and morphology of the nasal bones have been observed in other OM mouse models; the *Jeff* mutant (Hardisty-Hughes, Tateossian et al. 2006) and the *Tgif* knockout mouse (Tateossian, Morse et al. 2013). In the human population, OM is particularly prevalent in indigenous groups around the world (such as the Australian Aborigines (Sunderman and Dyer 1984), Greenlanders (Homøe 2001), Alaskan Eskimos (Maynard, Fleshman et al. 1972), and the American Indians (Wiet

1979)). It has been proposed that increased susceptibility is due to variables in ethnically associated craniofacial morphology and the resulting ET dysfunction (Beery, Doyle et al. 1980). However, recently it has been suggested that the increased susceptibility is more likely associated with the complex interactions in host-pathogen co-evolution, and the effects upon that of population geographic segregation (Bhutta and Burton 2013). Down syndrome and craniofacial abnormalities, such as cleft palate, affect both the anatomy and function of the ET and also predispose to CSOM (Nelson 1988). However, it is possible that the development of ME disease in these syndromes is achieved through unique pathobiological mechanisms that would not translate to non-syndromic OM (Bhutta and Burton 2013).

On a C3H/HeH background *Fbxo11*^{Jf/Jf} mice have cleft palate, among other severe developmental defects and are embryonic lethal, whereas *Fbxo11*^{Jf/+} mice demonstrate milder cranial defects with a shortened snout along with a smaller ET (Hardisty, Erven et al. 2003). Genes discovered from mouse models of OM have been suggested to fall into three categories; TLR4/MyD88 pathway genes that regulate innate immune function; those which are involved in craniofacial development and thereby ET morphology and function; and TGF- β pathway genes that modulate pro-inflammatory responses (Rye, Bhutta et al. 2011). The mild abnormal cranial morphology in *Jeff* mutants may contribute to the OM susceptibility seen in these mice, however the strong findings that FBXO11 impacts upon TGF- β signalling suggests that craniofacial defects are not the major mechanism in causing OM. The magnitude of the difference in cranial morphology between *Nisch*^{edsn/edsn} and wild-type mice was very small, at around 3%. Similarly, in *Tgif*^{-/-} mice the magnitude of the difference in cranial morphology was small (<2%), and no differences were observed in ET morphology (Tateossian, Morse et al. 2013). The ET morphology in *Nish*^{edsn/edsn} animals appeared normal, therefore it is likely that the craniofacial malformation does not impact upon ET function and hence, is probably not the major

mechanism of the *edison* OM phenotype. Further work would be required to establish if this is the case.

Another morphological difference observed in *edison* mice was the significantly smaller MEC in *Nisch^{edsn/edsn}* ears with chronic inflammation. Experimental AOM has been reported to induce a number of histopathological changes in the ME mucosa, including increased goblet cell density and massive osteoneogenesis (Cayé-Thomasen, Hermansson et al. 1995; Cayé-Thomasen, Hermansson et al. 1999). A number of inflammatory mediators stimulate both the resorption and formation of bone, such as TGF- β (Mundy, Boyce et al. 1995). Histopathological changes were observed in *Nisch^{edsn/edsn}* ears with OM, with new bone formation along the bulla wall the probable cause of the significantly smaller MEC. As *Nisch^{edsn/edsn}* ears without an OM phenotype displayed no changes in the bone morphology of the bulla wall it is likely that the osteoneogenesis observed was a subsequent effect of ME inflammation.

4.3.4 Upregulation of genes associated with inflammation and hypoxia in exudate of *edison* mice

A characteristic sequela of OM is the accumulation of fluid and inflammatory cells in the bulla and mucosal inflammation. This is observed in *edison* mice with large populations of foamy macrophages present within the *Nisch^{edsn/edsn}* ME exudate. The COM in *Tgif* KO mice is associated with raised levels of VEGF, TNF α and IL-1 β in ear fluids (Tateossian, Morse et al. 2013) and the inflamed ME of the *Fbxo11^{Jf/+}* and *Evi1^{lbo/+}* mice is also known to be hypoxic, with upregulation of HIF and VEGF pathways in bulla fluids of both mutants (Cheeseman, Tyrer et al. 2011). Sampling of ME fluids revealed upregulation of *Hif1- α* , *Il-1 β* , *Tnfa*, *Vegfa*, *Src* and *Evi1* in *Nisch^{edsn/edsn}* adult mice relative to blood WBC. Put simply, these genes are important

for inflammatory responses. Upregulation of *Il-1 β* , *Vegfa* and *Src* was also observed in ME fluids from 2wk *Nisch*^{*edsn/edsn*} mice, however again this is most likely due to delayed cavitation rather than ME inflammatory disease.

HIF1- α is involved in mediating responses to hypoxia to maintain tissue homeostasis. HIF signaling is also regulated by inflammation through inflammatory cytokines such as IL-1 β and TNF α (Frede, Berchner-Pfannschmidt et al. 2007; Dehne and Brüne 2009). VEGF facilitates angiogenesis leading to increased vascular permeability and recruitment of neutrophils and macrophages (Angelo and Kurzrock 2007; Nagy, Benjamin et al. 2008). With increased vascular permeability VEGF may contribute to COM through the accumulation of fluid and inflammatory cells within the MEC (Cheeseman, Tyrer et al. 2011). SRC can be activated by VEGF and its related signalling pathways are considered to be critical in regulating the structural changes involved in vascular permeability (Mucha, Myers et al. 2003; Okutani, Lodyga et al. 2006). Finally, EVI1 is a co-transcriptional repressor of SMAD3 that plays an important role in the pathogenesis of COM (Parkinson, Hardisty-Hughes et al. 2006). EVI1 has a novel role in negatively regulating NF- κ B-dependent inflammation (Xu, Woo et al. 2012), and the mutation in *Evi1* in *Junbo* mice may also exert effects via TGF- β signalling (Parkinson, Hardisty-Hughes et al. 2006). Additionally, gene expression analysis of *Fbxo11* showed no difference in regulation in the bulla fluids of *Nisch*^{*edsn/edsn*} mice relative to blood WBC. *Fbox11* is the gene mutated in *Jeff* mutant mice and impacts upon TGF- β signalling with important implications in the disease mechanisms of ME inflammatory disease (Tateossian, Hardisty-Hughes et al. 2009). Likewise, neither *Jeff* nor *Junbo* mice displayed a significant upregulation of *Fbxo11* expression in the bulla fluids relative to WBC (Cheeseman, Tyrer et al. 2011). The Low Oxygen in Otitis Media (LOOM) study was set up to collect glue ear samples from children undergoing grommet insertion for COME, specifically examining hypoxia signalling pathways and their similarities to the COM mouse models discovered at MRC Harwell. Gene expression analysis revealed a similar

pattern of gene expression in human ME fluid (Tyrer et al. data not published), suggesting *edison* as an excellent disease model for human COM.

4.3.5 No sensorineural component to the *edison* hearing loss

A mild to moderate conductive hearing loss usually accompanies COM, with inflammation of the ME that causes oedema of the ME lining and discharge (WHO 2004). There is some evidence that CSOM causes sensorineural hearing loss. A recent study in humans has shown loss of inner and outer hair cells in the basal turn of the cochlea in patients with CSOM (Cureoglu, Schachern et al. 2004). This is probably due to infiltration of infectious or inflammatory agents through the round window to produce a serous labyrinthitis (Meyerhoff, Kim et al. 1978; Paparella, Morizono et al. 1984). The ototoxic effects of some antibiotics used to treat the infection may add to these effects (Morizono 1990). Additionally, studies in animals have shown that inflammatory mediators, penetrating through the round window membrane into the inner ear, can cause the loss of hair cells in the cochlea (Huang, Dulon et al. 1990; Morizono and Tono 1991). In addition to COM, *Fbxo11*^{Jf/+} mutants in some cases display a sensorineural component to the hearing deficit (Hardisty, Erven et al. 2003).

Due to the profound elevation in hearing thresholds of some *Nisch*^{edsn/edsn} mice and an initial swim test deficit in one of the founder mice it was of interest to further examine the auditory function of these animals to decipher whether there was any additional sensorineural element confounding the hearing thresholds observed. Click-evoked ABR is characterised by its broad spectral content and rapid onset, making it a good choice for analysing conductive hearing loss. An alternative stimulus for ABR is a short duration frequency-specific tone-burst stimuli, more suited to analysing sensorineural hearing deficiencies. There were no obvious traits seen in the

frequency specific audiograms recorded from *edison* mice to suggest any additional sensorineural component to the conductive hearing loss caused by OM. This was confirmed by examining the hair cells in the inner ears of these mice. There was no evidence of inner ear damage found through examination of H&E stained inner ear sections, suggesting that the elevated ABR thresholds are not due to a sensorineural element. Furthermore, no structural defects were observed in hair cells in the inner ears of mice, implying again that it is unlikely these mice have any sensorineural hearing loss. As mentioned, an initial swim test deficit was observed in one of the *edison* founder mice, which can be a consequence of vestibular dysfunction. Further vestibular phenotyping on congenic *edison* littermates indicated that all *edison* genotypes displayed a functioning vestibular apparatus. Abnormal ME ossicles can result in conductive hearing loss, though analysis of ME ossicles in *edison* mice also showed no overt differences between genotypes. Analysis of cytokine profiles in the middle and inner ears of mice with induced AOM and unilateral chronic OM caused by a TLR4 mutation have suggested that inner ear inflammation occurs in both AOM and chronic OM (MacArthur, Pillers et al. 2011). This could potentially be a factor correlating to disease severity and hearing loss.

4.3.6 Dual lung phenotype in *Nisch^{edsn/edsn}* mice

Development of fully functional lungs requires the appropriate integration of extracellular signalling and transcriptional regulation to make certain that the correct morphological architecture and cellular differentiation occur (Perl and Whitsett 1999). Lung development is completed during the neonatal period and proper development during this period is crucial for the formation of fully functional adult lungs (Shi, Bellusci et al. 2007). Abnormal lung development leads to an increased propensity towards adult lung diseases, including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (Shi, Bellusci et al. 2007).

The reduced proportion of *Nisch*^{edsn/edsn} progeny produced from the breeding programme meant that generating mice for experimental work was challenging. It was first thought that the higher mortality was probably due to the significantly reduced size of mutant mice. The finding that mice with the *Nisch*^{edsn/edsn} allele were being found dead in increased proportions, before weaning age, added further weight to this hypothesis. However, in depth analysis of organs from these mice revealed an alternative explanation for this perinatal lethality. While no cleft palate or obvious respiratory distress was observed in these mice, analysis of organs revealed a lung phenotype that was visible before and after birth that may contribute to mortality through the presence of smaller airways and increased interstitial mesenchyme in *Nisch*^{edsn/edsn} mice compared to controls. In contrast, *Fbxo11*^{Jf/Jf} mice are perinatal lethal and display defects in palate, eyelid and importantly, lung development (Tateossian, Hardisty-Hughes et al. 2009). All of which involve growth of epithelial tissue and, in addition, the TGF- β signalling pathway is known to be important in these processes. Likewise, *Evi1*^{Jbo/Jbo} mice are embryonic lethal (Parkinson, Hardisty-Hughes et al. 2006), and *Evi1*^{Jbo/+} mice also display reduced survivorship that has been associated with the presence of fewer airways in *Evi1*^{Jbo/+} mice compared to controls, which is visible before birth and may contribute to mortality (Tyrer et al. data not published).

In adult mice, an in depth analysis of organs also confirmed a lung phenotype, although with no obvious effect on mortality. Adult lungs in *Nisch*^{edsn/edsn} mice displayed an emphysematous phenotype with increased airspace. Interestingly, the severity of the adult lung phenotype mirrored the severity of the OM phenotype, whereby mice with a bilateral OM had the most severe lung phenotype and mice with no OM phenotype had an almost normal lung phenotype. Emphysema is clinically characterised by a breakdown in the alveolar lung structure due to destruction of the alveolar wall (Barnes 2000). Recurrent and chronic inflammation, with cytokine production, protease activation, oxidative stress, and other mechanisms, results in

the destruction of the alveolar wall and subsequently increases alveolar size and fibrosis (Barnes 2000; Barnes 2003). The emphysema-like phenotype in *Nisch^{edsn/edsn}* mice cannot be compared to clinical emphysema and most likely arises from dysfunctional lung development, instead of destruction of the alveolar wall. The inflammatory cytokines TNF α , TGF- β and IL-1 β are found in respiratory tract lavage fluid and are associated with cultured whole lung tissue (Witschi and Last 2001). Increased lung inflammation and the overexpression of soluble mediators such as IL-1 β and VEGF are important in the pathogenesis of human as well as experimental emphysema (Lee, Link et al. 2004; Lappalainen, Whitsett et al. 2005). TNF-null mice exhibit significantly reduced inflammation and airspace enlargement in response to 6 months of exposure to cigarette smoke (Churg, Wang et al. 2004). In contrast, constitutive overexpression of TNF α in the lungs of mice produces a dual phenotype of emphysema and pulmonary fibrosis (Lundblad, Thompson-Figueroa et al. 2005). Upregulation of *Il-1 β* , *Tnfa* and *Vegfa* has been observed in the ME fluid of *Nisch^{edsn/edsn}* mice, and similarly if upregulated in lungs it would help explain the strong association seen between OM and emphysematous lungs in *edison*.

Whole body plethysmography indicated that the lung function of *edison* mice was considerably impacted by the emphysema-like phenotype. When exposed to hypoxic-hypercapnic conditions, both *Nisch^{edsn/edsn}* and wild-type mice showed a reduced breathing rate. However, the reduced breathing rate in response to hypoxia-hypercapnia exposure was significantly greater in *Nisch^{edsn/edsn}* mice. In recent work published, an increase in breathing rate is typically observed during hypoxia and hypercapnia (Bonora, Bernaudin et al. 2004; Adachi, Ogawa et al. 2006; Trapp, Tucker et al. 2011). MRC Harwell is one of the four mouse centres comprising EUMODIC (Simon, Greenaway et al. 2013), carrying out broad-based primary phenotyping of 500 mouse mutant knockout lines generated within the IKMC program (Skarnes, Rosen et al. 2011). As part of this work, MRC Harwell has been capturing extensive control data on the baseline phenotype of C57BL/6NTac.

Baseline data over 10 weeks for C57BL/6NTac mice shows similarly reduced breathing rates in response to hypoxia-hypercapnia exposure (normoxia-normocapnia: 469 breaths/min; hypoxia-hypercapnia: 384 breaths/min) (Brown, data not published). The whole body plethysmography system used looks at lung function in unrestrained mice – so the mice are free to move around the chamber. Based on observations during the procedure, the mice tend to sit still during the hypoxia-hypercapnia challenge in contrast to other stages of the protocol when the mice are quite active in the cages (sniffing, rearing, grooming etc). This may result in the displayed effect on the breathing rate. Additionally, sometimes if there are very low oxygen levels, the breathing rate decreases and the animals take in a larger volume of air, breathing less frequently but more deeply. An increase in tidal volume was recorded in both genotypes and could be an alternative explanation for the reduced breathing rate observed.

During the MCh challenge, the emphysematous lungs in *edison* also appeared to inhibit airway hyperresponsiveness, associated with a reduced Penh. This result could be confounded as during unchallenged conditions the *Nisch^{edsn/edsn}* lungs already had a significant increase in Penh. The *edison* lungs that display an emphysema-like phenotype could have critically impaired function and are therefore unresponsive to the increases in MCh concentration. Further work would be required to fully characterise the lung function of *edison* mice.

4.3.7 Conclusion

Unlike many other mouse mutants, *edison* mice do not show the wide-ranging pleiotropic effects often associated with ME inflammatory disease in other models. OM arises spontaneously in the postnatal period and develops into COM, often with a dense granulocyte-rich effusion, resulting in an associated conductive hearing loss.

Additionally, emphysematous lungs are observed in *edison* mice that cause a reduced ventilatory response under hypoxic-hypercapnic conditions. The *edison* mutant shares important features with COM found in human disease, and hence provides an important genetic disease model of COM.

CHAPTER 5: Validation of *Nischarin* as the causative gene in *edison*

5.1 Introduction

Highlighted in this chapter is the identification and subsequent phenotypic characterisation of additional *Nisch* alleles. Comprehensive characterisation revealed that one of the additional alleles identified spontaneously displayed a progressive COM with good resemblance to the *edison* mouse. Complementation testing between the alleles confirmed *Nisch* as the causal gene in the *edison* mutant.

5.1.1 ENU-DNA archive screen to generate an allelic series

In order to confirm an ENU-induced mutation, it is necessary to exclude the possibility that the phenotype of interest is caused by a mutation in a gene adjacent to the predicted causative gene identified. There are two direct ways to confirm an allele. Firstly, rescuing the phenotype by adding a functional copy of the gene of interest (e.g. BAC rescue) (Mburu, Mustapha et al. 2003), and secondly, showing failure of complementation by crossing the putative allele to a known allele of the same gene (Kile and Hilton 2005).

Gene targeting by homologous recombination (HR) to produce null mutations (knockout) has become a critical tool for functional gene analysis in biomedical research and has led to unprecedented insights into gene function including the genetic basis of human diseases (Hirano, Kodama et al. 2007; Leichtle, Hernandez et al. 2009; Leichtle, Hernandez et al. 2012; Tateossian, Morse et al. 2013). Knockout alleles can influence the behaviour of neighbouring loci (Maguire, Estabel et al. 2014) and can also be embryonic lethal. However, conditional null mutations can now eliminate the embryonic lethality of full knockout alleles. While null mutations are valuable, an important advantage of ENU mutagenesis is that it can generate a random allelic series for a particular gene, possibly generating a range of phenotypes from mutations in the same gene (Coghill, Hugill et al. 2002; Quwailid, Hugill et al.

2004). Approximately 64% of ENU-induced gene mutations are missense, producing a range of possible alleles, including hypomorphic, hypermorphic or neomorphic alleles (Justice, Noveroske et al. 1999). This complements and expands upon the typical result of gene inactivation by HR. These additional alleles can be invaluable in revealing previously unknown aspects of gene function. Showing failure of complementation can be achieved through crossing the putative allele to a null allele, however achieving this using an ENU allele is perhaps more elegant.

For the purpose of generating an allelic series in a gene of interest, the establishment of large DNA archives has allowed systematic screens for ENU-induced mutations (Coghill, Hugill et al. 2002). DNA may be obtained from either parallel archives of DNA and frozen sperm obtained from G₁ progenies of ENU mutagenised males (Quwailid, Hugill et al. 2004; Michaud, Culiati et al. 2005) or from mutagenized ES cells (Chen, Yee et al. 2000). Mutation detection can be carried out using techniques such as TGCE (temperature gradient capillary electrophoresis), DHPLC (denaturing HPLC), direct sequencing (Quwailid, Hugill et al. 2004) and more recently HRM (High Resolution Melting) analysis (Figure 5.1). Upon identification of a desired allele, mice carrying the ENU-induced mutation can be generated, from either frozen sperm or ES cells depending on which archive was screened. This is a powerful adjunct to conventional gene targeting strategies and has the advantage of generating a variety of alleles with potentially different phenotypic outcomes that facilitate the investigation of gene function and human disease (Yates, McMurray et al. 2009; Ricketts, McGoldrick et al. 2014).

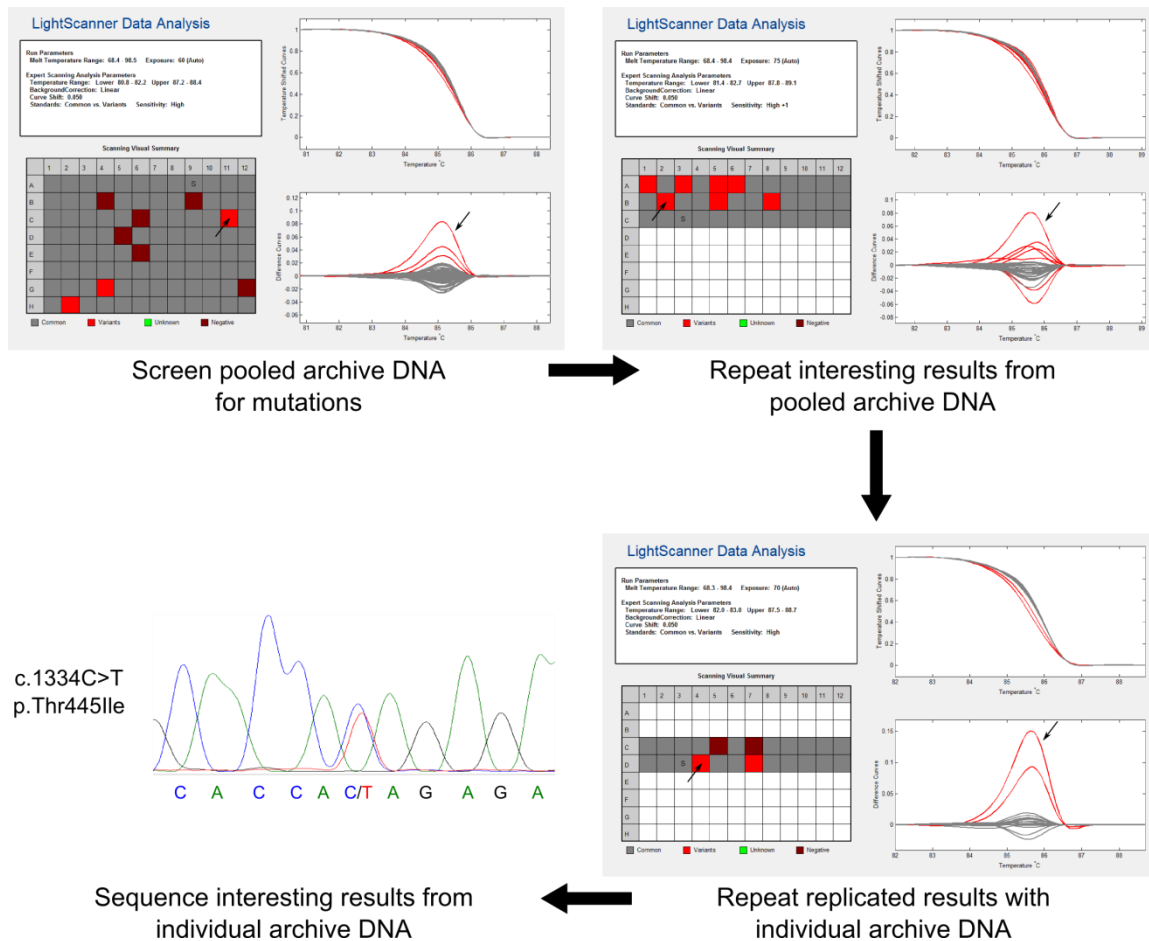


Figure 5.1| High Resolution Melting (HRM) analysis to screen ENU-DNA archive.

The ENU-DNA archive samples are pooled into sets of four to accelerate the primary screen for mutations. Pools are screened for SNV mutations using the LightScanner system and HRM analysis. Pooled samples that identify a possible mutation are repeated. If the positive result is replicated, the four individual DNA samples that make up the pools are tested to confirm in which sample the possible mutation is located. Once the individual DNA sample containing the possible mutation is known, the DNA sample is sent for Sanger sequencing to confirm whether a mutation is present.

5.2 Results

5.2.1 ENU-DNA archive screen recovered additional *Nisch* alleles

In order to confirm the putative mutation in *Nisch* as causal, I needed to use a known *Nisch* allele in a complementation test with *edison*. No *Nisch*-null alleles were available and consequently I utilised the ENU-DNA archive at MRC Harwell to identify additional *Nisch* alleles. The ENU-DNA archive consists of over 10,000 DNA samples from individual G₁ ENU mutagenised mice paralleled by frozen sperm samples. Mutations were identified by analysing PCR-amplified products of genomic DNA, spanning individual exons and at least 50bp of the flanking intronic regions, by using a LightScanner system to perform high throughput DNA melting analysis to detect variants.

Nisch consists of twenty one exons and I designed twenty eight primer sets to cover all exons in addition to the flanking intronic regions. After initial optimisation of all twenty eight primer sets, the ten most robust and consistent primer sets were chosen to screen the complete ENU-DNA archive. Exon 2, 4, 5, 7, 8, 9, 12, 15, 17 and 19 of *Nisch* were screened in 10,464 mutant mice employing HRM analysis to identify mutations. Eleven putative mutations were identified from the ENU-DNA archive screen (Table 5.1). With the use of Sanger sequencing, it was confirmed that one silent mutation, two intronic mutations and seven missense mutations, along with one false positive, had been identified (Figure 5.2). In total 22.31 Mbp of *Nisch* were screened, including 11.29 Mbp of coding sequence. Therefore, the mutation rate observed was one mutation per 2.23 Mbp screened, with one potentially functional change per 3.19 Mbp screened.

Table 5.1| Variants identified in *Nisch* from screening the ENU-DNA archive.

From eleven putative mutations found, ten mutations were confirmed with one false positive. Seven missense mutations were identified. The overall mutation rate was 1 in 2.23 Mbp screened, of which 1 in 3.19 Mbp were potentially functional. F/P, false positive.

<i>Nisch</i> exon	Base pairs	No. of mice	Total Mbp	Mutations found	AA change
2	182	10,464	1.90	1	1
4	155	10,464	1.62	0	0
5	270	10,464	2.83	2	2
7	206	10,464	2.16	1	1
(+ F/P)					
8	275	10,464	2.88	2	1
9	155	10,464	1.62	0	0
12	219	10,464	2.29	2	1
15	185	10,464	1.94	1	1
17	273	10,464	2.86	1	0
19	212	10,464	2.22	0	0
TOTAL	2132	104,640	22.31	10	7

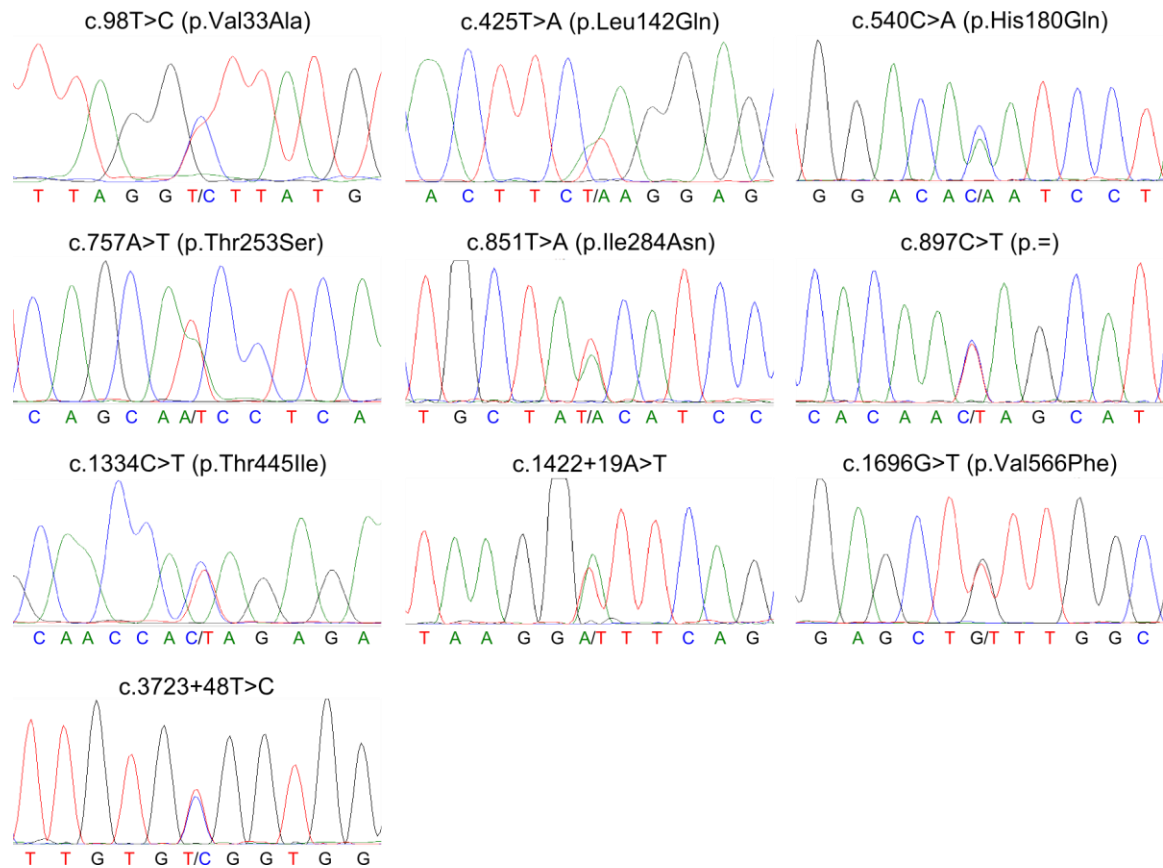


Figure 5.2| Sanger sequencing confirms the identification of 10 *Nisch* mutations from the ENU-DNA archive screen.

Sanger sequencing confirmed the identification of one silent mutation, two intronic mutations and seven missense mutations, along with one false positive.

Of the seven missense mutations identified, three were noted as being conservative and four were classified as non-conservative (Table 5.2). The three conservative mutations identified were Val33Ala, Thr253Ser and Val566Phe, and the four non-conservative mutations identified were Leu142Gln, His180Gln, Ile284Asn and Thr445Ile. Of the non-conservative mutations; Leu142Gln and Ile284Asn resulted in a nonpolar amino acid changed to an uncharged polar amino acid; His180Gln resulted in a charged basic polar amino acid changed to an uncharged polar amino acid; and Thr445Ile resulted in an uncharged polar amino acid changed to a nonpolar amino acid.

Table 5.2| Types of Nischarin mutations found within the archive

The ENU-DNA archive screen for additional Nischarin alleles identified 4 non-conservative (40%) and 3 conservative (30%) amino acid changes plus 1 mutation that did not change the amino acid (10%, silent). There were 2 intronic (20%) mutations and 1 false positive. ^A Nonpolar changed to uncharged polar. ^B Charged basic polar changed to uncharged polar. ^C Uncharged polar changed to nonpolar.

Nisch exon	Base mutation	Amino acid change	Type of Mutation
2	c.98T>C	Val33Ala	Conservative
5	c.425T>A	Leu142Gln	Non-conservative ^A
	c.540C>A	His180Gln	Non-conservative ^B
7	c.757A>T	Thr253Ser	Conservative
8	c.851T>A	Ile284Asn	Non-conservative ^A
	c.897C>T	Asn229Asn	Silent
12	c.1334C>T	Thr445Ile	Non-conservative ^C
	c.1442+19A>T	Intronic	Intron 12-13
15	c.1696G>T	Val566Phe	Conservative
17	c.3723+48T>C	Intronic	Intron 17-18

All seven of the potentially functional missense mutations were located in highly conserved regions of NISCH (data not shown). To establish which NISCH alleles to re-derive for preliminary characterisation, SIFT, SNAP and PROVEAN were utilised to predict whether the missense mutations identified from the ENU-DNA archive screen would have an impact on the biological function of NISCH (Table 5.3). SIFT analysis predicted that the Val33Ala, Leu142Gln, His180Gln and Thr445Ile substitutions were “not tolerated” (0.02, 0.00, 0.01 and 0.00 respectively); SNAP predicted that the Leu142Gln and Thr445Ile substitutions were “non-neutral”; and PROVEAN predicted that the Leu142Gln, His180Gln and Thr445Ile substitutions were “deleterious” (-4.361, -6.053 and -4.598 respectively).

Table 5.3| Functional prediction of additional Nischarin alleles identified from the ENU-DNA archive screen.

Three universally used *in silico* prediction tools were used to predict whether the seven missense mutations identified in the ENU-DNA archive screen would have an impact on the biological function of Nischarin. SIFT (Sorting Intolerant From Tolerant); SNAP (Screening for Non-Acceptable Polymorphisms); PROVEAN (Protein Variation Effect Analyzer).

Sequence variant	SIFT	Score	SNAP	Score	PROVEAN	Score
Val33Ala	Not tolerated	0.02	Neutral	-	Neutral	-2.163
Leu142Gln	Not tolerated	0.00	Non-neutral	-	Deleterious	-4.361
His180Gln	Not tolerated	0.01	Neutral	-	Deleterious	-6.053
Thr253Ser	Tolerated	0.17	Neutral	-	Neutral	-1.635
Ile284Asn	Tolerated	0.05	Neutral	-	Neutral	-2.469
Thr445Ile	Not tolerated	0.00	Non-neutral	-	Deleterious	-4.598
Val566Phe	Tolerated	0.10	Neutral	-	Neutral	-1.245

Using the functional prediction results and taking the location of each missense mutation into account, Val33Ala, Leu142Gln and Thr445Ile were selected for re-derivation from the parallel ENU sperm archive. Leu142Gln and Thr445Ile both were predicted, by all three *in silico* prediction software tools, to have a significant impact on the biological function of NISCH. Val33Ala was predicted to have an impact on the biological function of NISCH only when analysed through SIFT. However, Val33Ala was located within the PX domain (Figure 5.3). No other missense mutation was located within a known key functional domain of NISCH.

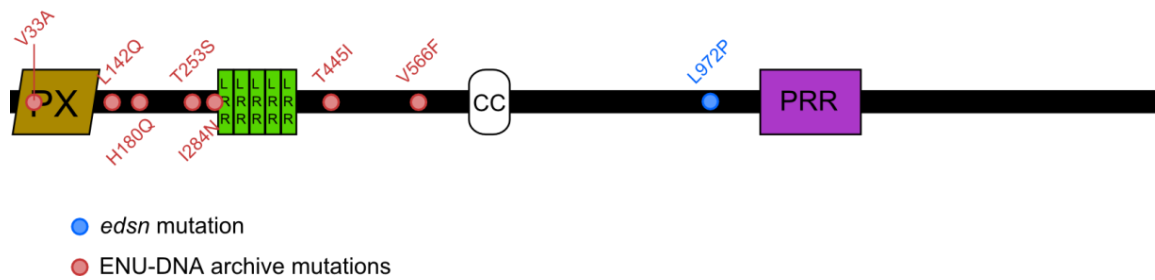


Figure 5.3| Location of additional *Nischarin* alleles within the protein structure.

Protein structure of NISCH showing the location of the L972P substitution found in *edison* (blue), along with the seven missense mutations identified from screening the ENU-DNA archive (red). PX, phox-homology domain; LRR, leucine-rich repeats; CC, coiled-coil domain; PRR, proline-rich region.

Once the mutations were selected for re-derivation, live animals were recovered by *in vitro* fertilisation (IVF) techniques using the frozen sperm samples and C3H/HeH eggs. The recovery rate for the Val33Ala, Leu142Gln and Thr445Ile *Nischarin* alleles was 100%. All were confirmed to carry the mutation identified in the ENU-DNA archive screening. Preliminary characterisation of these three re-derived *Nisch* alleles are further discussed below.

5.2.2 Alternative alleles from ENU-DNA archive confirm *Nisch* as the causative gene in *edison*

Three additional *Nischarin* alleles (*Nisch*^{V33A}, *Nisch*^{L142Q} and *Nisch*^{T445I}) were identified in the ENU-DNA archive at MRC Harwell, as discussed above. These alleles were re-derived by the FESA group at MRC Harwell. Preliminary characterisation of each allele was carried out to establish whether an OM phenotype could be observed. Two crosses were set up for analysis, first to homozygose the additional *Nischarin* alleles (e.g. *Nisch*^{V33A/+} × *Nisch*^{V33A/+}) and second to produce compound heterozygotes with the *edison* mutation (e.g. *Nisch*^{V33A/+} × *Nisch*^{edsn/+}). All genotypes produced from the

breeding crosses were phenotyped to control against and ascertain whether the mixed strain background had an effect on any significant phenovariants observed.

5.2.2.1 *Nisch*^{V33A} allele replicates hearing deficit caused by OM

5.2.2.1.1 Genotypic ratio analysis

As *edison* litters display reduced numbers of *Nisch*^{edsn/edsn} progeny, genotypic ratios from *Nisch*^{V33A/+} × *Nisch*^{V33A/+} crosses (*Nisch*^{V33A}|*Nisch*^{V33A}) were compared (Table 5.4). From the numbers of live mice observed at weaning age, there were fewer *Nisch*^{V33A/V33A} mice than were expected, however no significant differences were observed compared to expected Mendelian ratios ($P = 0.2684$). Similar observations were made when instead comparing genotypes from *Nisch*^{V33A/+} × *Nisch*^{edsn/+} crosses (*Nisch*^{V33A}|*Nisch*^{edsn}; Table 5.5). From the numbers of live mice observed at weaning age, no significant differences were observed between genotypes ($P = 0.8013$).

Table 5.4| Numbers of live mice from *Nisch*^{V33A/+} × *Nisch*^{V33A/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	20	16	30.8%	0.2684
<i>Nisch</i> ^{V33A/+}	34	33	52.3%	
<i>Nisch</i> ^{V33A/V33A}	11	16	16.9%	
TOTAL	65			

Table 5.5| Numbers of live mice from *Nisch*^{V33A/+} x *Nisch*^{edsn/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	6	6	25.0%	0.8013
<i>Nisch</i> ^{edsn/+}	7	6	29.2%	
<i>Nisch</i> ^{V33A/+}	4	6	16.7%	
<i>Nisch</i> ^{V33A/edsn}	7	6	29.2%	
TOTAL	24			

5.2.2.1.2 Weight

Analysis of weights across a longitudinal time course, from four to twelve weeks of age, demonstrated that no significant genotypic differences were observed in either gender at any time point in mice from *Nisch*^{V33A}|*Nisch*^{V33A} matings (Figure 5.4). This was also the case when mice from *Nisch*^{V33A}|*Nisch*^{edsn} crosses were analysed for weight across the same time course (Figure 5.5).

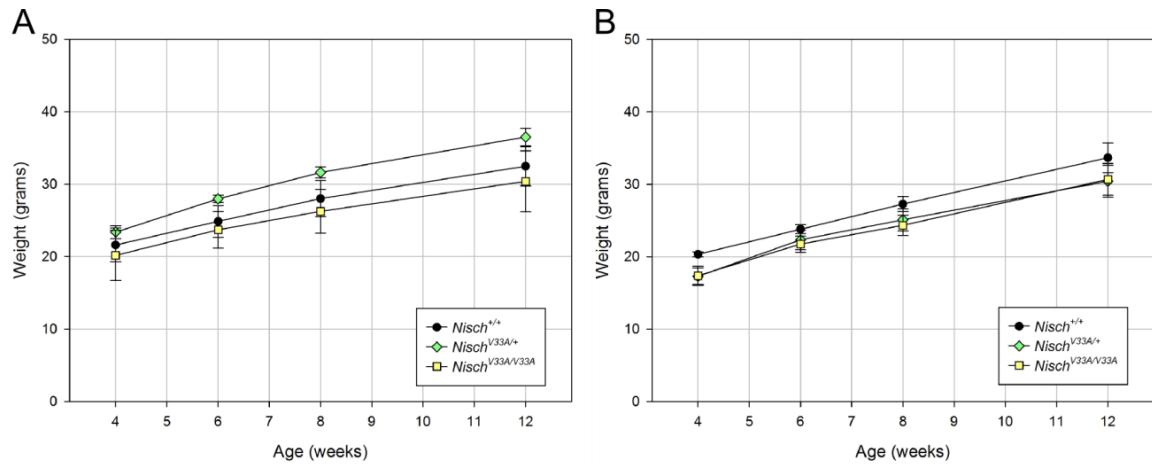


Figure 5.4| Weight data for *Nisch*^{V33A}|*Nisch*^{V33A} littermates over a longitudinal time course.

No genotypic differences in weight were observed in **(A)** male and **(B)** female mice from *Nisch*^{V33A}|*Nisch*^{V33A} litters. Statistics were conducted using one-way ANOVA's.

Nisch^{+/+} Male n = 4, Female n = 10; *Nisch*^{V33A/+} Male n = 8, Female n = 9; *Nisch*^{V33A/V33A} Male n = 4, Female n = 7.

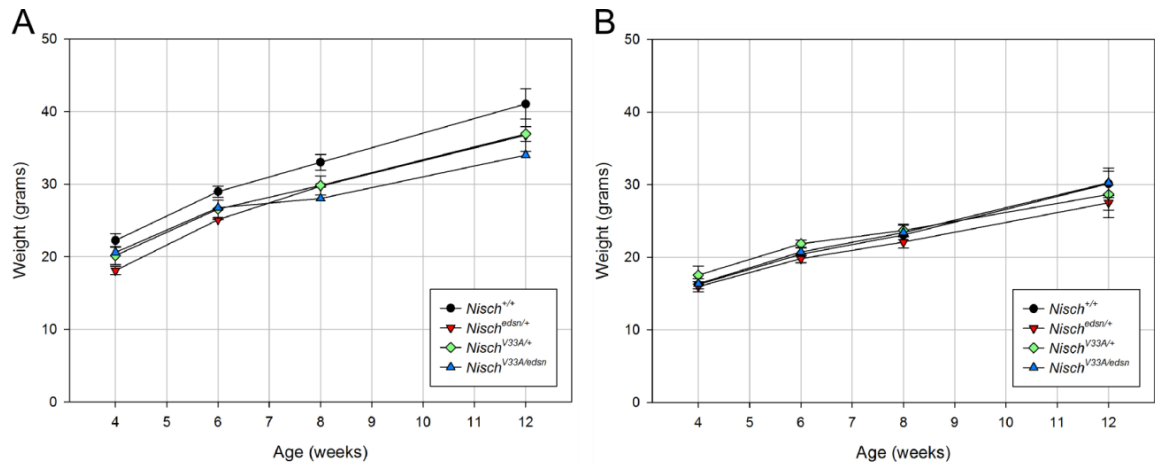


Figure 5.5| Weight data for *Nisch*^{V33A}|*Nisch*^{edsn} littermates over a longitudinal time course.

No genotypic differences in weight were observed in **(A)** male and **(B)** female mice from *Nisch*^{V33A}|*Nisch*^{edsn} litters. Statistics were conducted using one-way ANOVA's.

Nisch^{+/+} Male n = 4, Female n = 1; *Nisch*^{edsn/+} Male n = 2, Female n = 4; *Nisch*^{V33A/+} Male n = 2, Female n = 2; *Nisch*^{V33A/edsn} Male n = 1, Female n = 5.

5.2.2.1.3 ABR

In order to assess whether an abnormal hearing phenotype was displayed in *Nisch*^{V33A}/*Nisch*^{V33A} and *Nisch*^{V33A}/*Nisch*^{edsn} littermates, ABR tests for auditory function were conducted across a longitudinal time course with age matched littermates (Figure 5.6 and 5.7 respectively). Hearing assessments were conducted as summarised in Section 4.2.4.1.

Analysis of ABR thresholds found there was a significant difference in ABR thresholds between *Nisch*^{V33A/V33A} and *Nisch*^{+/+} mutant mice at 8 and 12wk ($P < 0.05$ and $P < 0.001$ respectively; Figure 5.6A). *Nisch*^{+/+} and *Nisch*^{V33A/+} littermates displayed normal, wild-type hearing thresholds of between 15-30 dB SPL. At 8wk, animals with the *Nisch*^{V33A/V33A} mutation were found to have slightly elevated auditory thresholds compared to wild-types, with a mean ABR threshold of 28.75 dB SPL (± 1.983). Even though the mean threshold fell within the expected wild-type threshold range, a slight significant difference was observed ($P = 0.028$). Hearing slightly deteriorated with age in *Nisch*^{V33A/V33A} mice, displayed by marginally elevated ABR thresholds. By 12wk, *Nisch*^{V33A/V33A} animals were recorded with a mean ABR threshold of 31.50 dB SPL (± 1.817), which was again significant compared to wild-type ($P < 0.001$). It is also worth noting that there was no obvious gender or left-right preference observed in ABR threshold data from all genotypes (Appendix, Figure 10.5).

The ABR threshold range for each genotype was analysed using box and whisker plots. For *Nisch*^{+/+} and *Nisch*^{V33A/+} littermates, the boxes (25th to 75th percentile) and whiskers (10th to 90th percentiles) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for normal hearing (Figure 5.6B and C). The ABR threshold range in *Nisch*^{V33A/V33A} mice slightly increased with age across the time course (Figure 5.6D). At 12wk, the threshold range was from 20 to 45 dB SPL, with a median of 30 dB SPL. The majority of ears recorded an ABR threshold in the

expected range for wild-type hearing, indicating that there was a low penetrance of OM in *Nisch*^{V33A/V33A} mice.

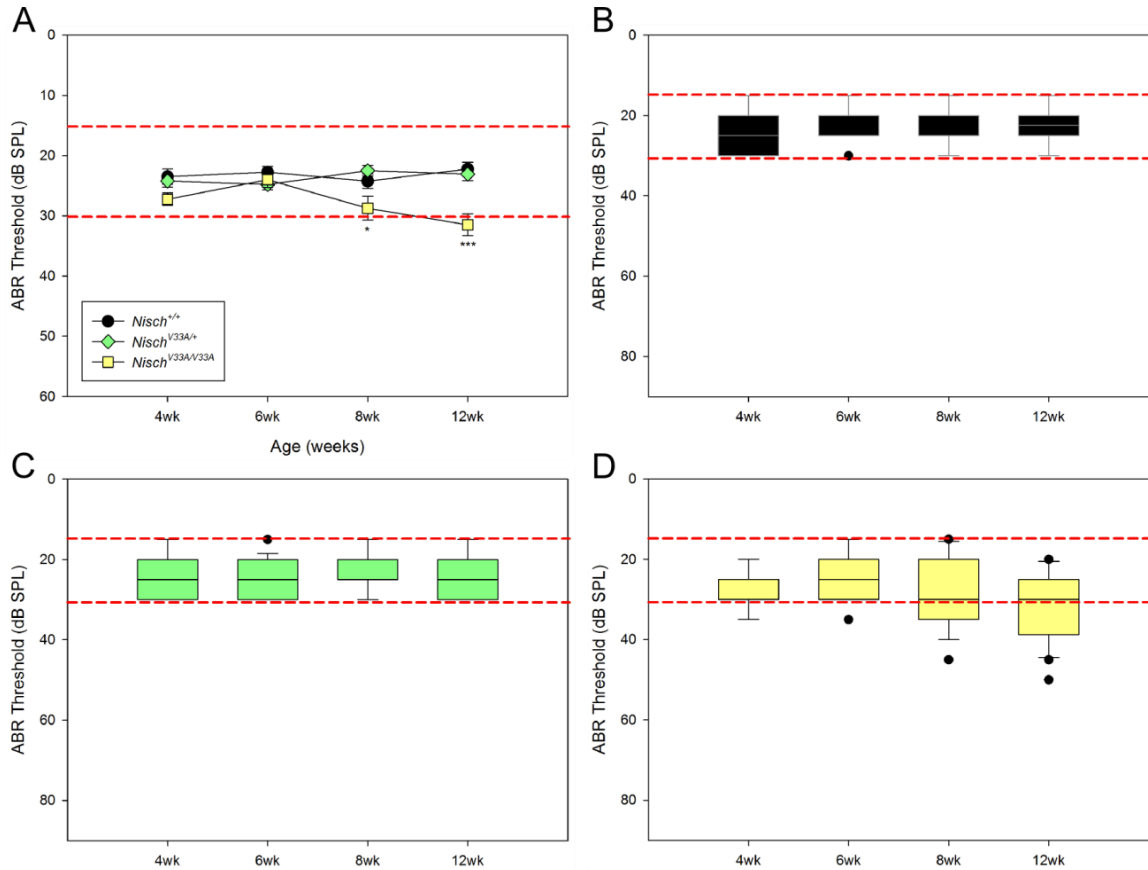


Figure 5.6| Progression of click-evoked ABR thresholds across a longitudinal time course for *Nisch*^{V33A}/*Nisch*^{V33A} littermates.

(A) Mice with the *Nisch*^{V33A/V33A} mutation have late onset elevated hearing thresholds due to OM. There is no difference in hearing thresholds between other littermates. (B-D) Box plots show the ABR threshold ranges of (B) *Nisch*^{+/+}, (C) *Nisch*^{V33A/+} and (D) *Nisch*^{V33A/V33A} mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (•) show any outliers. * $P < 0.05$; *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} $n = 20$; *Nisch*^{V33A/+} $n = 26$; *Nisch*^{V33A/V33A} $n = 20$.

The presence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was assessed across the time course for each genotype in

Nisch^{V33A}|*Nisch*^{V33A} littermates (Figure 5.7A-B). *Nisch*^{+/+} and *Nisch*^{V33A/+} animals recorded no ABR thresholds greater than 30 dB SPL. In *Nisch*^{V33A/V33A} mice there was a late onset in the prevalence of an increased ABR threshold from 8wk. At 4wk, 15% of *Nisch*^{V33A/V33A} ears tested had an increased ABR threshold, but no significance was observed (z-test, $P = 0.230$). When analysed at 8 and 12wk, 35% of *Nisch*^{V33A/V33A} ears tested had an increased ABR threshold, which was significantly increased compared to wild-type littermates (z-test, $P = 0.013$).

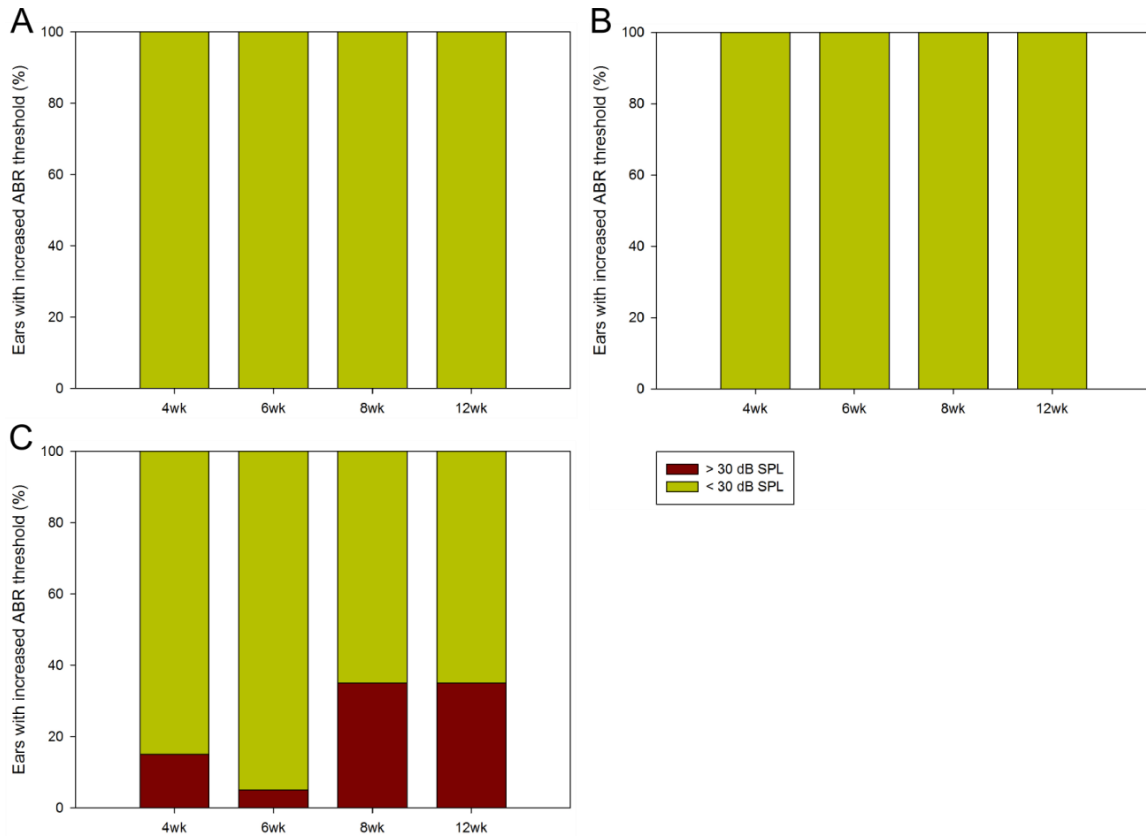


Figure 5.7| Presence of increased ABR thresholds in *Nisch*^{V33A}|*Nisch*^{V33A} litters across a time course.

(A-D) The presence or absence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was recorded in (A) *Nisch*^{+/+}, (B) *Nisch*^{V33A/+} and (C) *Nisch*^{V33A/V33A} mice. The prevalence of increased ABR thresholds above the normal range is increased in *Nisch*^{V33A/V33A} animals compared to littermates.

Analysis of ABR thresholds from *Nisch*^{V33A}/*Nisch*^{edsn} litters found there was a significant difference in ABR thresholds between *Nisch*^{V33A/edsn} and wild-type mice throughout the time course (Figure 5.8A). *Nisch*^{edsn/+} and *Nisch*^{V33A/+} littermates displayed normal, wild-type hearing thresholds of between 15-30 dB SPL. At 4wk, animals with the *Nisch*^{V33A/edsn} mutations were found to have elevated auditory thresholds compared to wild-types, with a mean ABR threshold of 29.17 dB SPL ($\pm$ 2.741). Even though the mean threshold fell within the expected wild-type threshold range, a significant difference was observed ($P = 0.028$). Hearing progressively deteriorated with age in *Nisch*^{V33A/edsn} mice, displayed by increasingly elevated ABR thresholds. By 12wk, *Nisch*^{V33A/edsn} animals were recorded with a mean ABR threshold of 38.75 dB SPL ($\pm$ 2.895), which was again significant compared to wild-type ($P < 0.001$). It is also worth noting that there was no obvious gender or left-right preference observed in ABR threshold data from all genotypes (Appendix, Figure 10.6).

The ABR threshold range for each genotype was analysed using box and whisker plots. For *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{V33A/+} littermates, the boxes (25th to 75th percentile) and whiskers (10th to 90th percentiles) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for normal hearing (Figure 5.8B-D). The ABR threshold range in *Nisch*^{V33A/edsn} mice marginally increased with age across the time course (Figure 5.8E). At 12wk, the threshold range was from 20 to 55 dB SPL, with a median of 40 dB SPL, indicating that there was variation in the severity of the OM phenotype in *Nisch*^{V33A/edsn} mice.

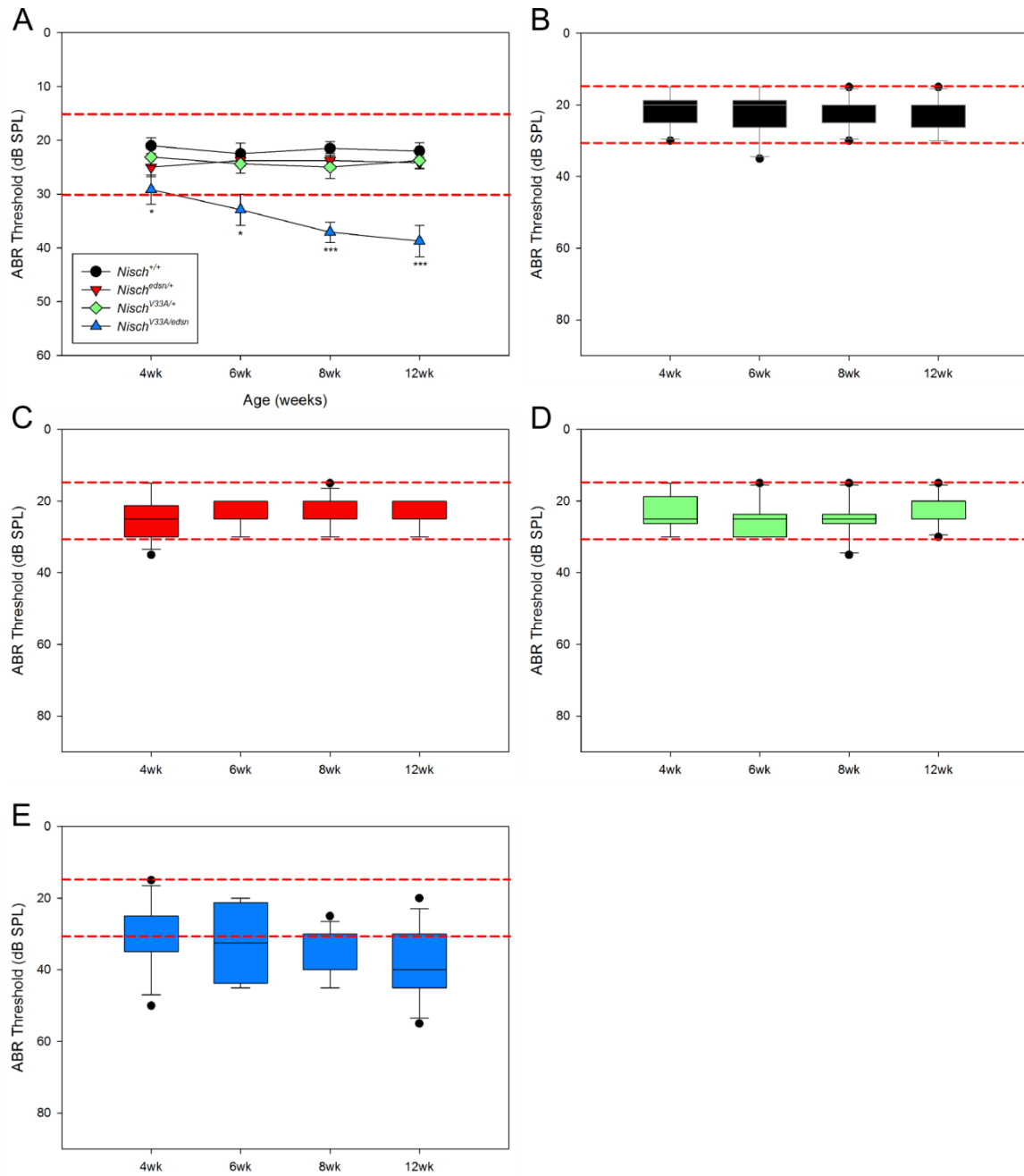


Figure 5.8| Progression of click-evoked ABR thresholds across a longitudinal time course for *Nisch*^{*V33A*}|*Nisch*^{*edsn*} litters.

(A) Mice with the *Nisch*^{*V33A*/*edsn*} mutations display a progressive elevation in hearing thresholds with onset from 4wk. There is no difference in hearing thresholds between other littermates. **(B-E)** Box plots show the ABR threshold ranges of (B) *Nisch*^{+/+}, (C) *Nisch*^{*edsn*/+} (D) *Nisch*^{*V33A*/+} and (E) *Nisch*^{*V33A*/*edsn*} mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (•) show any outliers. * $P < 0.05$; *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} $n = 10$; *Nisch*^{*edsn*/+} $n = 12$; *Nisch*^{*V33A*/+} $n = 8$; *Nisch*^{*V33A*/*edsn*} $n = 12$.

The presence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was assessed across the time course for each genotype in *Nisch*^{V33A}/*Nisch*^{edsn} littermates (Figure 5.9A-D). *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{V33A/+} animals showed a small incidence of an increased ABR threshold greater than 30 dB SPL. In *Nisch*^{V33A/edsn} mice there was onset in the prevalence of an increased ABR threshold from 4wk. At 4wk, 33% of *Nisch*^{V33A/edsn} ears tested had an increased ABR threshold, but no significance was observed (z-test, $P = 0.143$). By 6wk, 50% of *Nisch*^{V33A/edsn} ears tested had an increased ABR threshold, which was significantly increased compared to wild-type littermates (z-test, $P = 0.032$). When analysed at 8 and 12wk, 67% of *Nisch*^{V33A/edsn} ears tested had an increased ABR threshold, which was again significantly increased compared to wild-type littermates (z-test, $P = 0.005$).

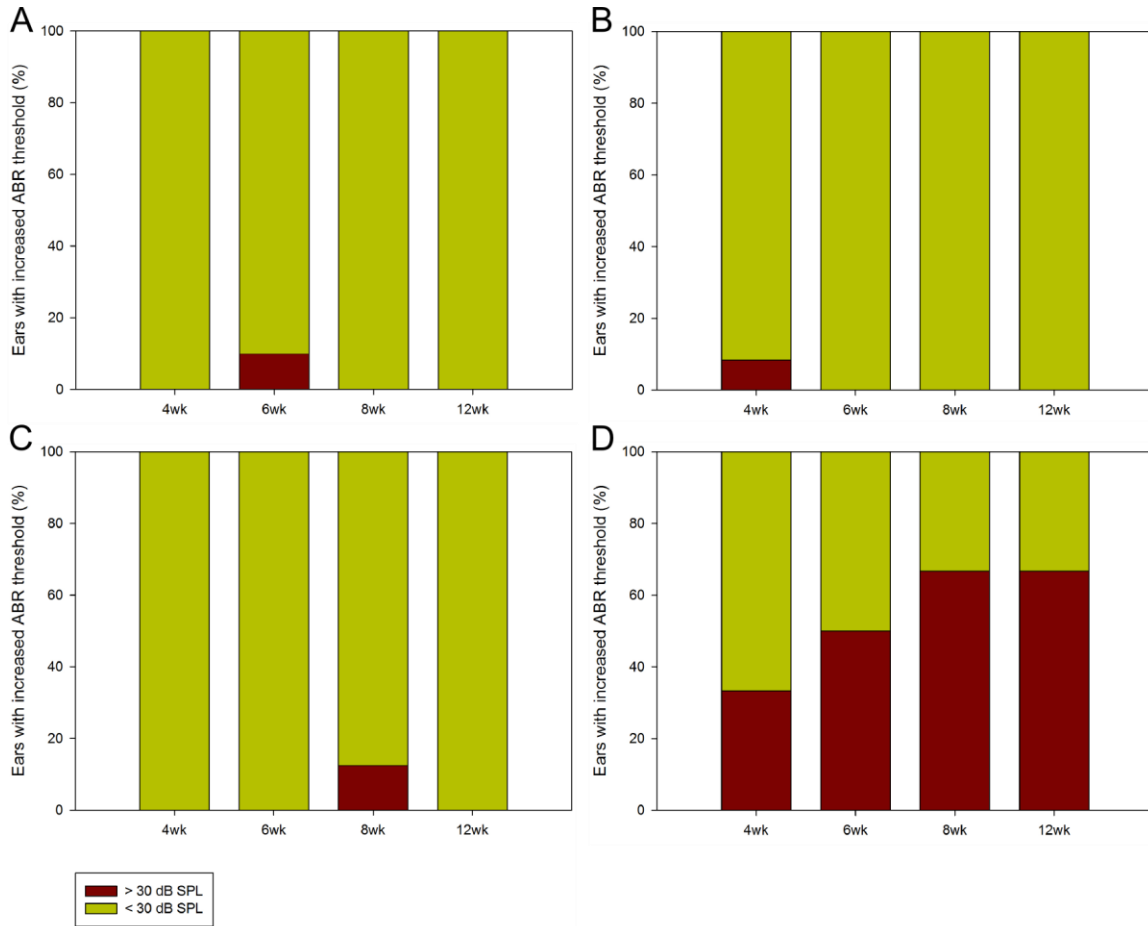


Figure 5.9| Presence of increased ABR thresholds in *Nisch*^{V33A}/*Nisch*^{edsn} litters across a time course.

(A-D) The presence or absence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was recorded in (A) *Nisch*^{+/+}, (B) *Nisch*^{edsn/+}, (C) *Nisch*^{V33A/+} and (D) *Nisch*^{V33A/edsn} mice. The prevalence of increased ABR thresholds above the normal range is increased in *Nisch*^{V33A/edsn} animals compared to littermates.

5.2.2.1.4 Histology

To confirm whether the hearing deficit observed in *Nisch*^{V33A/V33A} and *Nisch*^{V33A/edsn} mice was caused by an OM phenotype, the incidence of OM was verified from visual inspection of the tympanic membrane and the presence or absence of an inflamed eardrum, which is synonymous with OM (as previously described in Figure 4.12, section 4.2.4.2).

Analysis of eardrums from *Nisch*^{V33A}/*Nisch*^{V33A} litters showed there was a significant genotypic difference in the incidence of OM between *Nisch*^{V33A/V33A} mice and wild-type littermates (Figure 5.10). In mice with *Nisch*^{V33A/V33A} allele there was a significant increase in the incidence of OM compared to wild-type littermates ($P = 0.015$), with 10% of mice with bilateral OM, 50% with unilateral OM and 40% with no OM phenotype. There was a small incidence of unilateral OM seen in *Nisch*^{V33A/+} animals, 8%, compared to no incidence of OM seen in wild-types. No significant difference was observed between *Nisch*^{+/+} and *Nisch*^{V33A/+} mice ($P = 0.893$).

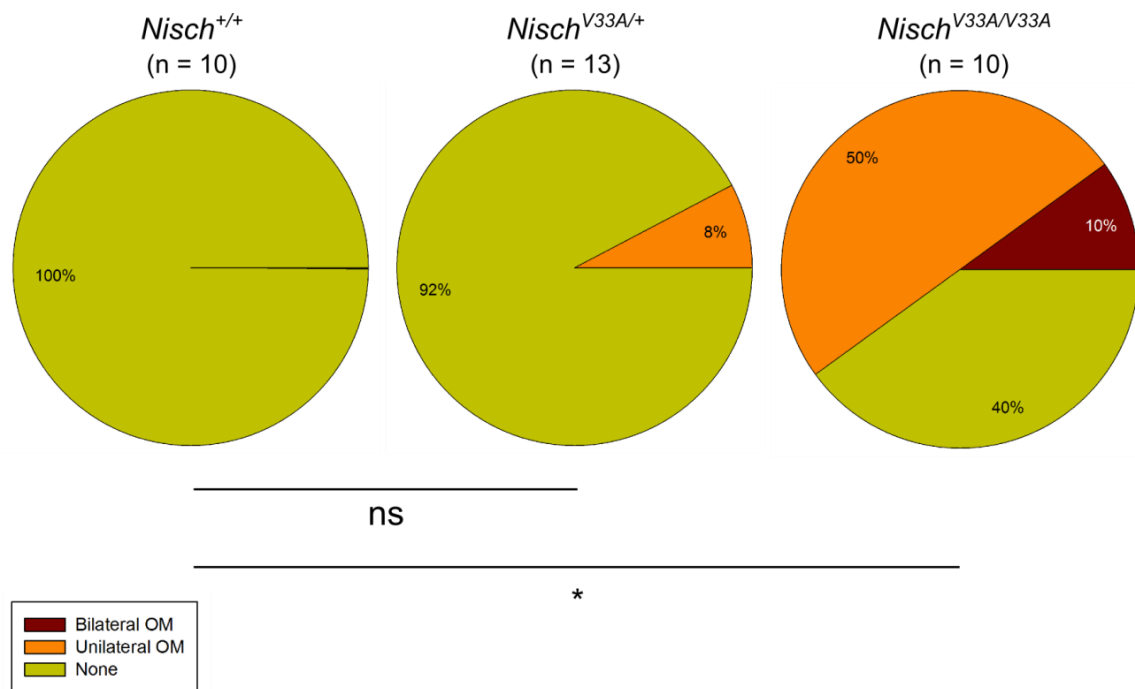


Figure 5.10| Incidence of OM in mice from *Nisch*^{V33A}/*Nisch*^{V33A} litters at 12wk.

The presence or absence of an inflamed eardrum was recorded from visual inspection after dissection. The incidence of OM was recorded in *Nisch*^{+/+}, *Nisch*^{V33A/+} and *Nisch*^{V33A/V33A} mice. OM in *Nisch*^{V33A/V33A} animals is significantly more prevalent compared to littermates. ns $P > 0.05$; * $P < 0.05$. Statistics were conducted using z-tests.

The incidence of OM observed in *Nisch*^{V33A}/*Nisch*^{V33A} littermates was replicated when eardrums were analysed in mice from *Nisch*^{V33A}/*Nisch*^{edsn} litters, whereby a

significant difference in the incidence of OM between *Nisch*^{V33A/edsn} mice and wild-type littermates was observed (Figure 5.11). In mice with the *Nisch*^{V33A/edsn} mutations there was a significant increase in the incidence of OM compared to wild-type littermates ($P = 0.012$), with 50% of mice with bilateral OM, 33% with unilateral OM and 17% with no OM phenotype. No significant difference was observed between *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{V33A/+} mice.

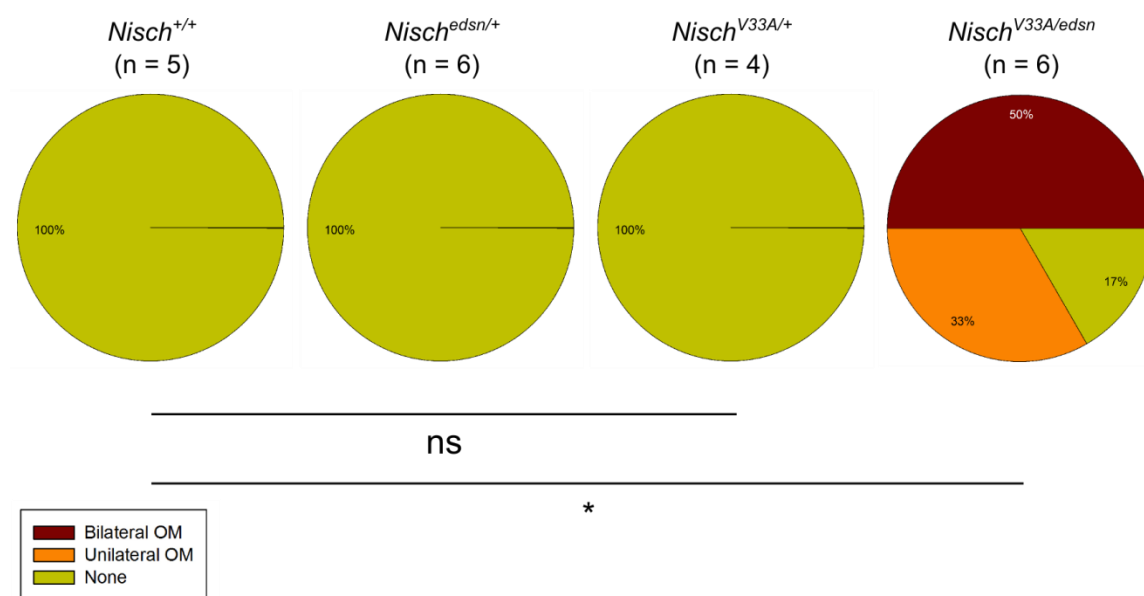


Figure 5.11 | Incidence of OM in *Nisch*^{V33A}/*Nisch*^{edsn} littermates at 12wk.

The presence or absence of an inflamed eardrum was recorded from visual inspection after dissection. The incidence of OM was recorded in *Nisch*^{+/+}, *Nisch*^{edsn/+}, *Nisch*^{V33A/+} and *Nisch*^{V33A/edsn} mice. OM in *Nisch*^{V33A/edsn} animals is significantly more prevalent compared to littermates. ns $P > 0.05$; * $P < 0.05$. Statistics were conducted using z-tests.

Histopathological examination of mice from *Nisch*^{V33A}/*Nisch*^{V33A} litters at 12wk confirmed that *Nisch*^{V33A/V33A} mice developed COM similar to *Nisch*^{edsn/edsn} mice (Figure 5.12B). The mucosal inflammation was diffuse and of moderate severity, with the presence of a mild cellular ME effusion (Figure 5.12D). Wild-type mice did not develop OM and had a ME free of effusion, with no thickening of the mucosal

lining (Figure 5.12A and C). The histology of *Nisch*^{V33A/+} mice was indistinguishable from wild-type littermates.

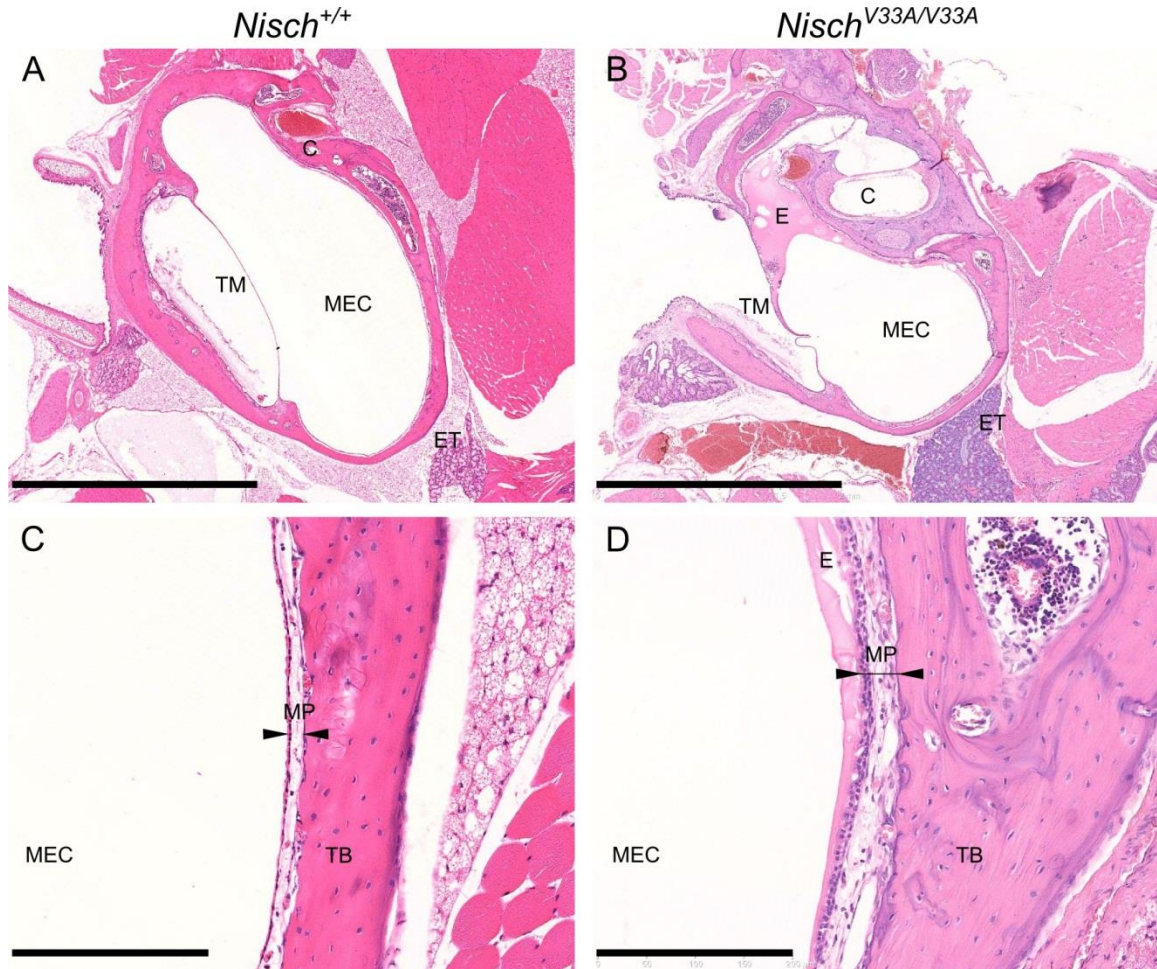


Figure 5.12| Characteristic ME histology in wild-type and *Nisch*^{V33A/V33A} animals at 12wk.

H&E stained transverse sections of the MEC and mucoperiosteum, in **(A & C)** *Nisch*^{+/+} and **(B & D)** *Nisch*^{V33A/V33A}, at 12wk. Histopathology of the MEC from *Nisch*^{V33A/V33A} mice demonstrates chronic inflammation with an exudate. Wild-type animals have no inflammation or exudate in the MEC and a thin mucoperiosteum covers the temporal bone. C, cochlea; ET, eustachian tube; E, exudate; MEC, middle ear cavity; MP, mucoperiosteum (arrowheads); TB, temporal bone; TM, tympanic membrane. (A & B) Scale bar = 2 mm; (C & D) Scale bar = 200 μ m.

Similar results were replicated in *Nisch*^{V33A}|*Nisch*^{edsn} littermates (Figure 5.13), where *Nisch*^{V33A/edsn} mice developed COM similar to *Nisch*^{edsn/edsn} mice (Figure 5.13B). Again,

the mucosal inflammation was diffuse and of moderate severity, with the presence of a cellular ME effusion (Figure 5.13D). Wild-type mice did not develop OM and had a ME free of effusion, with no thickening of the mucosal lining (Figure 5.13A and C). The histology of *Nisch^{edsn/+}* and *Nisch^{V33A/+}* mice was indistinguishable from wild-type littermates.

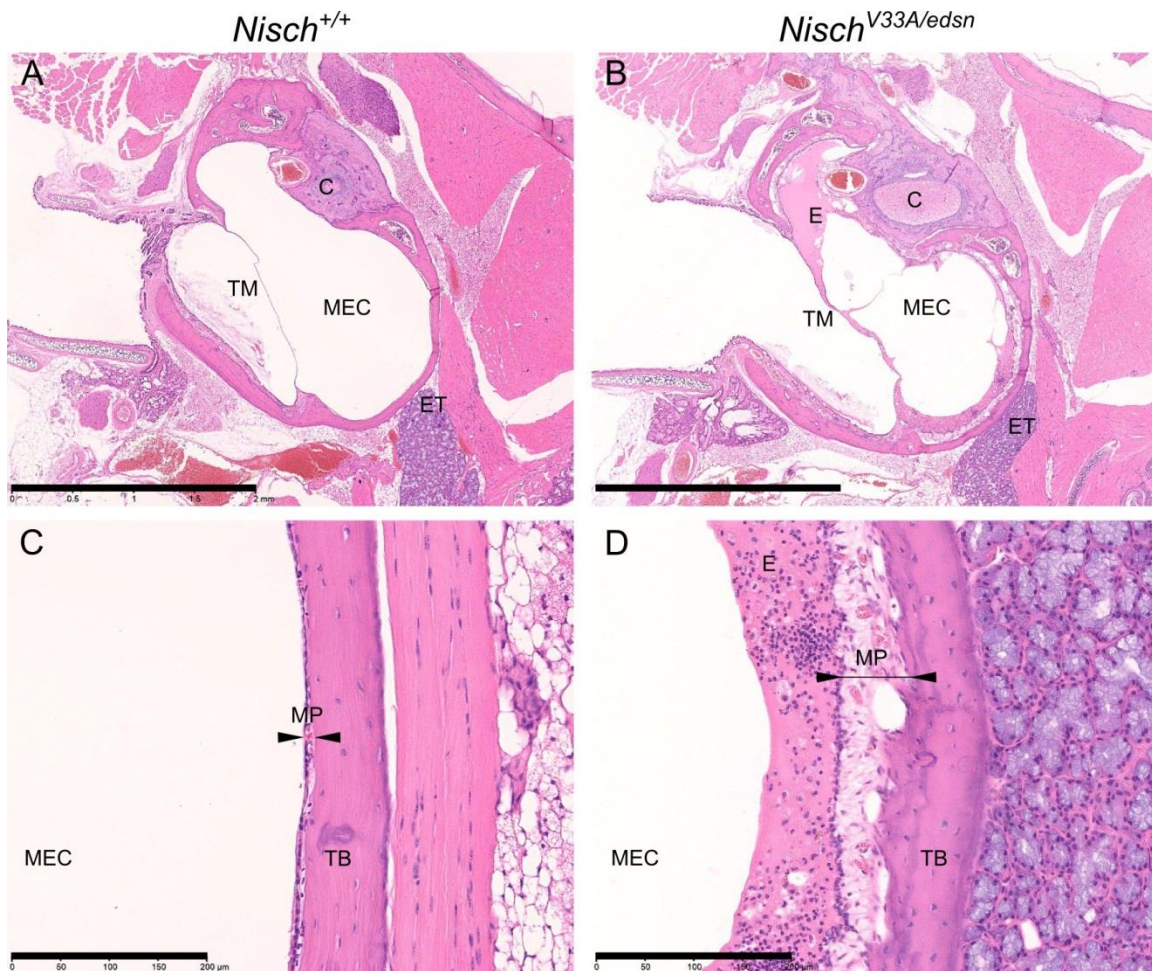


Figure 5.13| Characteristic ME histology in wild-type and *Nisch^{V33A/edsn}* animals at 12wk.

H&E stained transverse sections of the MEC and mucoperiosteum, in (A & C) *Nisch^{+/+}* and (B & D) *Nisch^{V33A/edsn}*, at 12wk. Histopathology of the MEC from *Nisch^{V33A/edsn}* mice demonstrates chronic inflammation with an exudate. Wild-type animals have no inflammation or exudate in the MEC and a thin mucoperiosteum covers the temporal bone. C, cochlea; ET, eustachian tube; E, exudate; MEC, middle ear cavity; MP, mucoperiosteum (arrowheads); TB, temporal bone; TM, tympanic membrane. (A & B) Scale bar = 2 mm; (C & D) Scale bar = 200 μm.

To quantify the chronic inflammation observed in *Nisch*^{V33A/V33A} mice and assess any subtle changes in the OM phenotype, blinded assessment of the mucoperiosteum thickness was carried out (Figure 5.14A). Comparable regions of ME were measured for each ear assessed. Consistent with inflammation, *Nisch*^{V33A/V33A} animals had a significantly thicker ME mucosa compared to wild-type littermates ($P = 0.018$). The mean mucosal thickness was 24.03 μm (± 3.601) in *Nisch*^{V33A/V33A} animals and 12.57 μm (± 0.922) for wild-types. As the OM phenotype was not fully penetrant in *Nisch*^{V33A/V33A} mice, the data was separated into ears that had an OM phenotype and ears that did not (Figure 5.14C). The criteria used to impartially categorise the ears into *Nisch*^{V33A/V33A} OM and *Nisch*^{V33A/V33A} Normal was from the subsequent visualisation of tympanic inflammation after dissection. *Nisch*^{V33A/V33A} ears with no OM had a mean mucosal thickness of 15.21 μm (± 0.716) and showed no significant difference compared to wild-type ($P = 0.101$). Whereas, *Nisch*^{V33A/V33A} ears with OM had a significantly thickened mucosa ($P = 0.002$), with a mean mucosal thickness of 41.68 μm (± 6.050).

Similar results were replicated in *Nisch*^{V33A/edsn} mice when mucoperiosteum thickness was measured (Figure 5.14B). *Nisch*^{V33A/edsn} animals had a significantly thicker ME mucosa compared to wild-type littermates ($P = 0.023$). The mean mucosal thickness was 37.23 μm (± 6.984) in *Nisch*^{V33A/edsn} animals and 14.44 μm (± 0.741) for wild-types. Similarly, as the OM phenotype was not fully penetrant in *Nisch*^{V33A/edsn} mice, the data was separated into ears that had an OM phenotype and ears that did not (Figure 5.14D). The criteria used to impartially categorise the ears into *Nisch*^{V33A/edsn} OM and *Nisch*^{V33A/edsn} normal was from the subsequent visualisation of tympanic inflammation after dissection. *Nisch*^{V33A/edsn} ears with no OM had a mean mucosal thickness of 17.11 μm (± 3.230) and showed no significant difference compared to wild-type ($P = 0.951$). Whereas, *Nisch*^{V33A/edsn} ears with OM had a significantly thickened mucosa ($P < 0.001$) with a mean mucosal thickness of 51.61 μm (± 8.104).

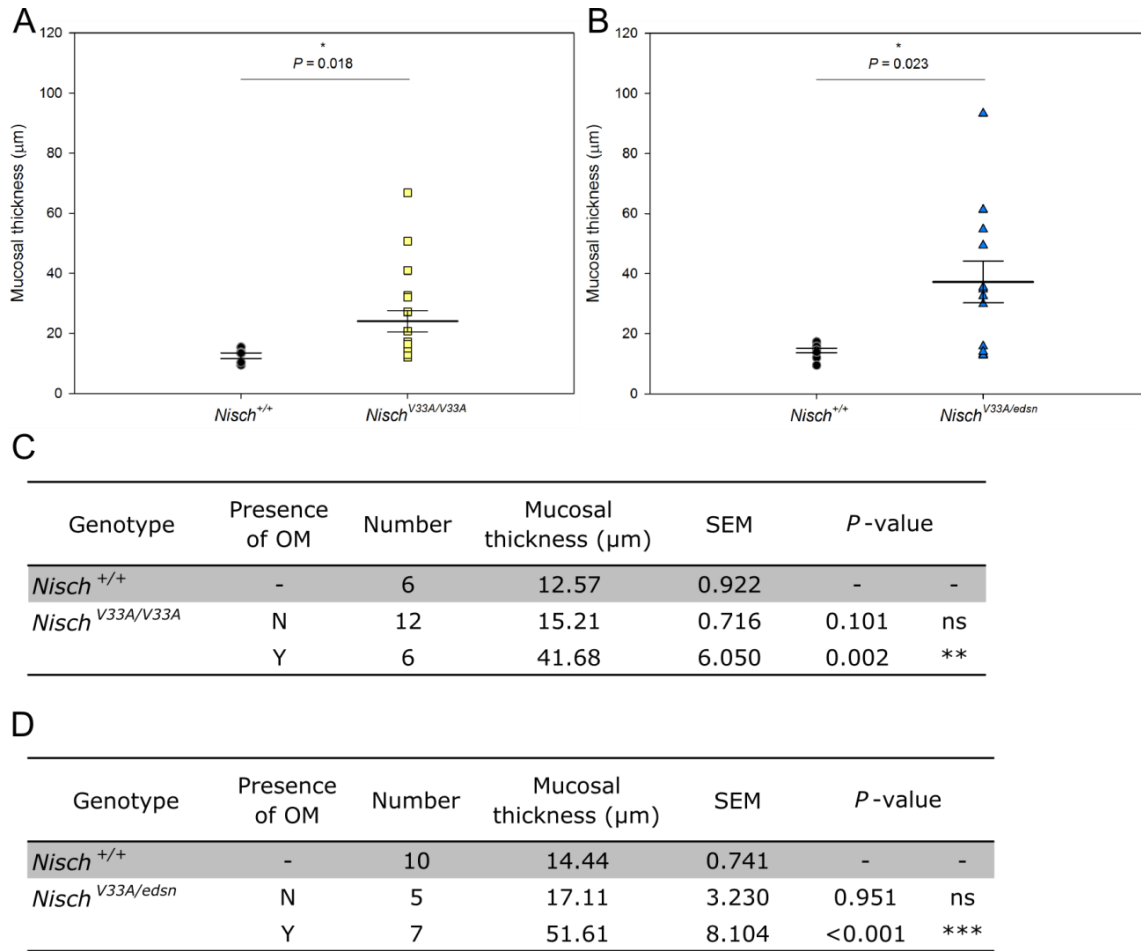


Figure 5.14| Analysis of mucosal thickness in the MEC of *Nisch*^{V33A/V33A} and *Nisch*^{V33A/edsn} mice.

H&E sections of ME were scanned into the Hamamatsu Pathology Imager and the mucosal thickness was averaged from 5 measurements of a standard 1000 μm length of ME mucosa (avoiding the cochlea and the region close to the ET). **(A)** Scatter plot showing mucosal thickness of the ME for *Nisch*^{+/+} and *Nisch*^{V33A/V33A} mice. Mice with the *Nisch*^{V33A/V33A} mutation show a significant increase in the mucosal thickness due to OM. **(B)** Scatter plot showing mucosal thickness of the ME for *Nisch*^{+/+} and *Nisch*^{V33A/edsn} mice. Mice with the *Nisch*^{V33A/edsn} mutations show a significant increase in the mucosal thickness due to OM. **(C)** Table showing the mean mucosal thickness of *Nisch*^{V33A/V33A} ears with or without OM, and corresponding *P*-values compared to wild-type littermates. **(D)** Table showing the mean mucosal thickness of *Nisch*^{V33A/edsn} ears with or without OM, and corresponding *P*-values compared to wild-type littermates. In scatter plots each point represents a single ear and thick black line indicates mean mucosal thickness ± SEM. * *P* < 0.05. Statistics were conducted using Mann Whitney Rank Sum tests.

(A) *Nisch*^{+/+} n = 6; *Nisch*^{V33A/V33A} n = 18. (B) *Nisch*^{+/+} n = 10; *Nisch*^{V33A/edsn} n = 12.

5.2.2.2 *Nisch*^{L142Q} allele displays no *edison* phenotypes**5.2.2.2.1 Genotypic ratio analysis**

Genotypes from *Nisch*^{L142Q/+} x *Nisch*^{L142Q/+} crosses (*Nisch*^{L142Q}|*Nisch*^{L142Q}) were compared in Table 5.6, to ascertain whether litters display reduced numbers of homozygous progeny as seen in *edison*. From the numbers of live mice observed at weaning age, there were fewer *Nisch*^{L142Q/L142Q} mice than were expected, however no significant genotypic differences were observed compared to expected Mendelian ratios ($P = 0.3329$). Similar observations were made when comparing genotypes from *Nisch*^{L142Q/+} x *Nisch*^{edsn/+} crosses (*Nisch*^{L142Q}|*Nisch*^{edsn}; Table 5.7). Again, from the numbers of live mice observed at weaning age, no significant genotypic differences were observed ($P = 0.7410$).

Table 5.6| Numbers of live mice from *Nisch*^{L142Q/+} x *Nisch*^{L142Q/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	12	10	30.0%	0.3329
<i>Nisch</i> ^{L142Q/+}	22	20	55.0%	
<i>Nisch</i> ^{L142Q/L142Q}	6	10	15.0%	
TOTAL	40			

Table 5.7| Numbers of live mice from *Nisch*^{L142Q/+} x *Nisch*^{edsn/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	19	16	29.7%	0.7410
<i>Nisch</i> ^{edsn/+}	17	16	26.6%	
<i>Nisch</i> ^{L142Q/+}	13	16	20.3%	
<i>Nisch</i> ^{L142Q/edsn}	15	16	23.4%	
TOTAL	64			

5.2.2.2.2 Weight

Analysis of weights across a longitudinal time course, from four to twenty weeks of age, demonstrated that no significant genotypic differences were observed in either gender at any time point from *Nisch*^{L142Q}|*Nisch*^{L142Q} littermates (Figure 5.15). This was also the case when mice from *Nisch*^{L142Q}|*Nisch*^{edsn} litters were analysed for weight across the same time course (Figure 5.16).

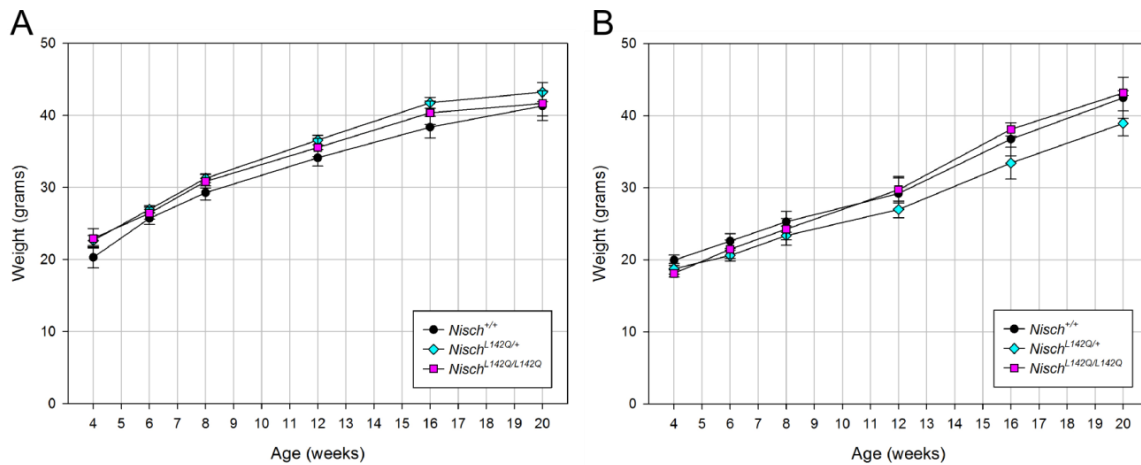


Figure 5.15| Weight data for *Nisch*^{L142Q}|*Nisch*^{L142Q} littermates over a longitudinal time course.

No genotypic differences in weight were observed in **(A)** male and **(B)** female mice from *Nisch*^{L142Q}|*Nisch*^{L142Q} litters. Statistics were conducted using one-way ANOVA's.

Nisch^{+/+} Male n = 6, Female n = 6; *Nisch*^{L142Q/+} Male n = 16, Female n = 4; *Nisch*^{L142Q/L142Q} Male n = 4, Female n = 2.

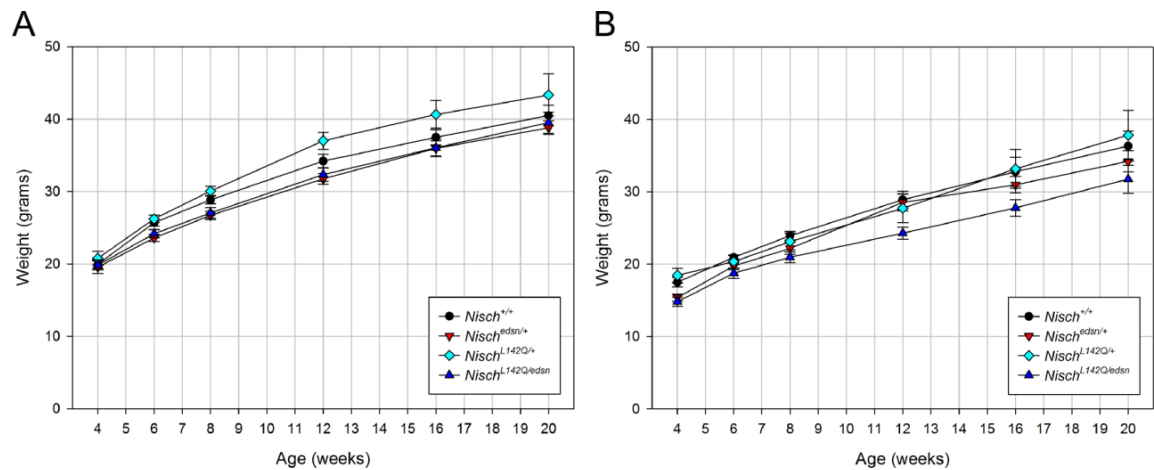


Figure 5.16| Weight data for mice from *Nisch*^{L142Q}|*Nisch*^{edsn} litters over a longitudinal time course.

No genotypic differences in weight were observed in **(A)** male and **(B)** female mice from *Nisch*^{L142Q}|*Nisch*^{edsn} litters. Statistics were conducted using one-way ANOVA's.

Nisch^{+/+} Male n = 11, Female n = 6; *Nisch*^{edsn/+} Male n = 9, Female n = 8; *Nisch*^{L142Q/+} Male n = 7, Female n = 5; *Nisch*^{L142Q/edsn} Male n = 8, Female n = 5.

5.2.2.2.3 ABR

In order to assess whether an abnormal hearing phenotype was displayed in mice from *Nisch*^{L142Q}|*Nisch*^{L142Q} and *Nisch*^{L142Q}|*Nisch*^{edsn} litters, ABR tests for auditory function were conducted across a longitudinal time course with age matched littermates (Figure 5.17 and 5.19 respectively). Hearing assessments were conducted as summarised in Section 4.2.4.1.

Analysis of ABR thresholds showed all mean thresholds from *Nisch*^{L142Q}|*Nisch*^{L142Q} littermates displayed normal, wild-type hearing thresholds of between 15-30 dB SPL (Figure 5.17A). No significant difference in ABR thresholds were observed between *Nisch*^{+/+}, *Nisch*^{L142Q/+} and *Nisch*^{L142Q/L142Q} mice across the time course. Likewise, no gender or left-right preference was observed in the ABR threshold data from each genotype (Appendix, Figure 10.7).

The ABR threshold range for each genotype was analysed using box and whisker plots. For *Nisch*^{+/+} and *Nisch*^{L142Q/+} littermates, the boxes (25th to 75th percentile) and

whiskers (10th to 90th percentiles) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for normal hearing (Figure 5.17B and C). A small number of outliers outside of the expected wild-type ABR threshold range were recorded in both genotypes. A greater variation in the ABR threshold range was observed in *Nisch*^{L142Q/L142Q} mice; with an increased number of ABR thresholds recorded above the expected wild-type threshold range across the time course (Figure 5.17D). An increased number of outliers were also observed in *Nisch*^{L142Q/L142Q} mice. The majority of ears recorded an ABR threshold in the expected range for wild-type hearing, indicating that there was a very low incidence of abnormal hearing thresholds in *Nisch*^{L142Q/L142Q} mutants.

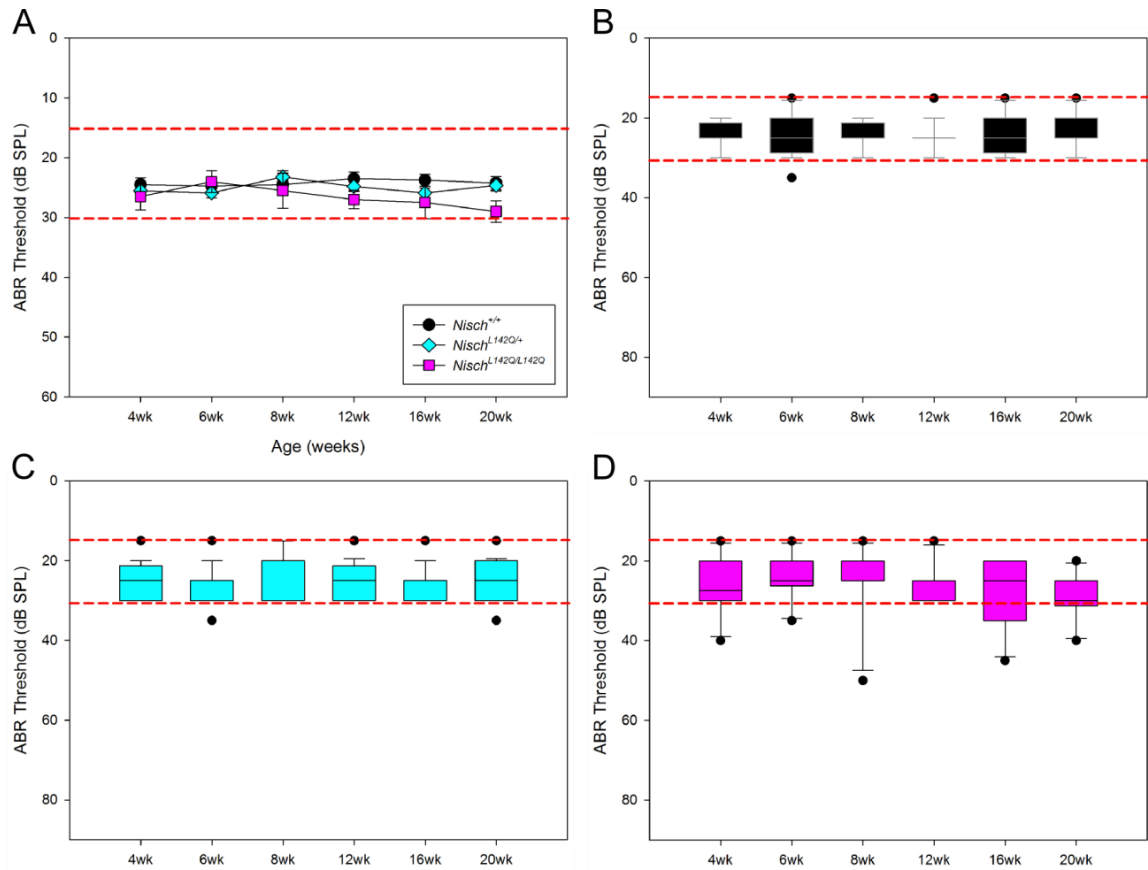


Figure 5.17| Progression of click-evoked ABR thresholds across a longitudinal time course for *Nisch*^{L142Q}/*Nisch*^{L142Q} littermates.

(A) Mice with the *Nisch*^{L142Q/L142Q} mutation display normal mean hearing thresholds compared to littermates. No significant genotypic differences were observed. (B-D) Box plots show the ABR threshold ranges of (B) *Nisch*^{+/+}, (C) *Nisch*^{L142Q/+} and (D) *Nisch*^{L142Q/L142Q} mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (•) show any outliers. *Nisch*^{+/+} n = 20; *Nisch*^{L142Q/+} n = 28; *Nisch*^{L142Q/L142Q} n = 10. Statistics were performed using Kruskal-Wallis tests.

The presence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was assessed across the time course for each genotype in litters from *Nisch*^{L142Q}/*Nisch*^{L142Q} crosses (Figure 5.18A-D). *Nisch*^{+/+} and *Nisch*^{L142Q/+} animals showed a small incidence of an increased ABR threshold. In *Nisch*^{L142Q/L142Q} mice the prevalence of an increased ABR threshold appeared to increase with age. At 4wk, 10% of *Nisch*^{L142Q/L142Q} ears tested had an increased ABR threshold (z-test, *P* = 0.719). By 16wk, the prevalence of an increased ABR threshold was increased

compared to wild-type littermates, where 30% of *Nisch*^{L142Q/L142Q} ears tested had an increased ABR threshold (z-test, $P = 0.053$). However, no significant differences between genotypes were observed in the presence of increased ABR thresholds throughout the time course.

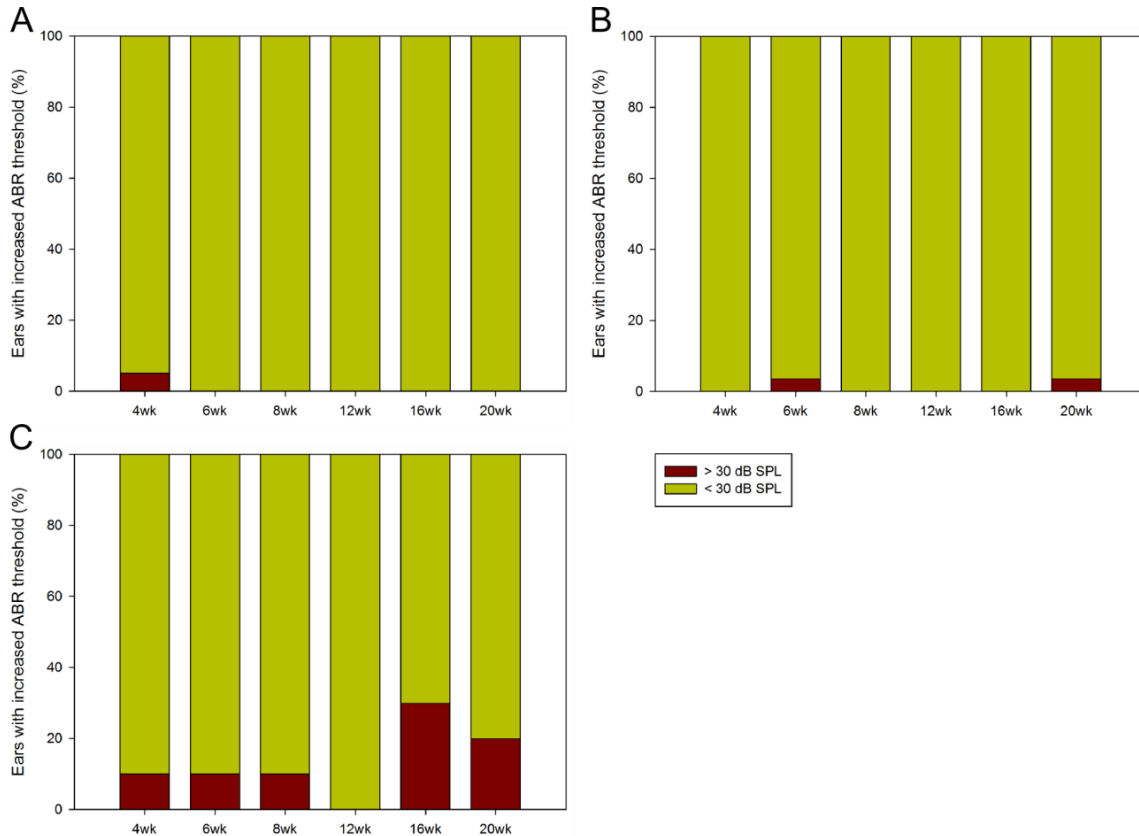


Figure 5.18| Presence of increased ABR thresholds in *Nisch*^{L142Q}/*Nisch*^{L142Q} litters across a time course.

(A-D) The presence or absence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was recorded in (A) *Nisch*^{+/+}, (B) *Nisch*^{L142Q/+} and (C) *Nisch*^{L142Q/L142Q} mice. The prevalence of increased ABR thresholds above the normal range is increased in *Nisch*^{L142Q/L142Q} animals compared to littermates.

Analysis of ABR thresholds found there were no significant genotypic differences in mean ABR thresholds of mice from *Nisch*^{L142Q}/*Nisch*^{edsn} litters throughout the time course (Figure 5.19A). All *Nisch*^{L142Q}/*Nisch*^{edsn} littermates displayed a normal, wild-

type hearing threshold between 15-30 dB SPL. Also, there was no gender or left-right preference observed in ABR threshold data for all genotypes from *Nisch*^{L142Q}|*Nisch*^{edsn} litters (Appendix, Figure 10.8).

The ABR threshold range for each genotype was analysed using box and whisker plots. For *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{L142Q/+} littermates, the boxes (25th to 75th percentile) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for normal hearing (Figure 5.19B-D). A small number of outliers were recorded in each genotype outside of the expected wild-type ABR threshold range. The ABR threshold range in *Nisch*^{L142Q/edsn} mice slightly increased with age across the time course (Figure 5.19E). At 12wk and 20wk, the threshold range was from 15 to 50 dB SPL, but with a median of 25 dB SPL. The majority of ears recorded an ABR threshold in the expected range for wild-type hearing, indicating that there was a very low incidence of abnormal hearing thresholds in *Nisch*^{L142Q/edsn} compound mutants.

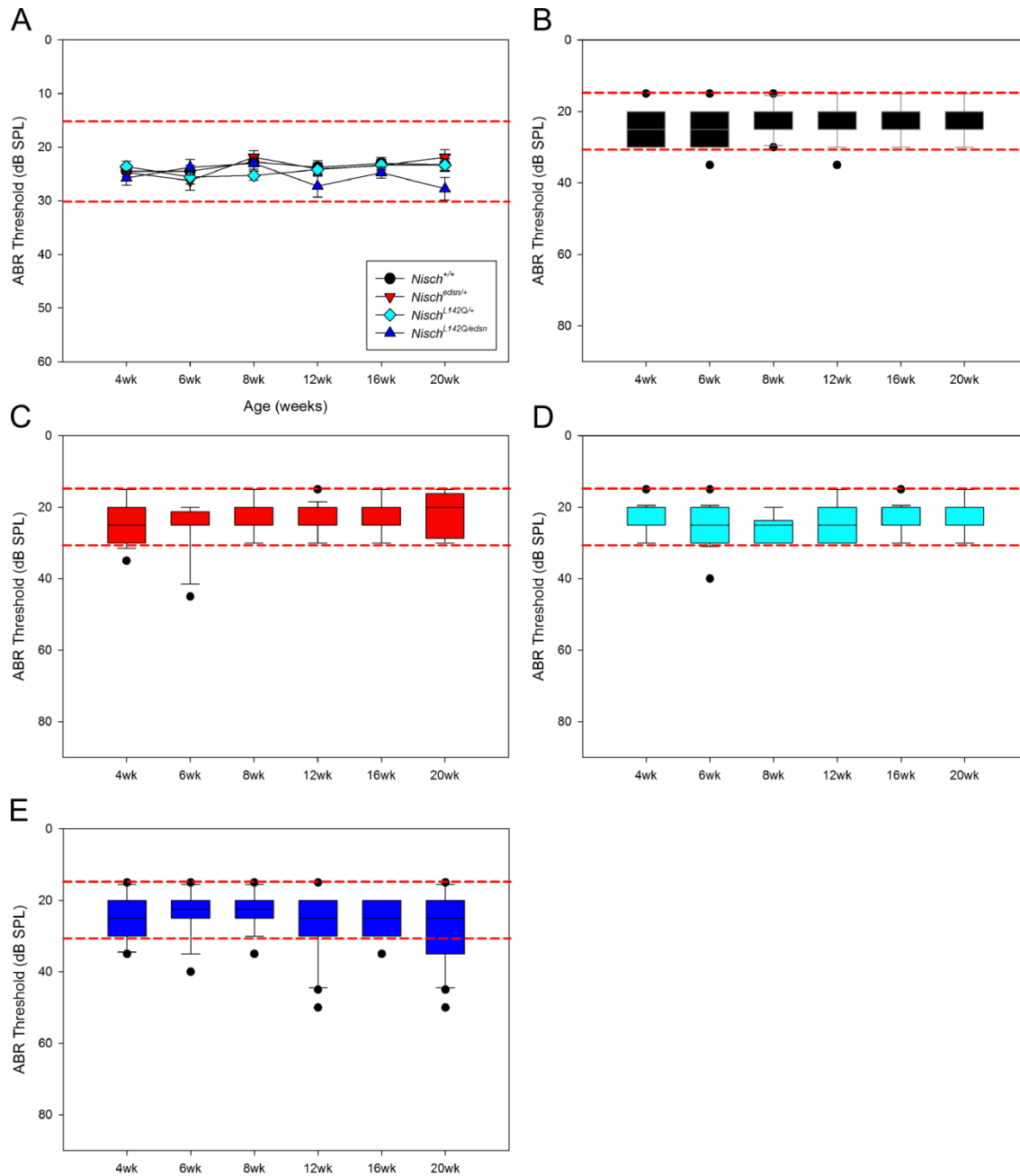


Figure 5.19| Progression of click-evoked ABR thresholds across a longitudinal time course for *Nisch*^{L142Q}|*Nisch*^{edn} littermates.

(A) Mice with the *Nisch*^{L142Q/edn} mutation display normal mean hearing thresholds compared to littermates. No significant genotypic differences were observed. **(B-E)** Box plots show the ABR threshold ranges of (B) *Nisch*^{+/+}, (C) *Nisch*^{edn/+} (D) *Nisch*^{L142Q/+} and (E) *Nisch*^{L142Q/edn} mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (●) show any outliers. *Nisch*^{+/+} n = 20; *Nisch*^{edn/+} n = 16; *Nisch*^{L142Q/+} n = 18; *Nisch*^{L142Q/edn} n = 20. Statistics were performed using Kruskal-Wallis tests.

The presence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was assessed across the time course for each genotype in mice from *Nisch*^{L142Q}|*Nisch*^{edsn} litters (Figure 5.20A-D). *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{L142Q/+} animals showed a small incidence of an increased ABR threshold. In *Nisch*^{L142Q/edsn} mice the prevalence of an increased ABR threshold was present at each time point and increased with age. At 4wk, 10% of *Nisch*^{L142Q/edsn} ears tested had an increased ABR threshold (z-test, $P = 0.468$). By 20wk, the prevalence of an increased ABR threshold was significantly increased compared to wild-type littermates, where 30% of *Nisch*^{L142Q/edsn} ears tested had an increased ABR threshold (z-test, $P = 0.027$). No other time point showed a significant genotypic difference in the presence of increased ABR thresholds.

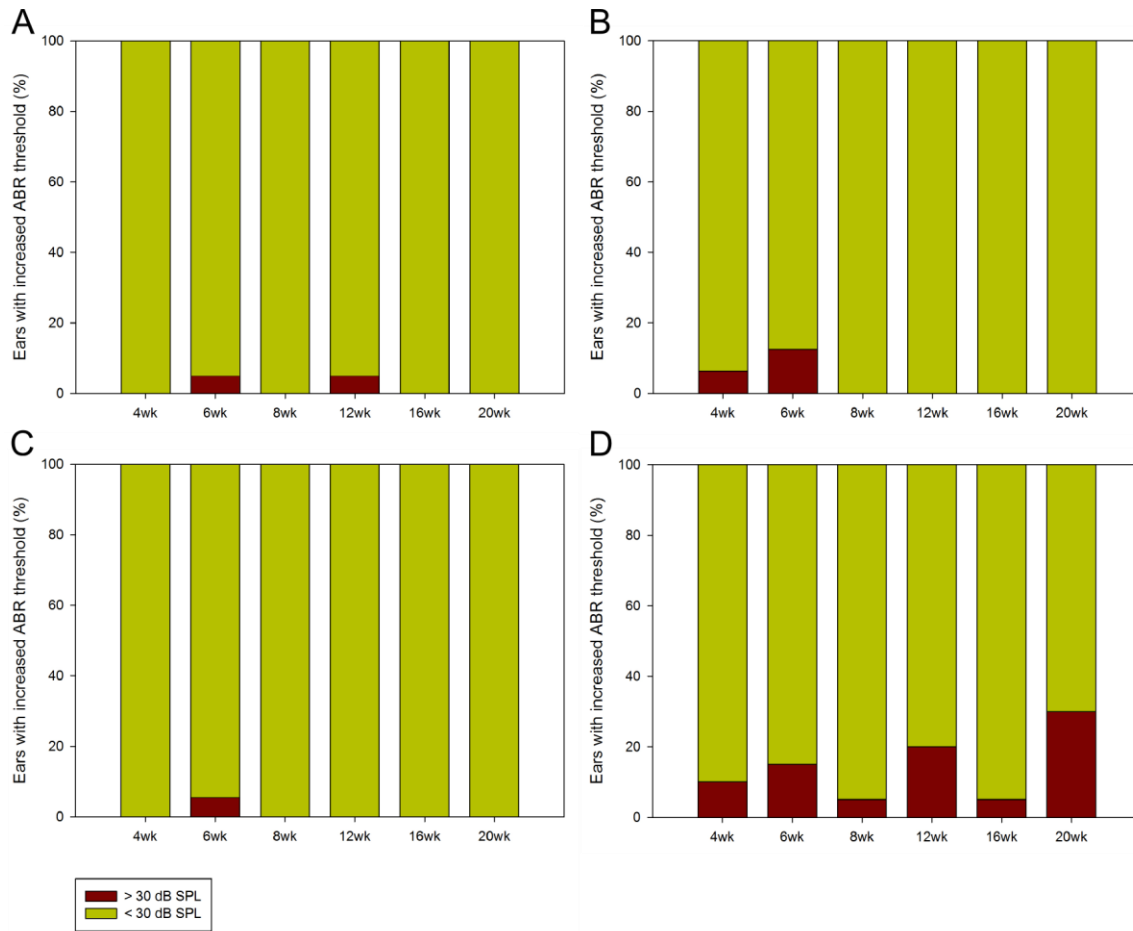


Figure 5.20| Presence of increased ABR thresholds in *Nisch*^{L142Q}/*Nisch*^{edsn} litters across a time course.

(A-D) The presence or absence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was recorded in (A) *Nisch*^{+/+}, (B) *Nisch*^{edsn/+}, (C) *Nisch*^{L142Q/+} and (D) *Nisch*^{L142Q/edsn} mice. The prevalence of increased ABR thresholds above the normal range is increased in *Nisch*^{L142Q/edsn} animals compared to littermates.

5.2.2.2.4 Histology

As the prevalence of an increased ABR threshold was greater in *Nisch*^{L142Q/L142Q} and *Nisch*^{L142Q/edsn} mice compared to littermates, the incidence of OM was analysed from visual inspection of the tympanic membrane and the presence or absence of an inflamed eardrum, which is synonymous with OM (as previously described in Figure 4.12, section 4.2.4.2).

Analysis of eardrums from all genotypes of *Nisch*^{L142Q}/*Nisch*^{L142Q} crosses showed there was no incidence of OM throughout littermates (Figure 5.21). *Nisch*^{L142Q}/*Nisch*^{edsn} littermates were also investigated (Figure 5.22), and in *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{L142Q/edsn} mice again there was no incidence of OM. A small incidence of unilateral OM was observed in *Nisch*^{L142Q/+} animals (8%), however there were no significant differences between genotypes ($P = 0.926$).

Histopathological examination of mice from *Nisch*^{L142Q}/*Nisch*^{L142Q} and *Nisch*^{L142Q}/*Nisch*^{edsn} litters at 20wk confirmed that the majority of mice did not develop OM (data not shown). They had a ME free of effusion, with no thickening of the mucosal lining. Histology confirmed only one *Nisch*^{L142Q/+} animal displayed an OM phenotype and in one ear. The histology of most *Nisch*^{L142Q}/*Nisch*^{L142Q} and *Nisch*^{L142Q}/*Nisch*^{edsn} genotypes was indistinguishable from that of wild-type littermates.

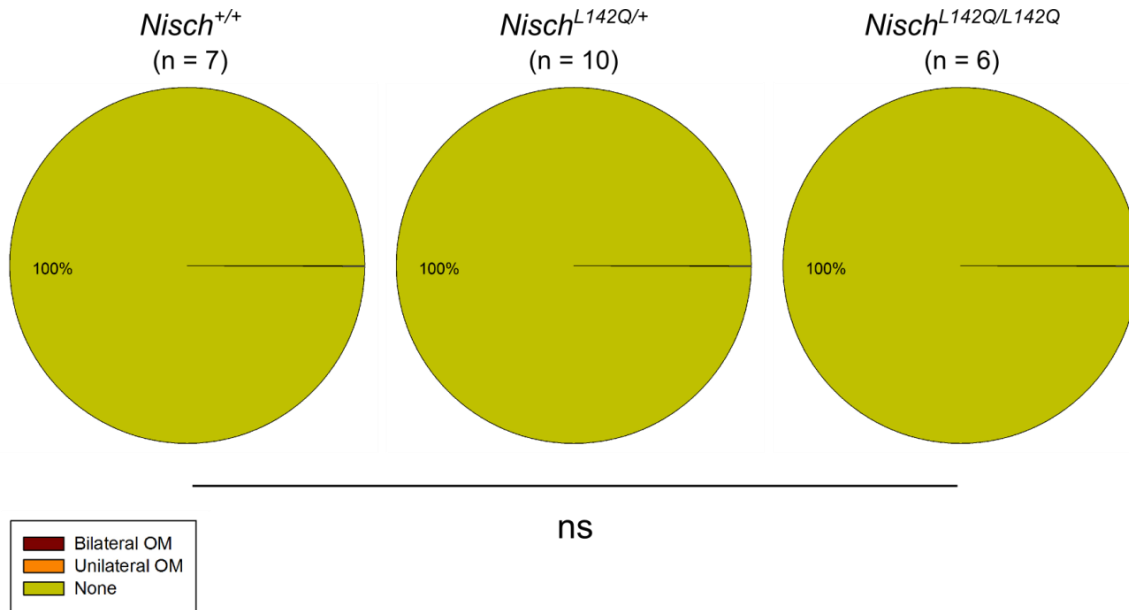


Figure 5.21 | Incidence of OM in mice from *Nisch*^{L142Q} | *Nisch*^{L142Q} litters at 20wk.

The presence or absence of an inflamed eardrum was recorded from visual inspection after dissection. The incidence of OM was recorded in *Nisch*^{+/+}, *Nisch*^{L142Q/+} and *Nisch*^{L142Q/L142Q} mice. No differences between genotypes were observed. ns $P > 0.05$. Statistics were conducted using z-tests.

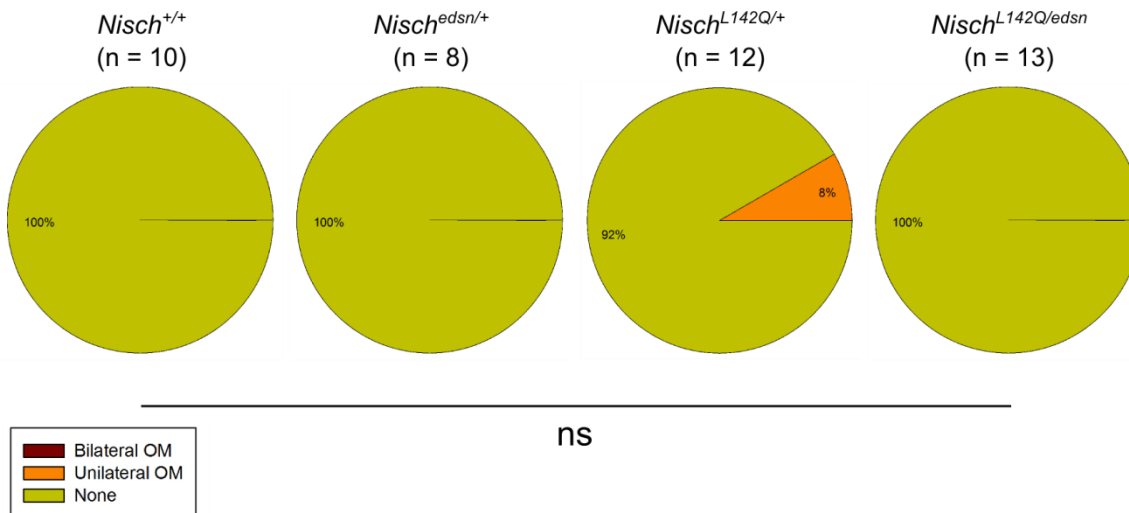


Figure 5.22 | Incidence of OM in *Nisch*^{L142Q} | *Nisch*^{edsn} littermates at 20wk.

The presence or absence of an inflamed eardrum was recorded from visual inspection after dissection. The incidence of OM was recorded in *Nisch*^{+/+}, *Nisch*^{edsn/+}, *Nisch*^{L142Q/+} and *Nisch*^{L142Q/edsn} mice. No significant differences between genotypes were observed between littermates. ns $P > 0.05$. Statistics were conducted using z-tests.

To evaluate whether inflammation was observed in the ME of mice from *Nisch*^{L142Q}/*Nisch*^{L142Q} litters and to assess any subtle sub-clinical changes, blinded assessment of the mucoperiosteum thickness was carried out (Figure 5.23A). Comparable regions of ME were measured for each ear assessed. Consistent with the absence of tympanic inflammation, *Nisch*^{L142Q/L142Q} animals showed no significant changes in ME mucosa compared to wild-type littermates ($P = 0.665$). The mean mucosal thickness was 16.16 μm (± 1.362) in *Nisch*^{L142Q/L142Q} animals and 15.32 μm (± 1.172) in wild-types. Similar results were replicated in *Nisch*^{L142Q}/*Nisch*^{edsn} littermates when mucoperiosteum thickness was measured (Figure 5.23B). No significant genotypic difference was observed in the ME mucosa ($P = 0.524$), where the mean mucosal thickness was 12.42 μm (± 0.560) in *Nisch*^{L142Q/edsn} animals and 13.11 μm (± 1.012) in wild-type littermates.

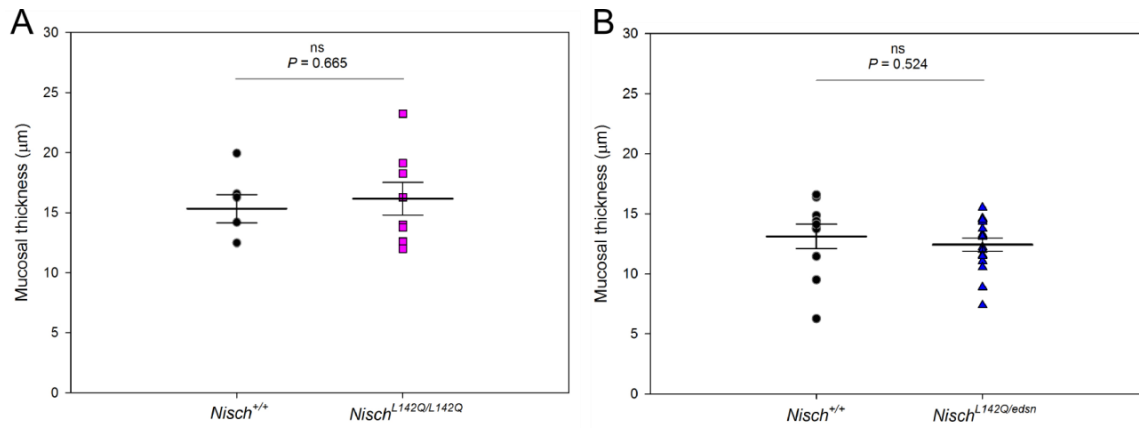


Figure 5.23| Analysis of mucosal thickness in the MEC of *Nisch*^{L142Q/L142Q} and *Nisch*^{L142Q/edsn} mice.

H&E sections of ME were scanned into the Hamamatsu Pathology Imager and the mucosal thickness was averaged from 5 measurements of a standard 1000 μm length of ME mucosa (avoiding the cochlea and the region close to the ET). **(A)** Scatter plot showing mucosal thickness of the ME for *Nisch*^{+/+} and *Nisch*^{L142Q/L142Q} mice. No significant genotypic difference in mucosal thickness is observed. **(B)** Scatter plot showing mucosal thickness of the ME for *Nisch*^{+/+} and *Nisch*^{L142Q/edsn} mice. No significant genotypic difference in mucosal thickness is observed. Each point represents a single ear and thick black line indicates mean mucosal thickness ± SEM. ns $P > 0.05$. Statistics were conducted using Student's t-tests.

(A) *Nisch*^{+/+} n = 6; *Nisch*^{L142Q/L142Q} n = 8. (B) *Nisch*^{+/+} n = 10; *Nisch*^{L142Q/edsn} n = 16.

5.2.2.3 *Nisch*^{T445I} allele also displays no edison phenotypes

5.2.2.3.1 Genotypic ratio analysis

Genotypic ratios were compared, in litters from *Nisch*^{T445I/+} × *Nisch*^{T445I/+} crosses (*Nisch*^{T445I}|*Nisch*^{T445I}), to assess if there were any abnormal reductions from the expected progeny (Table 5.8). From the numbers of live mice observed at weaning age, interestingly there were fewer *Nisch*^{+/+} mice than were expected, however no significant genotypic differences were observed compared to expected Mendelian ratios ($P = 0.5674$). Similar observations were made when instead comparing genotypes from *Nisch*^{T445I/+} × *Nisch*^{edsn/+} crosses (*Nisch*^{T445I}|*Nisch*^{edsn}; Table 5.9). Even though there were fewer *Nisch*^{+/+} mice than were expected from the numbers of live mice observed at weaning age, again no significant genotypic differences were observed ($P = 0.3340$).

Table 5.8| Numbers of live mice from *Nisch*^{T445I/+} x *Nisch*^{T445I/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	9	11	20.0%	0.5674
<i>Nisch</i> ^{T445I/+}	22	23	48.9%	
<i>Nisch</i> ^{T445I/T445I}	14	11	31.1%	
TOTAL	45			

Table 5.9| Numbers of live mice from *Nisch*^{T445I/+} x *Nisch*^{edsn/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Nisch</i> ^{+/+}	5	10	12.5%	0.3340
<i>Nisch</i> ^{edsn/+}	12	10	30.0%	
<i>Nisch</i> ^{T445I/+}	11	10	27.5%	
<i>Nisch</i> ^{T445I/edsn}	12	10	30.0%	
TOTAL	40			

5.2.2.3.2 Weight

Analysis of weights across a longitudinal time course, from four to twenty weeks of age, demonstrated that no significant genotypic differences were observed in male from *Nisch*^{T445I}|*Nisch*^{T445I} litters at any time point. Female mice with the *Nisch*^{T445I/+} mutation were marginally, but significantly larger than their wild-type littermates from 16wk and older ($P < 0.05$) (Figure 5.24). Female *Nisch*^{T445I/T445I} animals showed no significant difference to wild-type littermates throughout the time course. However, this was not replicated in *Nisch*^{T445I}|*Nisch*^{edsn} littermates, which displayed no significant genotypic differences in either gender across the time course (Figure 5.25).

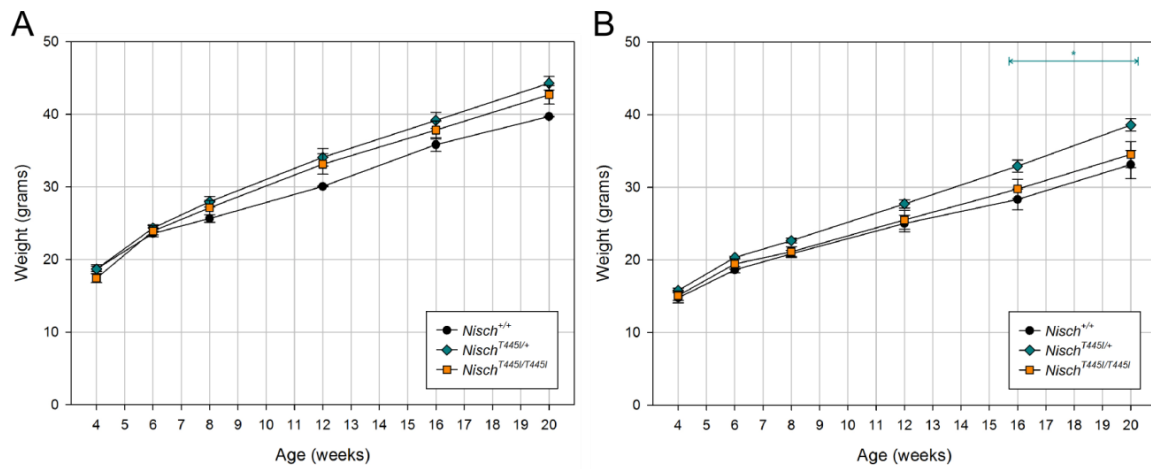


Figure 5.24| Weight data for mice from *Nisch*^{T445I}|*Nisch*^{T445I} litters over a longitudinal time course.

(A) No genotypic differences in weight are observed in male mice from *Nisch*^{T445I}|*Nisch*^{T445I} litters. **(B)** Female *Nisch*^{T445I/+} animals are slightly larger than wild-type littermates from 16wk. No genotypic differences in weight are observed between female *Nisch*^{+/+} and *Nisch*^{T445I/T445I} littermates. * $P < 0.05$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} Male $n = 2$, Female $n = 7$; *Nisch*^{T445I/+} Male $n = 8$, Female $n = 11$; *Nisch*^{T445I/T445I} Male $n = 8$, Female $n = 5$.

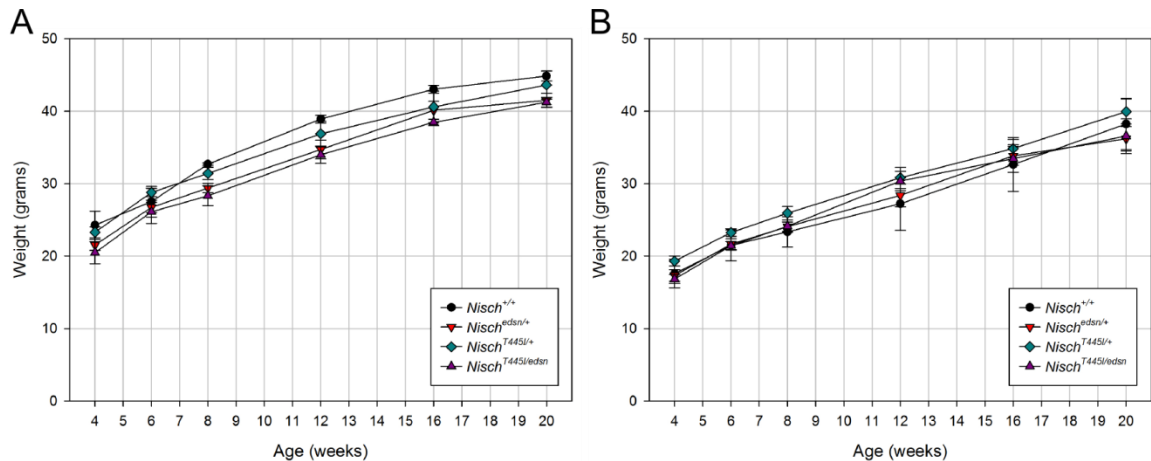


Figure 5.25| Weight data for *Nisch*^{T445I}|*Nisch*^{edsn} littermates over a longitudinal time course.

No genotypic differences in weight were observed in **(A)** male and **(B)** female mice from *Nisch*^{T445I}|*Nisch*^{edsn} litters. Statistics were conducted using one-way ANOVA's.

Nisch^{+/+} Male n = 3, Female n = 2; *Nisch*^{edsn/+} Male n = 7, Female n = 5; *Nisch*^{T445I/+} Male n = 5, Female n = 6; *Nisch*^{T445I/edsn} Male n = 3, Female n = 8.

5.2.2.3.3 ABR

ABR tests for auditory function were again conducted across a longitudinal time course with age matched littermates in order to assess whether an abnormal hearing deficit was displayed in mice from *Nisch*^{T445I}|*Nisch*^{T445I} and *Nisch*^{T445I}|*Nisch*^{edsn} litters (Figure 5.26 and 5.28 respectively). Hearing assessments were conducted as summarised in Section 4.2.4.1.

Analysis of ABR thresholds showed mean thresholds from all *Nisch*^{T445I}|*Nisch*^{T445I} littermates displayed normal, wild-type hearing thresholds between 15-30 dB SPL (Figure 5.26A). No significant difference in ABR thresholds were observed between *Nisch*^{+/+} and *Nisch*^{L142Q/+} mice across the time course. *Nisch*^{T445I/T445I} animals displayed a slightly significant increase in ABR thresholds at 4 and 6wk, however from 8wk this increase was no longer significant compared to wild-type littermates. At 4wk, mutants with the *Nisch*^{T445I/T445I} mutation were found to have slightly elevated auditory thresholds compared to wild-types, with a mean ABR threshold of

28.25 dB SPL (± 1.508). Even though the mean threshold fell within the expected wild-type threshold range, a significant difference was observed ($P = 0.030$). This was observed again at 6wk, where *Nisch*^{T445I/T445I} mice had a mean ABR threshold of 28.50 dB SPL (± 1.543), which was again a significant increase compared to wild-type ($P = 0.006$). From 8wk onwards no significant difference in ABR thresholds were observed between genotypes. Additionally, no gender or left-right preference was observed in the ABR threshold data from each genotype (Appendix, Figure 10.9).

The ABR threshold range for each genotype was analysed using box and whisker plots. For *Nisch*^{+/+} and *Nisch*^{T445I/+} littermates, the boxes (25th to 75th percentile) and whiskers (10th to 90th percentiles) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for normal hearing (Figure 5.26B and C). A small number of outliers outside of the expected wild-type ABR threshold range were recorded in both genotypes. A greater variation in the ABR threshold range was observed in *Nisch*^{T445I/T445I} mice; with an increased number of ABR thresholds recorded above the expected wild-type threshold range across the time course (Figure 5.26D). An increased number of outliers were also observed in *Nisch*^{T445I/T445I} mice. At 6wk, where the greatest significance was observed between mean ABR thresholds, the threshold range was from 20 to 45 dB SPL with a median of 27.5 dB SPL. The majority of ears recorded an ABR threshold in the expected range for wild-type hearing, indicating that there was a very low incidence of abnormal hearing thresholds in *Nisch*^{T445I/T445I} mutants.

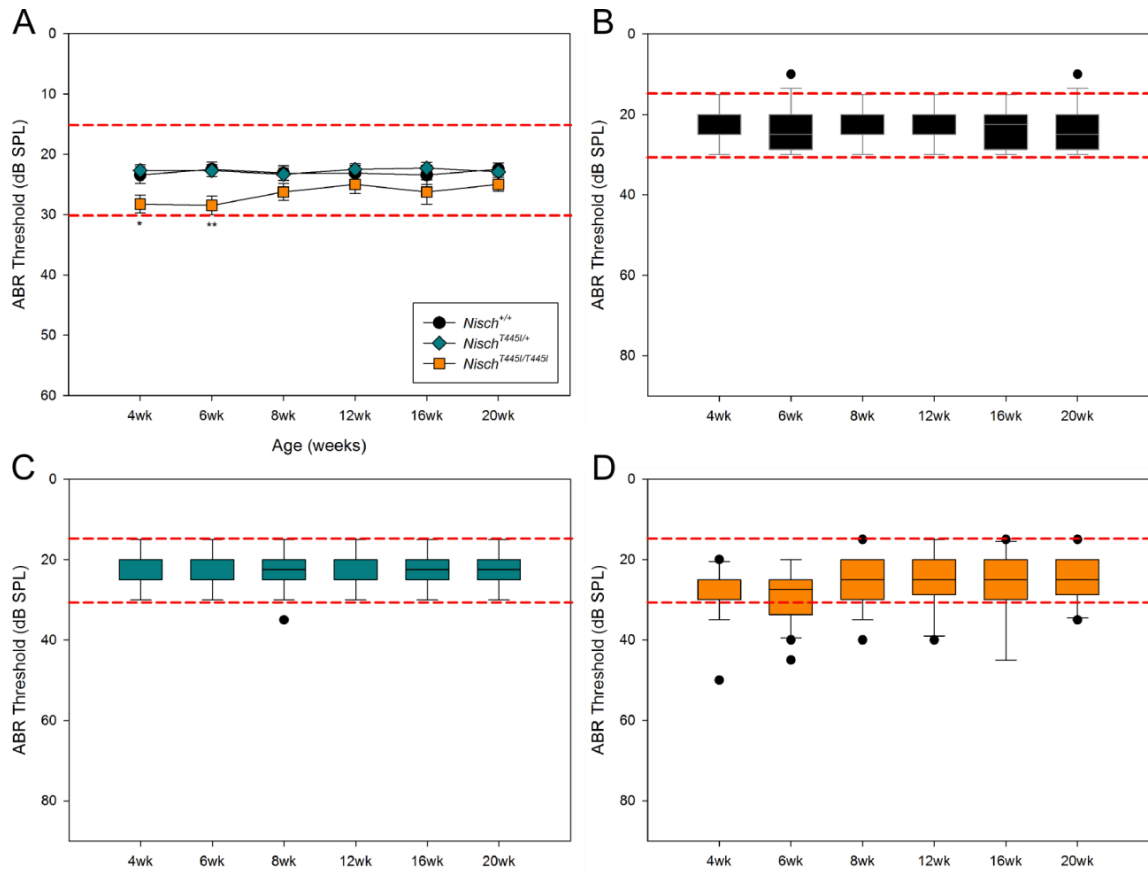


Figure 5.26| Progression of click-evoked ABR thresholds across a longitudinal time course for *Nisch*^{T445I}/*Nisch*^{T445I} littermates.

(A) Mice with the *Nisch*^{T445I/T445I} mutation have elevated hearing thresholds at 4 and 6wk. There is no difference in hearing thresholds between genotypes at any other time point. **(B-D)** Box plots show the ABR threshold ranges of (B) *Nisch*^{+/+}, (C) *Nisch*^{T445I/+} and (D) *Nisch*^{T445I/T445I} mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (•) show any outliers. * $P < 0.05$; ** $P < 0.01$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} $n = 16$; *Nisch*^{T445I/+} $n = 24$; *Nisch*^{T445I/T445I} $n = 20$.

The presence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was assessed across the time course for each genotype from *Nisch*^{T445I}/*Nisch*^{T445I} littermates (Figure 5.27A-C). *Nisch*^{+/+} animals recorded no ABR thresholds greater than 30 dB SPL, whereas *Nisch*^{T445I/+} mice showed a small incidence of an increased ABR threshold. In *Nisch*^{T445I/T445I} mice the prevalence of an increased ABR threshold was greater at each time point, however no time point

showed a significant genotypic difference compared to wild-type. At 6wk, 25% of *Nisch*^{T445I/T445I} ears tested had an increased ABR threshold, but no significance was observed (z-test, $P = 0.095$).

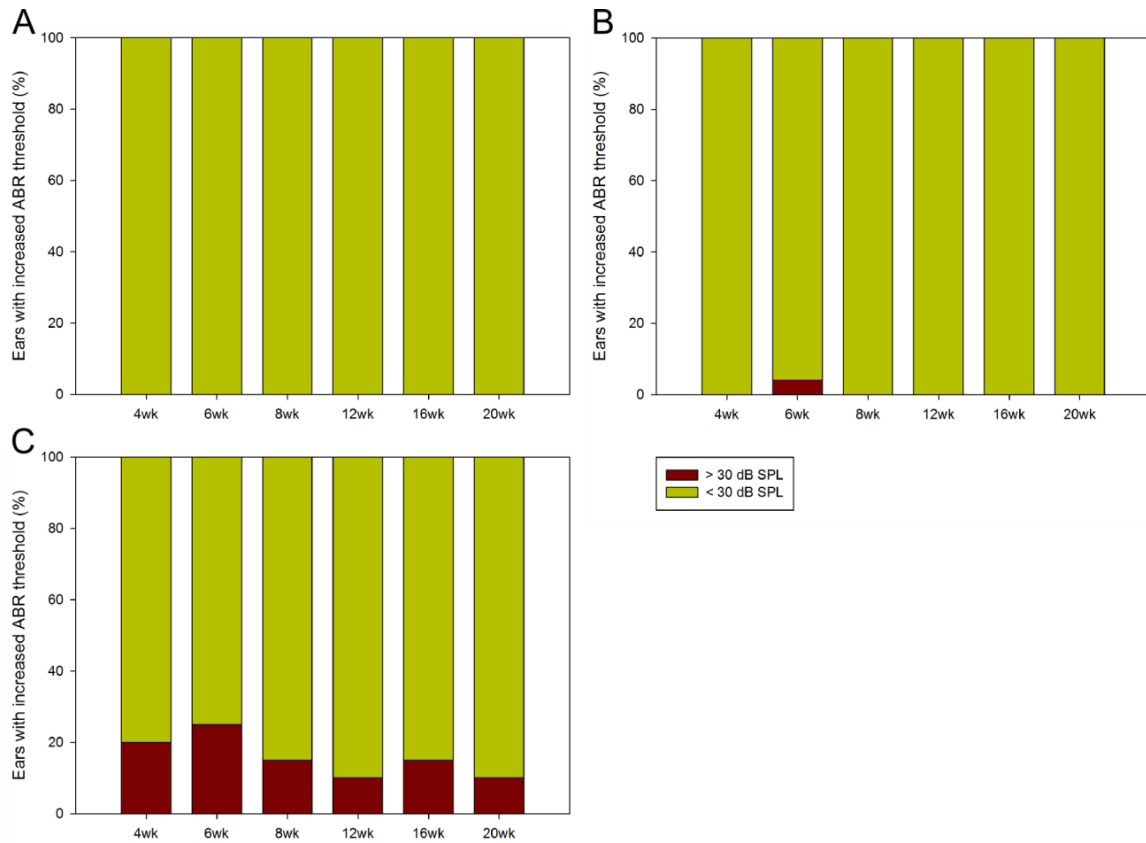


Figure 5.27| Presence of increased ABR thresholds in *Nisch*^{T445I}/*Nisch*^{T445I} littermates across a time course.

(A-C) The presence or absence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was recorded in (A) *Nisch*^{+/+}, (B) *Nisch*^{T445I/+} and (C) *Nisch*^{T445I/T445I} mice. The prevalence of increased ABR thresholds above the normal range is increased in *Nisch*^{T445I/T445I} animals compared to littermates.

Analysis of ABR thresholds showed mean thresholds from all *Nisch*^{T445I}/*Nisch*^{edsn} littermates displayed normal, wild-type hearing thresholds between 15–30 dB SPL throughout the time course (Figure 5.28A), except at 8wk where *Nisch*^{T445I/edsn} mice showed a significant increase above the expected threshold range ($P = 0.026$). At

8wk, *Nisch*^{T445I/edsn} mutants had a mean ABR threshold of 30.83 dB SPL ($\pm$ 1.991), compared to wild-type mice with a mean ABR threshold of 23.75 dB SPL ($\pm$ 3.146). This difference was not observed at any other time point. No significant difference in ABR thresholds were observed between *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{T445I/+} mice across the time course. Additionally, no gender or left-right preference was observed in the ABR threshold data from each genotype (Appendix, Figure 10.10).

The ABR threshold range for each genotype was analysed using box and whisker plots. For *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{T445I/+} littermates, the boxes (25th to 75th percentile) and whiskers (10th to 90th percentiles) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for normal hearing (Figure 5.28B and C). A small number of outliers outside of the expected wild-type ABR threshold range were recorded in *Nisch*^{edsn/+} animals. A greater variation in the ABR threshold range was observed in *Nisch*^{T445I/edsn} mice; with an increased number of ABR thresholds recorded above the expected wild-type threshold range across the time course (Figure 5.28D). An increased number of outliers were also observed in *Nisch*^{T445I/edsn} mice. At 8wk, where the greatest significance was observed between mean ABR thresholds, the threshold range was from 20 to 50 dB SPL with a median of 30 dB SPL. The majority of ears recorded an ABR threshold in the expected range for wild-type hearing, indicating that there was a very low incidence of abnormal hearing thresholds in *Nisch*^{T445I/edsn} mutants.

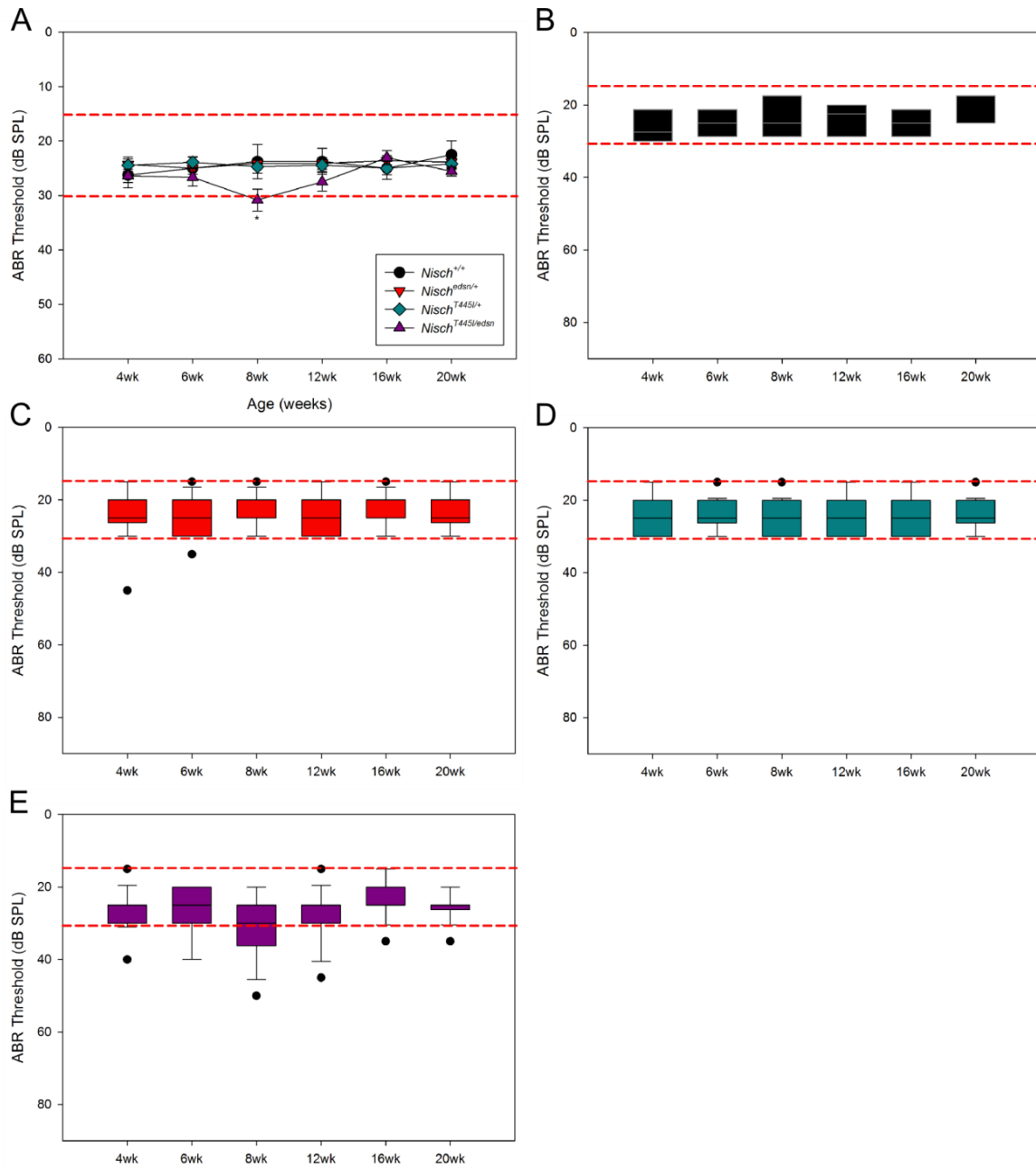


Figure 5.28| Progression of click-evoked ABR thresholds across a longitudinal time course for *Nisch*^{T445I}/*Nisch*^{edsn} littermates.

(A) *Nisch*^{T445I/edsn} mice have elevated hearing thresholds at 8wk. There is no difference in hearing thresholds observed between genotypes at any other time point. (B-E) Box plots show the ABR threshold ranges of (B) *Nisch*^{+/+}, (C) *Nisch*^{edsn/+} (D) *Nisch*^{T445I/+} and (E) *Nisch*^{T445I/edsn} mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (●) show any outliers. * $P < 0.05$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} $n = 4$; *Nisch*^{edsn/+} $n = 22$; *Nisch*^{L142Q/+} $n = 18$; *Nisch*^{L142Q/edsn} $n = 18$.

The presence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was assessed across the time course for each genotype from *Nisch*^{T445I}/*Nisch*^{edsn} litters (Figure 5.29A-D). *Nisch*^{+/+} and *Nisch*^{T445I/+} animals recorded no ABR thresholds greater than 30 dB SPL, whereas *Nisch*^{edsn/+} mice showed a small incidence of an increased ABR threshold. In *Nisch*^{T445I/edsn} mice the prevalence of an increased ABR threshold was present at each time point and peaked at 8wk, however, no time point showed a significant genotypic difference compared to wild-type. At 8wk, 27.8% of *Nisch*^{T445I/edsn} ears tested had an increased ABR threshold (z-test, $P = 0.589$).

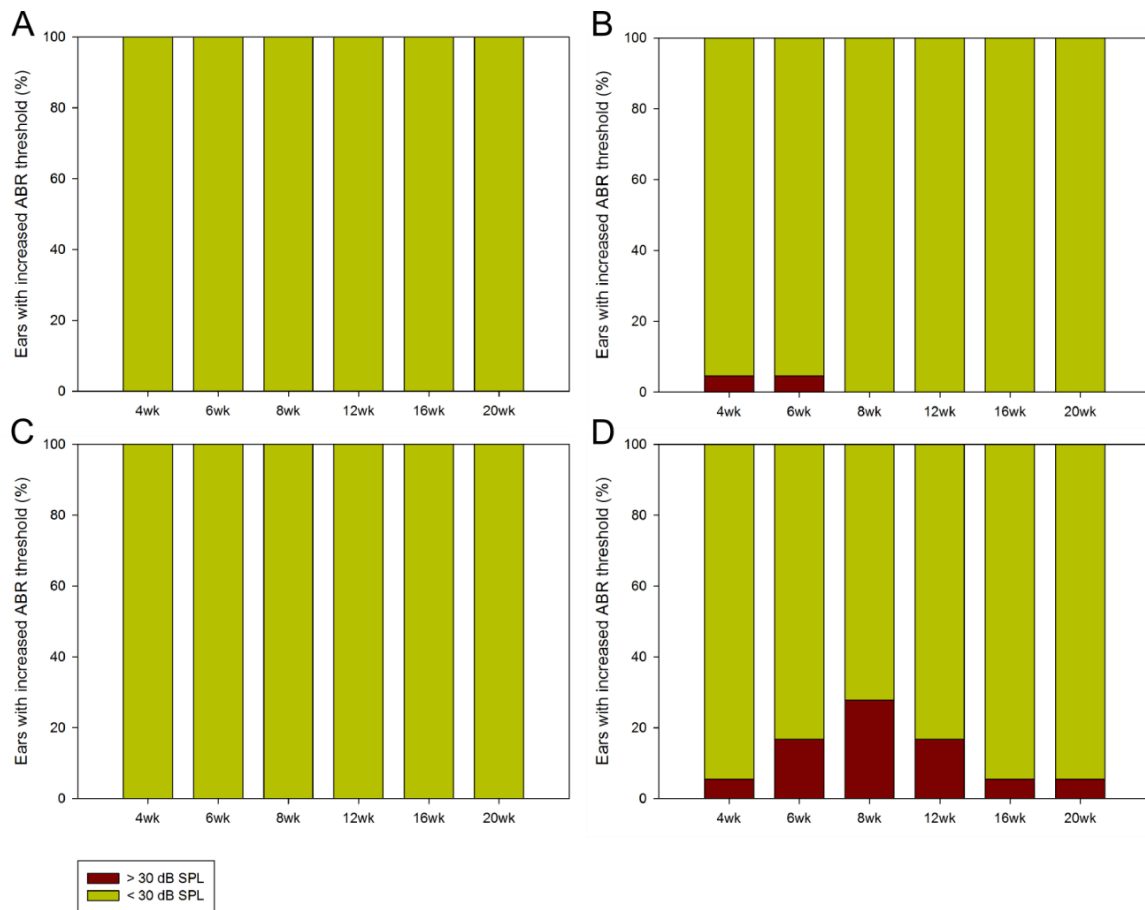


Figure 5.29| Presence of increased ABR thresholds in mice from *Nisch*^{T445I}/*Nisch*^{edsn} litters across a time course.

(A-D) The presence or absence of an increased ABR threshold above the normal threshold range (15–30 dB SPL) was recorded in (A) *Nisch*^{+/+}, (B) *Nisch*^{edsn/+}, (C) *Nisch*^{T445I/+} and (D) *Nisch*^{T445I/edsn} mice. The prevalence of increased ABR thresholds above the normal range is increased in *Nisch*^{T445I/edsn} animals compared to littermates.

5.2.2.3.4 Histology

As the prevalence of an increased ABR threshold was greater in *Nisch*^{T445I/T445I} and *Nisch*^{T445I/edsn} mice compared to littermates, the incidence of OM was analysed from visual inspection of the tympanic membrane and the presence or absence of an inflamed eardrum, which is synonymous with OM (as previously described in Figure 4.12, section 4.2.4.2).

Analysis of eardrums from *Nisch*^{T445I}|*Nisch*^{T445I} littermates showed there was no incidence of OM in *Nisch*^{+/+}, *Nisch*^{T445I/+} or *Nisch*^{T445I/T445I} mice (Figure 5.30). Mice from *Nisch*^{T445I}|*Nisch*^{edsn} litters were also investigated (Figure 5.31), and in *Nisch*^{+/+}, *Nisch*^{edsn/+} and *Nisch*^{T445I/+} mice again there was no incidence of OM, however a small incidence of unilateral OM was observed in *Nisch*^{T445I/edsn} animals (8%). Similarly, there was no significant genotypic difference ($P = 0.641$).

Histopathological examination of mice from *Nisch*^{T445I}|*Nisch*^{T445I} and *Nisch*^{T445I}|*Nisch*^{edsn} litters at 20wk confirmed that the majority of mice did not develop OM (data not shown). They had a ME free of effusion, with no thickening of the mucosal lining. Histology confirmed only one *Nisch*^{T445I/edsn} animal displayed an OM phenotype and it was unilateral only. The histology of most *Nisch*^{T445I}|*Nisch*^{T445I} and *Nisch*^{T445I}|*Nisch*^{edsn} genotypes was indistinguishable from wild-type littermates.

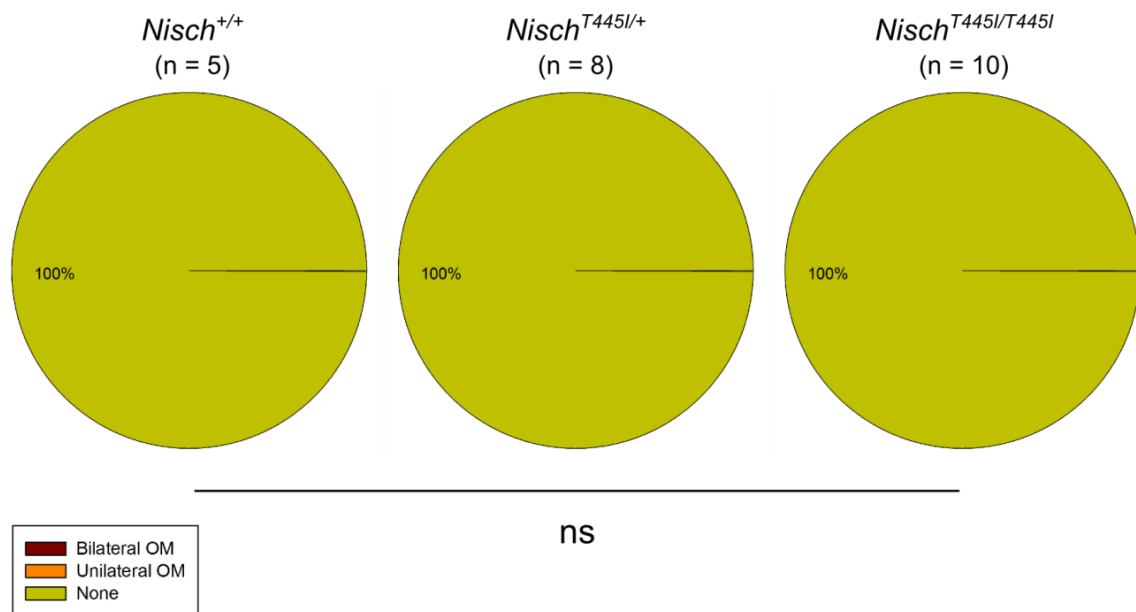


Figure 5.30| Incidence of OM in *Nisch*^{T445I}|*Nisch*^{T445I} littermates at 20wk.

The presence or absence of an inflamed eardrum was recorded from visual inspection after dissection. The incidence of OM was recorded in *Nisch*^{+/+}, *Nisch*^{T445I/+} and *Nisch*^{T445I/T445I} mice. No genotypic differences were observed. ns $P > 0.05$. Statistics were conducted using z-tests.

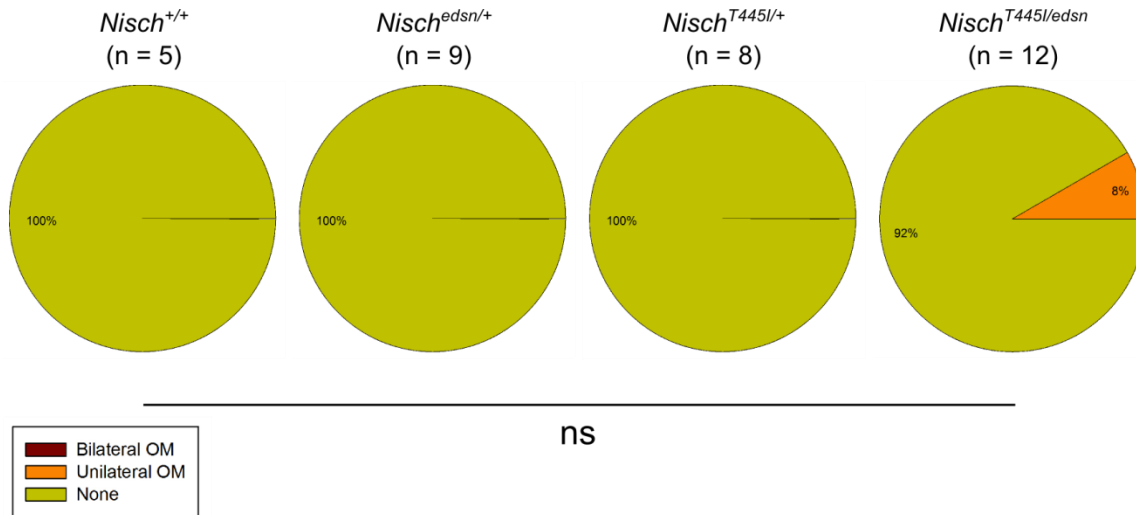


Figure 5.31 | Incidence of OM in mice from *Nisch*^{T445I}|*Nisch*^{edsn} litters at 20wk.

The presence or absence of an inflamed eardrum was recorded from visual inspection after dissection. The incidence of OM was recorded in *Nisch*^{+/+}, *Nisch*^{edsn/+}, *Nisch*^{T445I/+} and *Nisch*^{T445I/edsn} mice. No significant genotypic differences were observed between littermates. ns $P > 0.05$. Statistics were conducted using z-tests.

To evaluate whether inflammation was observed in the ME of mice from *Nisch*^{T445I}|*Nisch*^{T445I} litters, blinded assessment of the mucoperiosteum thickness was undertaken (Figure 5.32A). Comparable regions of ME were measured for each ear assessed. Consistent with the absence of tympanic inflammation, *Nisch*^{T445I/T445I} animals showed no significant changes in ME mucosa compared to wild-type littermates ($P = 0.857$). The mean mucosal thickness was 12.90 μm (± 0.720) in *Nisch*^{T445I/T445I} animals and 13.17 μm (± 1.600) in wild-types. Similar results were replicated in *Nisch*^{T445I}|*Nisch*^{edsn} littermates when mucoperiosteum thickness was measured (Figure 5.32B). No significant genotypic difference was observed in the ME mucosa ($P = 0.285$), where the mean mucosal thickness was 18.70 μm (± 2.553) in *Nisch*^{T445I/edsn} animals and 14.47 μm (± 0.791) in wild-type littermates. Even though no significant difference was observed, there were two occurrences of mucosal inflammation in *Nisch*^{T445I/edsn} animals. When omitted from the mucosal measurements, the mean mucosal thickness was reduced to 15.38 μm (± 1.062) in *Nisch*^{T445I/edsn} mice (Figure 5.32C). The mean mucosal thickness in the two ears with

inflammation was $41.98 \mu\text{m}$ (± 7.760) and, although not significant, was notably increased compared to wild-type ($P = 0.071$).

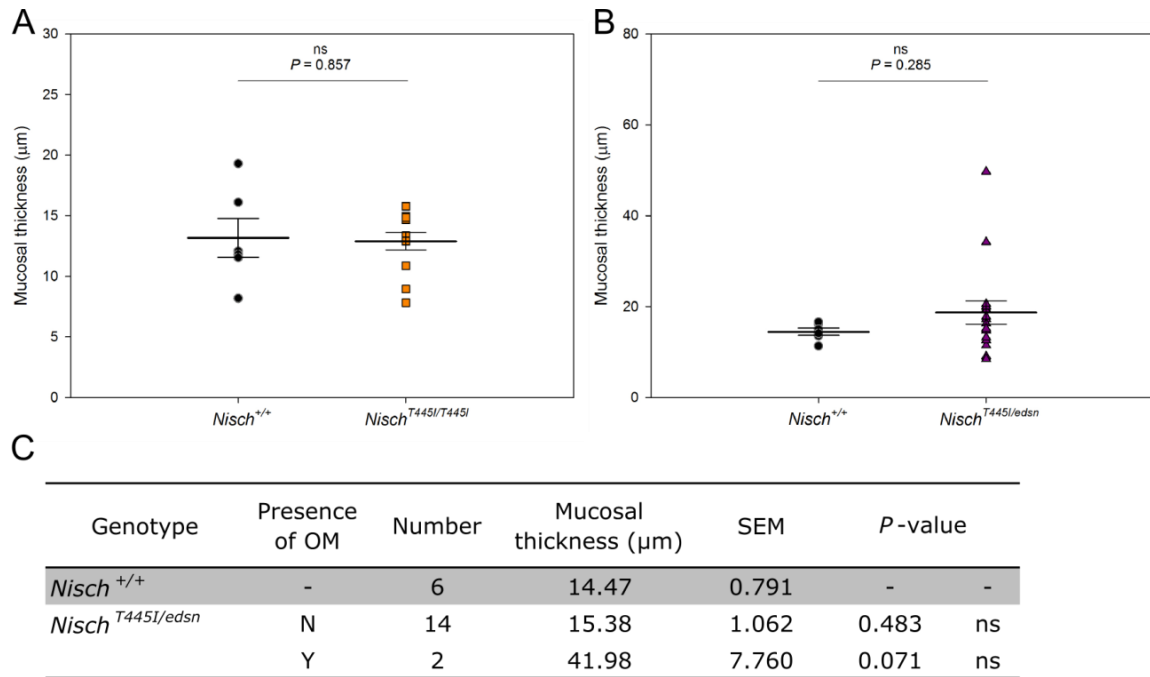


Figure 5.32| Analysis of mucosal thickness in the MEC of $Nisch^{T445I/T445I}$ and $Nisch^{T445I/edsn}$ mice.

H&E sections of ME were scanned into the Hamamatsu Pathology Imager and the mucosal thickness was averaged from 5 measurements of a standard $1000 \mu\text{m}$ length of ME mucosa (avoiding the cochlea and the region close to the ET). **(A)** Scatter plot showing mucosal thickness of the ME for $Nisch^{+/+}$ and $Nisch^{T445I/T445I}$ mice. No significant genotypic difference in mucosal thickness is observed. **(B)** Scatter plot showing mucosal thickness of the ME for $Nisch^{+/+}$ and $Nisch^{T445I/edsn}$ mice. No significant genotypic difference in mucosal thickness is observed, however there were two occurrences of OM in $Nisch^{T445I/edsn}$ animals. **(C)** Table showing the mean mucosal thickness of $Nisch^{T445I/edsn}$ ears with or without OM, and corresponding P -values compared to wild-type littermates. In scatter plots each point represents a single ear and thick black line indicates mean mucosal thickness $\pm$ SEM. ns $P > 0.05$. Data in panel A was analysed by Student's t-test. $Nisch^{T445I/edsn}$ data (panel B and C) was not normally distributed and Mann Whitney Rank Sum tests were performed.

(A) $Nisch^{+/+}$ $n = 6$; $Nisch^{T445I/T445I}$ $n = 12$. (B) $Nisch^{+/+}$ $n = 6$; $Nisch^{T445I/edsn}$ $n = 16$.

5.3 Discussion

5.3.1 Summary

The ENU-DNA archive at MRC Harwell was utilised to identify additional *Nisch* alleles for complementation testing with the *Nisch^{edsn}* allele. Of the alleles identified, three were re-derived for characterisation, resulting in the validation of *Nisch* as the causative gene in *edison* mice.

5.3.2 Identification of additional Nischarin alleles

In order to confirm the underlying mutation in *edison* was the ENU-induced mutation in *Nisch*, it was necessary to exclude the possibility that the reduced startle response was caused by a mutation in an adjacent gene. I wanted to confirm this by showing failure of complementation by crossing the putative *Nisch* allele in *edison* to a known *Nisch* allele. Unfortunately, when required no additional *Nisch* alleles were available for import.

An allelic series for *Nisch* was generated by utilising the ENU-DNA archive at MRC Harwell. I screened ten exons of *Nisch* employing HRM analysis of 10,464 mutant mice and identified ten additional point mutations. Seven of these mutations resulted in a non-synonymous change in an amino acid residue. The per-base-pair overall mutation rate (1/2.23 Mbp) was around half of the mutation rate observed by Quwailid *et al.*, 2004 (1/1.01 Mbp). This was also observed with the potentially functional mutation rate (1/3.19 Mbp compared to 1/1.82Mbp).

Using prediction software for coding variants, three alleles (Val33Ala, Leu142Gln and Thr445Ile) were selected as the most probable to have an impact on the biological function of NISCH. The Leu142Gln and Thr445Ile alleles were the only two alleles to be predicted by all three prediction tools to affect protein function. The Leu142Gln

allele was not located in a known functional domain of NISCH. In contrast, the Thr445Ile allele was located within the region of amino acid residues shown to strongly bind PAK1 (amino acids 246 – 660) (Alahari, Reddig et al. 2004). This same region of amino acids has been shown to interact with both RAC1 and phospho-RAC1 (Reddig, Xu et al. 2005; Kuijl, Pilli et al. 2013). Therefore, interaction of NISCH with PAK1 and RAC1 could be affected by the Thr445Ile allele. The Val33Ala allele was only predicted to affect protein function when analysed through SIFT, however the substitution was located in the PX domain of NISCH. This allele could impact upon association of NISCH with PI(3)P-enriched endosomal membranes and the redistribution of ITGA5 from the cell membrane to intracellular endosomes.

5.3.3 Additional Nischarin alleles confirm *Nisch* as causative gene in *edison* mice

As mentioned above, DNA and sperm archives derived from ENU mutagenesis programmes (Quwailid, Hugill et al. 2004) were utilised to identify seven additional alleles at the *Nisch* locus. Three alleles were selected for isolation and preliminary characterisation; *Nisch*^{V33A}, *Nisch*^{L142Q} and *Nisch*^{T445I}. After re-derivation of an allele from the ENU-DNA archive, congenic strains containing the allele are typically produced. However, due to time constraints the three additional alleles I identified were characterised on a mixed background (C57BL/6; C3H-HeH). When using animal models, the strain is important to consider. Certain phenotypic traits, such as weight, are polygenic and different mouse strains vary from each other in these phenotypic traits. An important example of strain differences has been seen in the *Junbo* mouse model, where on the C57BL/6 strain 96% of *Evi1*^{Jbo/+} ears (n = 46) have an OM phenotype by 2 months, whereas C3H-HeH and BALB/c mice with the same mutation show a significantly lower incidence of OM, at 79% and 64% respectively (C3H-HeH n = 38 ears; BALB/c n = 36 ears) (Tyrrer et al. data not

published). Genetic background can be used to exacerbate the phenotype of interest, but it can also obscure underlying differences; therefore, strain choice should be planned carefully and appropriate controls used when using a mixed genetic background. All genotypes produced from the crosses in this chapter were characterised for use as appropriate controls.

Both *Nisch*^{V33A/V33A} and *Nisch*^{V33A/edsn} mice display an OM phenotype that shows a good resemblance to the *edison* mouse. This result unreservedly validates *Nisch* as the causative gene in *edison* mice. However, phenotyping the multiple *Nisch* alleles has revealed a disparity in OM severity. In particular, *Nisch*^{V33A/V33A} animals develop a milder form of the disease compared to *Nisch*^{edsn/edsn} mice, resulting in better auditory function and limited disease progression. In SPF conditions, mice with the *Nisch*^{V33A/V33A} and *Nisch*^{V33A/edsn} mutations develop a spontaneous COM, with an associated elevation in ABR hearing thresholds. The reduced hearing deficit is progressive, with onset observed at 8wk in *Nisch*^{V33A/V33A} mice and as early as 4wk in *Nisch*^{V33A/edsn} mice. This better auditory function is probably due to a smaller volume of fluid being present in the ME, as a consequence of reduced ME inflammation and vascular leakage, allowing better sound transmission from the outer to the inner ear. The incidence of OM in *Nisch*^{V33A/edsn} mice was assessed and is comparable to the incidence of OM observed in age-matched *Nisch*^{edsn/edsn} animals (Table 5.10). In contrast, *Nisch*^{V33A/V33A} mice display a reduced incidence of OM, with fewer cases of bilateral disease, and an increased incidence of either unilateral disease or no affected ears. Furthermore, histological analysis demonstrated that *Nisch*^{V33A/V33A} ears with an OM phenotype have a reduced mucosal thickening compared to *edison* (Table 5.10). In *Nisch*^{V33A/edsn} mice, changes in the ME mucosa due to COM were again more comparable to changes seen in *Nisch*^{edsn/edsn} animals. There is a clear phenotypic gradient in the severity of OM seen in *Nisch*^{V33A/V33A}, *Nisch*^{V33A/edsn} and *Nisch*^{edsn/edsn} mice.

Table 5.10| Summary of OM phenotypes in *Nisch*^{V33A/V33A}, *Nisch*^{V33A/edsn} and *Nisch*^{edsn/edsn} mice at 12wk.

Table summarising the incidence of OM for *Nisch*^{V33A/V33A}, *Nisch*^{V33A/edsn} and *Nisch*^{edsn/edsn} mice; and the mean mucosal thickness for *Nisch*^{V33A/V33A}, *Nisch*^{V33A/edsn} and *Nisch*^{edsn/edsn} ears with an OM phenotype.

Incidence: *Nisch*^{V33A/V33A} n = 6; *Nisch*^{V33A/edsn} n = 7; *Nisch*^{edsn/edsn} n = 8.

Mucosal thickness: *Nisch*^{V33A/V33A} n = 10; *Nisch*^{V33A/edsn} n = 6; *Nisch*^{edsn/edsn} n = 19.

OM mouse mutant	Bilateral OM	Unilateral OM	None	Mucosal thickness (µm)
<i>Nisch</i> ^{V33A/V33A}	10%	50%	40%	41.68 (± 6.050)
<i>Nisch</i> ^{V33A/edsn}	50%	33%	17%	51.61 (± 8.104)
<i>Nisch</i> ^{edsn/edsn}	53%	26%	21%	69.63 (± 4.873)

No ME disease was detected in *Nisch*^{L142Q/L142Q}, *Nisch*^{L142Q/edsn} or *Nisch*^{T445I/T445I} mice at 20wk; however a small incidence of unilateral OM was displayed in *Nisch*^{T445I/edsn} mice. For each genotype, at earlier time points there were recorded instances of ABR thresholds greater than the expected wild-type threshold range for normal hearing, which is indicative of a conductive hearing loss. As discussed in chapter 4, resolution of hearing deficit was observed in some *Nisch*^{edsn/edsn} ears, possibly due to the hypomorphic nature of the *edison* allele. Therefore, if the *Nisch*^{L142Q} and *Nisch*^{T445I} alleles are more hypomorphic than the *Nisch*^{edsn} allele, it is feasible that the occurrences of increased ABR thresholds observed may be due to mild ME inflammation that later resolves. This may warrant further investigation that was not undertaken here.

Onset of disease could be a contributing factor in the difference in severity between alleles. However, it is more likely that *Nisch*^{edsn/edsn} mice develop a more aggressive form of disease. This significant difference is due to the differences in the nature of the mutation between *edison* and the *Nisch*^{V33A} allele. *Nisch*^{L142Q} and *Nisch*^{T445I} do not develop OM, suggesting they are more hypomorphic alleles at the *Nisch* locus or the

mutations have no impact on protein function. With the four *Nisch* alleles characterised, it is clear that mutant alleles of various strengths have been isolated. Further investigation will be required to fully understand the impact that the three additional alleles identified have towards the susceptibility of ME inflammatory disease.

5.3.4 Conclusion

Three *Nisch* alleles were identified from the ENU-DNA archive and re-derived for characterisation. Phenotypic analysis of the *Nisch*^{V33A} allele revealed the identification of an additional mouse model of COM. Complementation testing between the *Nisch*^{V33A} and *Nisch*^{edsn} alleles validated *Nisch* as the causative gene in *edison* mice. Analysis of the molecular mechanisms by which *Nisch* and its various alleles cause spontaneous COM will contribute further to our knowledge of the pathogenesis of human ME disease.

CHAPTER 6: Investigating genetic and molecular roles of Nischarin

6.1 Introduction

Mouse models of OM have highlighted that deficiencies in NF- κ B or TGF- β signalling could be two mechanisms that impact on susceptibility to COM, leading to the overactive pro-inflammatory response seen (Tateossian, Hardisty-Hughes et al. 2009; Xu, Woo et al. 2012; Tateossian, Morse et al. 2013). With the discovery of the *edison* mouse and identification of *Nisch* as the causative gene, the emphasis of this chapter was to attempt to elucidate the molecular mechanism by which NISCH impacts on COM in *edison* mice. A genetic cross-interaction between *Nisch* and *Itga5* mutants further exacerbated the OM phenotype seen in *edison*, indicating a functional interaction. Analysis of downstream pathways points towards changes in RAC1 signalling and TGF- β /SMAD signalling.

6.1.1 Integrins

Integrins are cation-dependent transmembrane heterodimers that provide links to the extracellular and intracellular environments, mediating signal transduction. Upon binding of extracellular matrix (ECM) proteins, integrins aggregate to enhance signal transduction and form cytoplasmic focal adhesions (Wozniak, Modzelewska et al. 2004). Integrins have been implicated in many important cellular functions; specifically they have been shown to play an important role in both blood and lymphatic vessel development (Hynes 2007; Avraamides, Garmy-Susini et al. 2008; Astrof and Hynes 2009; Tammela and Alitalo 2010; Chen, Alexander et al. 2012). Multiple integrins, including $\alpha 5\beta 1$, have been implicated in angiogenesis.

6.1.1.1 ITGA5 crosstalk with VEGF and TGF- β pathways

Both VEGF and TGF- β are key regulators of angiogenesis. Increased vascular permeability and endothelial cell growth are important in the pathogenesis of OME and VEGF is known to play an important role in the increased vascular permeability and angiogenesis (Senger, Galli et al. 1983; Ferrara and Henzel 1989). VEGF is highly expressed in samples of ME fluids taken from human patients with OME and COME (Jung, Kim et al. 1999), and therefore appears to play a significant role in the pathogenesis of OM. It has been suggested also that VEGF expression in the ME is linked with the production of mucoid ME exudates (Sekiyama, Ohori et al. 2011). A recent study at MRC Harwell has highlighted the HIF-VEGF pathways to be critical for the OM phenotype seen in the OM mouse models *Jeff* and *Junbo* (Cheeseman, Tyrer et al. 2011). Inhibitors of VEGFR signalling and HSP90 were shown to moderate hearing loss, angiogenesis, and lymphangiogenesis in *Junbo* mice. The mutation in *Fbxo11* gene found in the *Jeff* mouse is suggested to affect signalling of the TGF- β superfamily, via SMAD proteins (Tateossian, Hardisty-Hughes et al. 2009). Recent work published on the *Tgif* KO mouse has further highlighted the important role that TGF- β signalling plays in the susceptibility to COM (Tateossian, Morse et al. 2013).

Cross-talk between VEGF and integrins has been shown to be a critical factor in the regulation of angiogenesis and vascularization (De, Razorenova et al. 2005). During angiogenesis, a significant upregulation of $\alpha 5\beta 1$ on activated vascular endothelium has been identified (Brooks, Clark et al. 1994; Kim, Bell et al. 2000). In contrast, inhibition of ITGA5 has been shown to cause selective inhibition of lymphangiogenesis (Dietrich, Onderka et al. 2007). Complete absence of ITGA5 in KO mice causes mesodermal and vascular defects, and the mice are embryonic lethal (Yang, Rayburn et al. 1993). VEGFR-3 plays an essential role in lymphangiogenesis (Alitalo 2011), and *in vitro* $\alpha 5\beta 1$ is required for activation of VEGFR-3-mediated cell signalling (Zhang, Groopman et al. 2005). Processes involved in the adhesion of cells to extracellular matrix and adjacent cells are known to play a key role in vascular cell

migration and angiogenesis. Induction of integrins by Fibronectin-coated beads has shown that integrins regulate VE-cadherin via the activation of SRC (Wang, Jin et al. 2006). Also it has been shown that FAK catalytic activity is required when $\alpha 5\beta 1$ integrin stimulates SRC activation through FAK phosphorylation (Wu, Bernard-Trifilo et al. 2008). Recently the importance of FAK activity in regulation of adheren junctions has been shown, as FAK inhibition prevents VEGF-stimulated vascular permeability (Chen, Nam et al. 2012).

There is also extensive interplay between TGF- β signalling and integrins. TGF- β controls the transcription of genes that encode numerous integrins and in most cases TGF- β stimulates integrin expression (Margadant and Sonnenberg 2010). Expression of TGF- β s can also be regulated by integrins (Ortega-Velázquez, Díez-Marqués et al. 2003). TGF- β is secreted in an inactive form in a complex with latency associated peptide (LAP) and latent TGF- β binding protein (LTBP). Activation of TGF- β requires dissociation from the complex, which can occur through the action of several integrins. Like many ECM proteins such as fibronectin, LAP contains an Arg-Gly-Asp (RGD) motif and binds to integrin heterodimers (Derynck and Rhee 1987; Ruoslahti 1996; Munger, Harpel et al. 1998; Lu, Munger et al. 2002; Ludbrook, Barry et al. 2003). Recently, transgenic mice with a knock-in mutation of the RGD sequence to RGE in LAP were shown to exhibit a phenotype very similar to the TGF- $\beta 1$ null mice by displaying defects in vasculogenesis and enhanced inflammation (Yang, Mu et al. 2007). Integrin $\alpha 5\beta 1$ binds the RGD motif in fibronectin and is thought to bind the RGD sequence present in LAP, implicating a possible role in TGF- β activation (Wipff and Hinz 2008; Margadant and Sonnenberg 2010). Integrins can also indirectly control the expression of components of the TGF- β pathway. Ectopic expression of ITGA5 has been shown to induce TGF- β RII expression rendering cells responsive to TGF- β and is further potentiated by $\alpha 5\beta 1$ ligation to fibronectin (Wang, Sun et al. 1999). Functional studies have also revealed that the upregulation of ITGA5

expression plays a crucial role by which VEGF and TGF- β RI-kinase inhibitors achieve their synergistic angiogenic response (Liu, Kobayashi et al. 2009).

6.1.1.2 Possible role of ITGA5 in edison

NISCH preferentially binds to the cytoplasmic domain of ITGA5 (Alahari, Lee et al. 2000). Elevated levels of NISCH are thought to mediate the translocation of ITGA5 from the cell membrane to endosomes (Lim and Hong 2004). Restoring *NISCH* expression in MDA-MB-231 cells was shown to decrease ITGA5 expression and reduce FAK phosphorylation; whereas in contrast, shRNA-mediated silencing of *NISCH* expression in MCF-7 cells increased both ITGA5 expression and FAK phosphorylation (Baranwal, Wang et al. 2011). As I discussed above ITGA5 is known to be involved in the activation of SRC-family protein tyrosine kinases (PTKs), as well as FAK PTKs, both of which are involved with mediating VEGF-induced vascular leak (De, Razorenova et al. 2005; Chen, Nam et al. 2012). Hypoxia and VEGF-induced signalling underlie the OM phenotypes in *Jeff* and *Junbo* (Cheeseman, Tyrer et al. 2011); with *Vegfa* levels raised in ME fluids from both OM mouse models. Additionally, upregulation of *Vegfa* is also observed in *edison* ME fluids.

The *edison* mutation is in a highly conserved region of *Nisch* and was predicted to impact upon the biological function of NISCH. It was hypothesised the *edison* mutation could result in increased expression of ITGA5 and therefore drive downstream regulators of angiogenesis and vascular permeability, such as VEGF and TGF- β , causing the OM phenotype seen in *edison* mice (possible mechanism summarised in Figure 6.1).

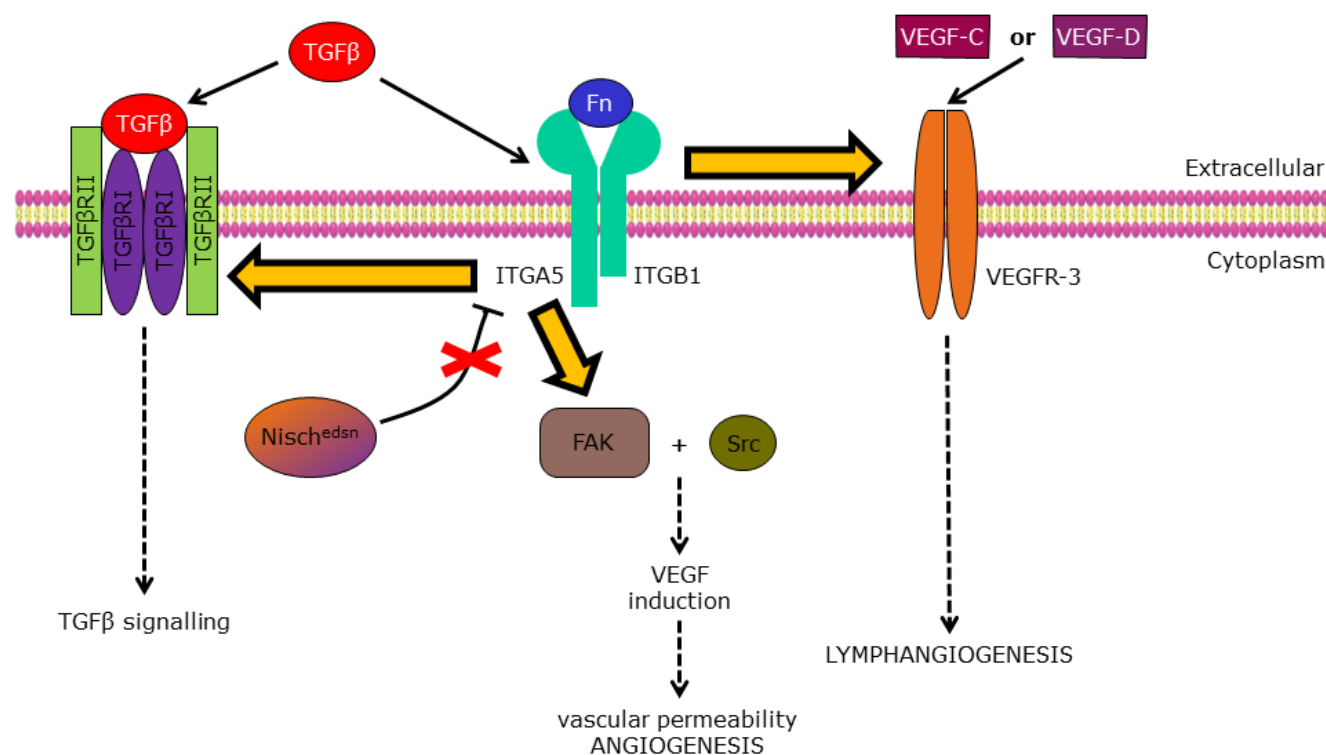


Figure 6.1| Possible pathways linked to OM proneness in *edison*.

Schematic indicating the potential associations of NISCH and ITGA5 that may lead to spontaneous COM. NISCH is thought to regulate ITGA5 expression through redistribution to intracellular endosomal structures. In *edison* mice the Leu972Pro causative mutation identified was predicted to impact upon the biological function of NISCH, resulting in upregulation of ITGA5 expression. Integrin $\alpha 5 \beta 1$ can stimulate SRC activation through FAK phosphorylation, leading to VEGF-stimulated vascular permeability. Additionally, integrin $\alpha 5 \beta 1$ appears to be essential for VEGFR-3-mediated cell signalling, contributing to lymphangiogenesis. Extensive crosstalk occurs between the TGF- β pathway and integrins, specifically TGF- β can result in upregulation of integrin $\alpha 5 \beta 1$. Furthermore, the ectopic expression of ITGA5 can induce TGF- β RII expression, which is potentiated further by $\alpha 5 \beta 1$ ligation to fibronectin, rendering cells responsive to TGF- β signalling. See the text for a more detailed description of pathways.

6.2 Results

6.2.1 Genetic cross-interaction between *Nisch* and *Itga5* in OM

A genetic-cross interaction between *Nisch* and *Itga5* was set up to assess whether addition of the *Itga5*^{tm1Hyn} KO allele would ameliorate the OM phenotype seen in *edison* mice as predicted. Alternatively, disrupted expression of ITGA5 in *edison* mice may exacerbate the OM phenotype observed. Two breeding crosses were set up to analyse how the addition of the *Itga5*^{tm1Hyn} allele impacts upon the phenotype observed in mice with the *Nisch*^{edsn} allele. Initially, double heterozygotes were generated by crossing *Itga5*^{tm1Hyn/+} mice with *Nisch*^{edsn/+} mice to see if addition of the *Itga5*^{tm1Hyn} allele would exacerbate the *Nisch*^{edsn/+} phenotype resulting in COM. Next, *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} double heterozygote mice were crossed again with *Nisch*^{edsn/+} mice to produce *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} offspring. Characterisation of *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice would demonstrate whether addition of the *Itga5*^{tm1Hyn} allele would ameliorate or exacerbate the OM phenotype seen in *Nisch*^{edsn/edsn} animals. All genotypes produced from both breeding crosses were phenotyped as controls.

6.2.1.1 Characterisation of *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice

6.2.1.1.1 Genotypic ratio analysis

As *edison* litters display reduced numbers of *Nisch*^{edsn/edsn} progeny, genotypic ratios from *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} × *Itga5*^{+/+} *Nisch*^{edsn/+} crosses were compared (Table 6.1 – 6.3). From the numbers of live mice observed at weaning age, no significant genotypic differences were observed compared to expected Mendelian ratios ($P = 0.3430$). Between the expected genotypes in the dead pups, from the same crosses, it was found that again there was no significant difference ($P = 0.7477$). Also, when the numbers of live mice were combined with the numbers of dead pups, as

anticipated there was no significant difference between the expected genotypes ($P = 0.5210$).

Table 6.1| Numbers of live mice from *Itga5^{tm1Hyn/+} Nisch^{+/+}* x *Itga5^{+/+} Nisch^{edsn/+}* crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Itga5^{+/+} Nisch^{+/+}</i>	35	30	29.2%	0.3430
<i>Itga5^{+/+} Nisch^{edsn/+}</i>	25	30	20.8%	
<i>Itga5^{tm1Hyn/+} Nisch^{+/+}</i>	35	30	29.2%	
<i>Itga5^{tm1Hyn/+} Nisch^{edsn/+}</i>	25	30	20.8%	
TOTAL	120			

Table 6.2| Numbers of dead pups from *Itga5^{tm1Hyn/+} Nisch^{+/+}* x *Itga5^{+/+} Nisch^{edsn/+}* crosses

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Itga5^{+/+} Nisch^{+/+}</i>	2	2	22.2%	0.7477
<i>Itga5^{+/+} Nisch^{edsn/+}</i>	3	2	33.3%	
<i>Itga5^{tm1Hyn/+} Nisch^{+/+}</i>	1	2	11.1%	
<i>Itga5^{tm1Hyn/+} Nisch^{edsn/+}</i>	3	2	33.3%	
TOTAL	9			

Table 6.3| Numbers of mice from *Itga5^{tm1Hyn/+} Nisch^{+/+}* x *Itga5^{+/+} Nisch^{edsn/+}* crosses including dead pups

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Itga5^{+/+} Nisch^{+/+}</i>	37	32	28.7%	0.5210
<i>Itga5^{+/+} Nisch^{edsn/+}</i>	28	32	21.7%	
<i>Itga5^{tm1Hyn/+} Nisch^{+/+}</i>	36	32	27.9%	
<i>Itga5^{tm1Hyn/+} Nisch^{edsn/+}</i>	28	32	21.7%	
TOTAL	129			

6.2.1.1.2 Weight

Analysis of weights across a longitudinal time course, from four to twenty weeks of age, demonstrated that male mice with the *Itga5*^{+/+} *Nisch*^{edsn/+} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mutations were significantly smaller than their wild-type littermates from 8wk and older ($P < 0.001$; Figure 6.2). Male *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} animals showed no significant difference to wild-type littermates throughout the time course. *Itga5*^{+/+} *Nisch*^{edsn/+} male mice were on average 13% smaller compared with their wild-type littermates, with *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} male mice 15% smaller. No significant genotypic differences were observed in female mice at any time point.

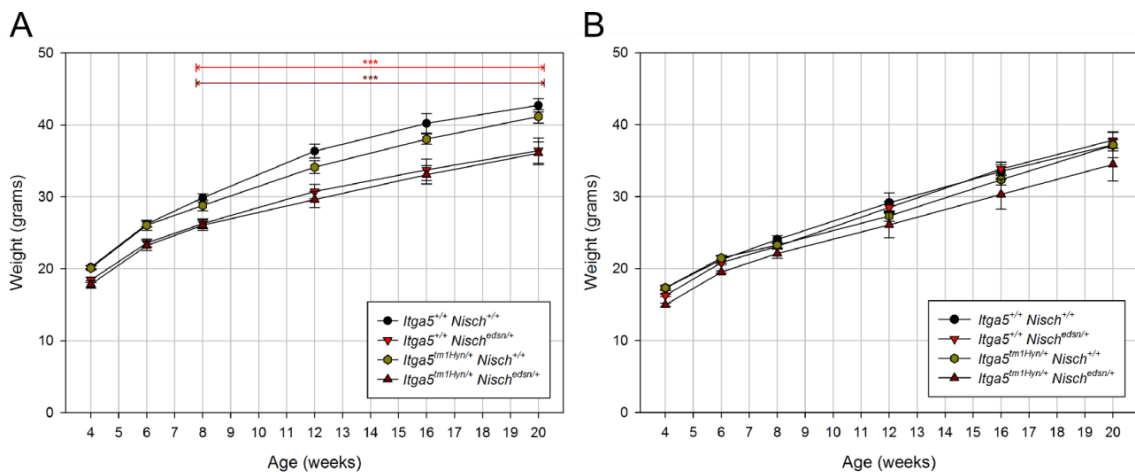


Figure 6.2| Weight data for *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} litters over a longitudinal time course.

(A) Male *Itga5*^{+/+} *Nisch*^{edsn/+} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice are significantly smaller than wild-type littermates from 8wk. No significant difference was observed between *Itga5*^{+/+} *Nisch*^{+/+} and *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} mice. **(B)** No genotypic differences in weight are observed in female mice from *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} litters. *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis. *Itga5*^{+/+} *Nisch*^{+/+} Male $n = 7$, Female $n = 4$; *Itga5*^{+/+} *Nisch*^{edsn/+} Male $n = 7$, Female $n = 9$; *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} Male $n = 6$, Female $n = 12$; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} Male $n = 8$, Female $n = 5$.

6.2.1.1.3 ABR

In order to assess whether an abnormal hearing phenotype was displayed by *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice, ABR tests for auditory function were conducted across a time course with cohorts of age matched littermates to record the progression in hearing thresholds (Figure 6.3). Hearing assessments were conducted with cohorts at 4, 6, 8, 12, 16 and 20wk. Mice were anesthetized and hearing thresholds determined using a click-evoked ABR ranging from 90 dB SPL to 5 dB SPL in decreasing 5 dB increments. The ABR threshold was measured for each ear.

Analysis of ABR thresholds found there was a significant difference in ABR thresholds between *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} and *Itga5*^{+/+} *Nisch*^{+/+} carrier mice from 12wk and onwards ($P < 0.05$; Figure 6.3A). *Itga5*^{+/+} *Nisch*^{edsn/+} and *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} littermates displayed normal, wild-type hearing thresholds of between 15-30 dB SPL. At 12wk, compound mutants with the *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} double mutation were found to have slightly elevated auditory thresholds compared to wild-types, with a mean ABR threshold of 26.54 dB SPL (± 0.952). Even though the mean threshold fell within the expected wild-type threshold range, a significant difference was observed ($P = 0.030$). Hearing appeared to slightly deteriorate with age in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice, displayed by marginally elevated ABR thresholds. By 20wk, *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} animals were recorded with a mean ABR threshold of 28.08 dB SPL (± 1.041), which was again significant compared to wild-type ($P = 0.002$). It is also worth noting that there was no obvious gender or left-right preference observed in ABR threshold data from all genotypes (Appendix, Figure 10.11).

The ABR threshold range for each genotype was analysed using box and whisker plots. For *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/+} and *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} littermates, the boxes (25th to 75th percentile) and whiskers (10th to 90th percentiles) at each time point fell within the expected hearing threshold range, of 15-30 dB SPL, for normal hearing (Figure 6.3B-D). A small number of outliers were recorded in both

genotypes outside of the expected wild-type ABR threshold range. The ABR threshold range in *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice slightly increased with age across the time course (Figure 6.3E). At 16wk and 20wk, the threshold range was from 15 to 40 dB SPL, with a median of 30 dB SPL. The majority of ears recorded an ABR threshold in the expected range for wild-type hearing, indicating that there was a very low penetrance of OM in *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* compound mutants.

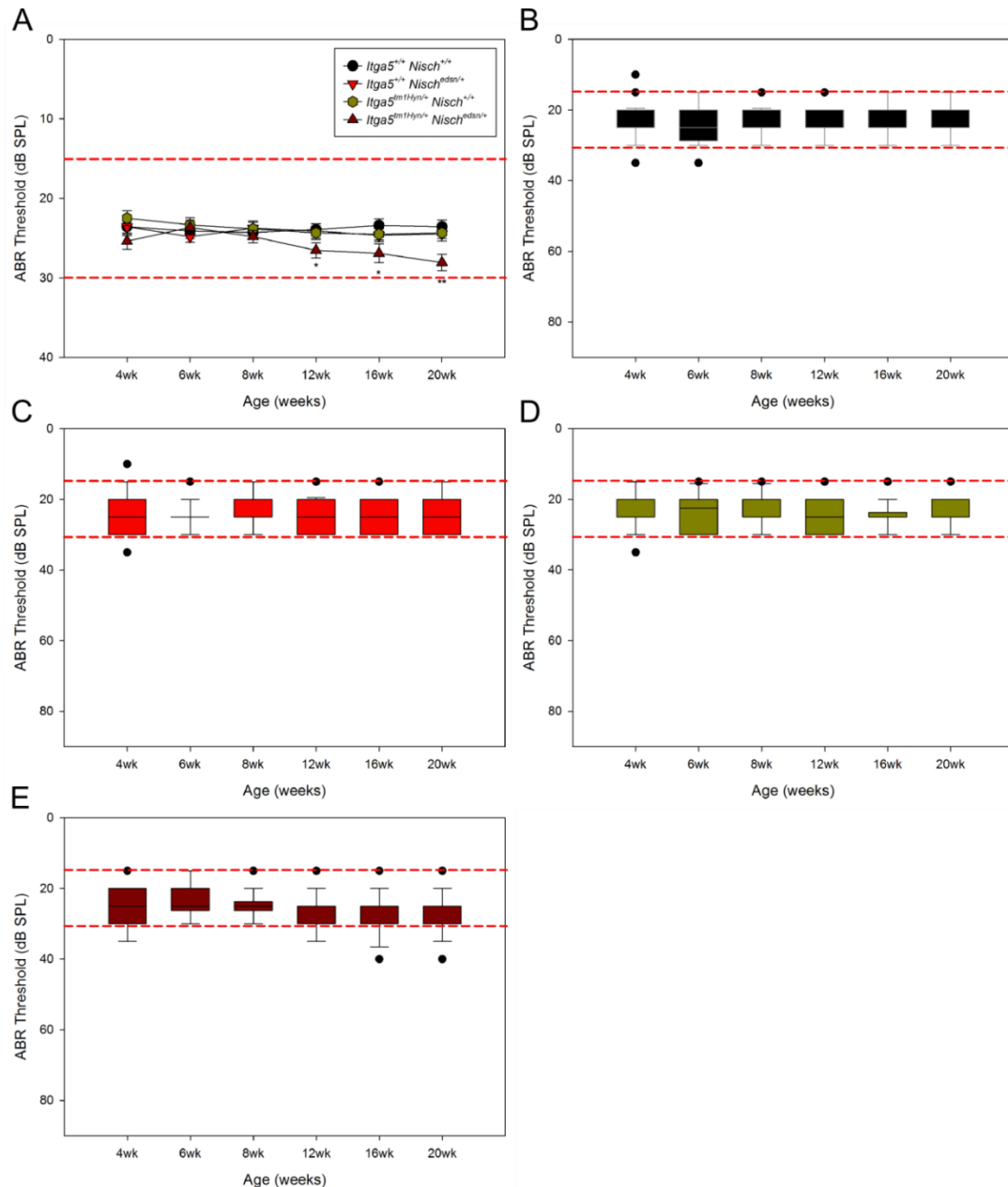


Figure 6.3| Progression of click-evoked ABR thresholds across a cohort time course for *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} litters.

(A) Mice with the *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mutation have late onset elevated hearing thresholds due to OM. There is no difference in hearing thresholds between other littermates. (B-E) Box plots show the ABR threshold ranges of (B) *Itga5*^{+/+} *Nisch*^{+/+}, (C) *Itga5*^{+/+} *Nisch*^{edsn/+} (D) *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} and (E) *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (●) show any outliers. * *P* < 0.05; ** *P* < 0.01. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Itga5^{+/+} *Nisch*^{+/+} *n* = 28; *Itga5*^{+/+} *Nisch*^{edsn/+} *n* = 28; *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} *n* = 30; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} *n* = 26.

6.2.1.1.4 Presence of OM

The incidence of OM was also assessed across a time course for each genotype (Figure 6.4A-D). *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/+} and *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} animals showed a small incidence of unilateral OM at 4wk, one mouse in each genotype. In *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice there was a late onset in the prevalence of OM from 12wk. At 4wk, 77% of *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice had bilateral clear ears with no OM phenotype present and 23% displayed unilateral OM (z-test, $P = 0.533$). OM is not recorded again in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice until 12wk, where 15% had unilateral OM and 85% had no OM phenotype (z-test, $P = 0.430$). The prevalence of OM increased onwards from 12wk, where at 20wk 31% had unilateral OM and 69% had no OM phenotype (z-test, $P = 0.032$).

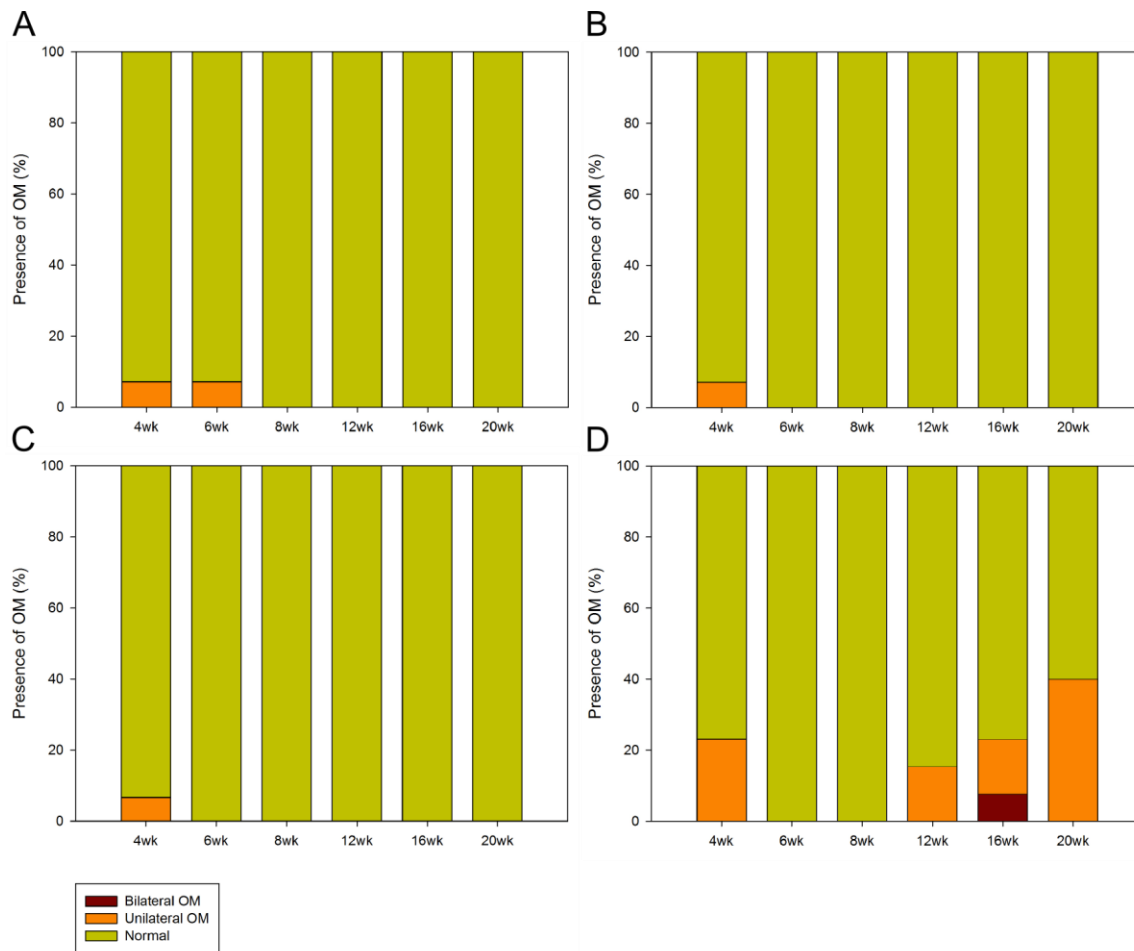


Figure 6.4| Incidence of OM in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} litters across a time course.

The presence or absence of OM for each mouse was recorded from visual inspection in (A) *Itga5*^{+/+} *Nisch*^{+/+}, (B) *Itga5*^{+/+} *Nisch*^{edsn/+} (C) *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} and (D) *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice. Prevalence of OM in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} animals slightly increases with age compared to littermates.

Itga5^{+/+} *Nisch*^{+/+} n = 14; *Itga5*^{+/+} *Nisch*^{edsn/+} n = 14; *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} n = 15; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} n = 13.

6.2.1.1.5 Histology

Histopathology was used to examine the morphology of the ME in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice. H&E sections at 20wk confirmed that *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice developed COM similar to *Nisch*^{edsn/edsn} mice (Figure 6.5B). The mucosal inflammation was diffuse and of mild severity, with the presence of a cellular ME effusion (Figure 6.5D). Wild-type mice did not develop OM and had a ME free of effusion, with no thickening of the mucosal lining (Figure 6.5A and C). The histology of *Itga5*^{+/+}

Nisch^{edsn/+} and *Itga5^{tm1Hyn/+} Nisch^{+/+}* mice was indistinguishable from wild-type littermates.

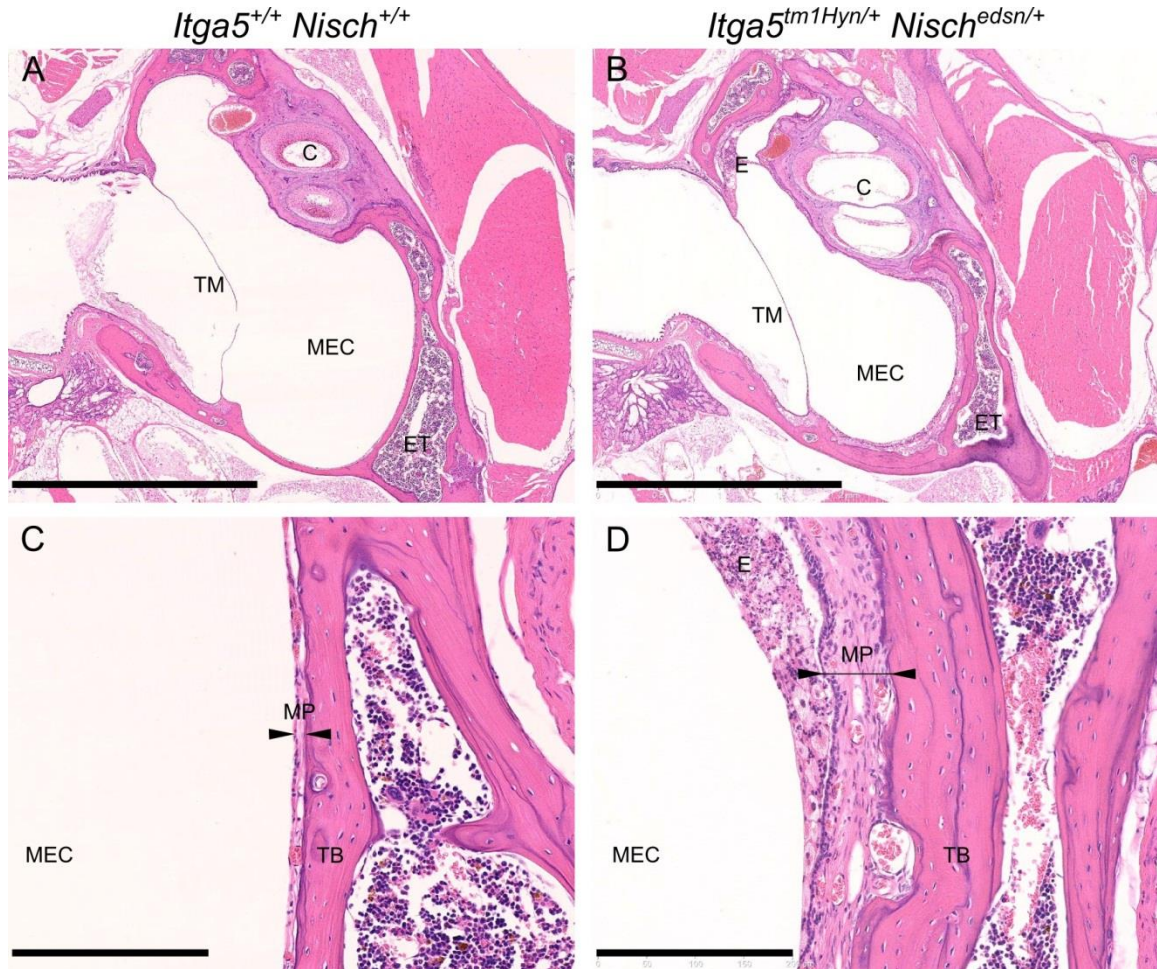


Figure 6.5| Characteristic ME histology in wild-type and *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* animals at 20wk.

H&E stained transverse sections of the MEC and mucoperiosteum, in **(A & C)** *Itga5^{+/+} Nisch^{+/+}* and **(B & D)** *Itga5^{tm1Hyn/+} Nisch^{edsn/+}*, at 20wk. Histopathology of the MEC from *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice demonstrates chronic inflammation with an exudate. Wild-type animals have no inflammation or exudate in the MEC and a thin mucoperiosteum covers the temporal bone. C, cochlea; ET, eustachian tube; E, exudate; MEC, middle ear cavity; MP, mucoperiosteum (arrowheads); TB, temporal bone; TM, tympanic membrane. (A & B) Scale bar = 2 mm; (C & D) Scale bar = 200 μ m.

From the nine *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice sacrificed at 20wk, there were three ears that contained fluid in the MEC with a thickened epithelial lining (17%, n = 18 ears).

Two ears exhibited a cellular effusion with the presence of macrophages and PMNs, and one ear had a thin watery effusion. No inflammatory phenotype was displayed in *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/+} or *Itga5*^{tm1Hyn/+} *Nisch*^{+/+} littermates (n = 8, n = 10 and n = 14 ears respectively).

To quantify the chronic inflammation observed in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice and assess any subtle changes in the OM phenotype, blinded assessment of the mucoperiosteum thickness was carried out (Figure 6.6A). Comparable regions of ME were measured for each ear assessed. Consistent with the increased presence of tympanic inflammation, *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} animals had a significantly thicker ME mucosa compared to wild-type littermates ($P = 0.005$). The mean mucosal thickness was 25.68 μm (± 4.248) in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} animals and 15.09 μm (± 0.637) for wild-types. As the OM phenotype was not fully penetrant in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} mice, the data was categorised into ears that had an OM phenotype and ears that did not (Figure 6.6B). To remain unbiased, the presence of tympanic inflammation, previously recorded by visual assessment after dissection, was used to categorise ears with an OM phenotype. *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} ears with no OM were similar to wild-type, although still had a significantly thickened mucosa ($P = 0.015$), with a mean mucosal thickness of 18.13 μm (± 1.015). Whereas, *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} ears with OM had a greatly increased mean mucosal thickness of 63.44 μm (± 5.368) and showed a greater significant difference compared to wild-type ($P < 0.001$).

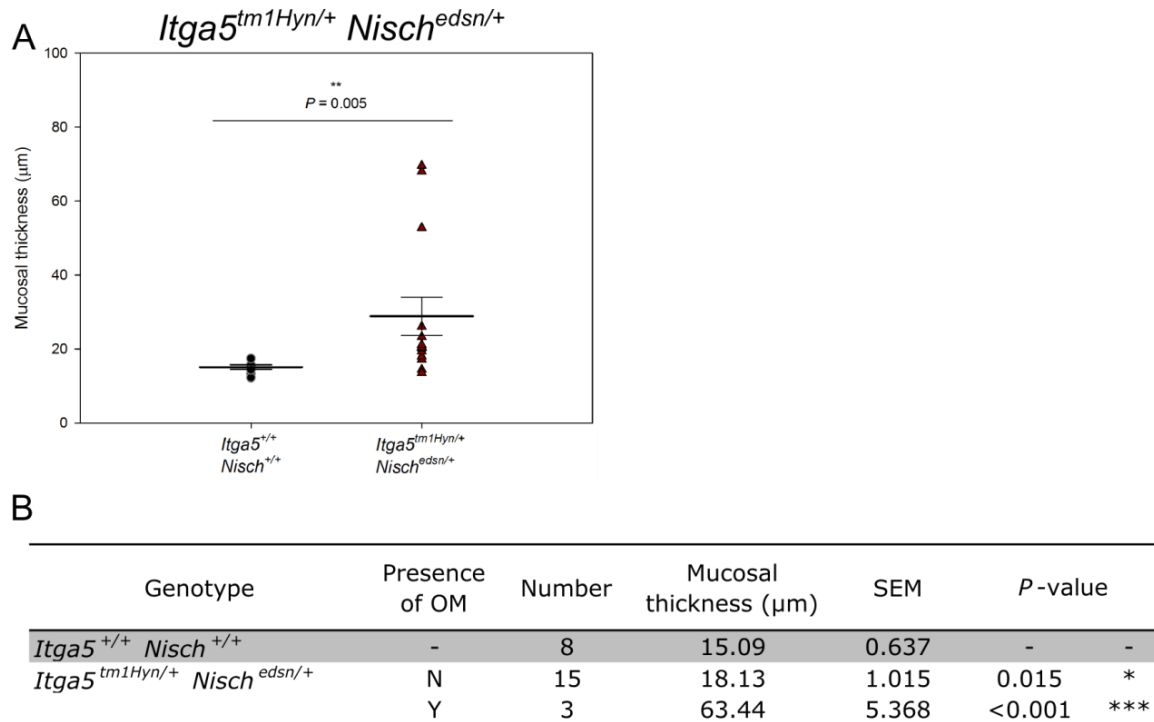


Figure 6.6| Analysis of mucosal thickness in the MEC of *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice.

H&E sections of ME were scanned into the Hamamatsu Pathology Imager and the mucosal thickness was averaged from 5 measurements of a standard 1000 μm length of ME mucosa (avoiding the cochlea and the region close to the ET). **(A)** Scatter plot showing mucosal thickness of the ME for *Itga5^{+/+} Nisch^{+/+}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice. *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice had significantly thicker mucosa compared to wild-type. **(B)** Table showing the mean mucosal thickness of *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* ears with or without OM, and corresponding *P*-values compared to wild-type littermates. In scatter plot each point represents a single ear and thick black line indicates mean mucosal thickness ± SEM. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001. Statistics were conducted using Mann Whitney Rank Sum tests.

Itga5^{+/+} Nisch^{+/+} n = 8; *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* n = 18.

6.2.1.2 Characterisation of *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice

All genotypes from *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* x *Itga5^{+/+} Nisch^{edsn/+}* crosses were phenotyped, however I have excluded data from *Itga5^{+/+} Nisch^{edsn/+}*, *Itga5^{tm1Hyn/+} Nisch^{+/+}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* littermates in this section as the data replicated results already observed in section 6.2.1.

6.2.1.2.1 Genotypic ratio analysis

There was a reduced number of *Nisch*^{edsn/edsn} progeny from *edison* litters, therefore genotypic ratios were assessed for progeny from *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} x *Itga5*^{+/+} *Nisch*^{edsn/+} crosses (Table 6.4 – 6.6). From the numbers of live mice observed at weaning age, there was no significant difference between observed and expected genotypes ($P = 0.6023$). However, when analysing the expected genotypes in the dead pups, from the same litters, it was found that there was a significant difference ($P = 0.0033$). An increased proportion of *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} pups were being found dead before weaning. As to be expected, when the numbers of live mice were combined with the numbers of dead pups, there was also no significant difference between the expected genotypes ($P = 0.8491$).

Table 6.4| Numbers of live mice from *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} x *Itga5*^{+/+} *Nisch*^{edsn/+} crosses at weaning age

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{+/+}	14	13	14.0%	0.6023
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{edsn/+}	26	25	26.0%	
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{+/+}	17	13	17.0%	23.0%
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/+}	23	25	23.0%	
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{edsn/edsn}	8	13	8.0%	12.0%
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/edsn}	12	13	12.0%	
TOTAL	100			

Table 6.5| Numbers of dead pups from *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} x *Itga5*^{+/+} *Nisch*^{edsn/+} crosses

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{+/+}	0	2	0.0%	0.0033
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{edsn/+}	3	4	18.8%	
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{+/+}	0	2	0.0%	0.0033
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/+}	2	4	12.5%	
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{edsn/edsn}	5	2	31.3%	0.0033
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/edsn}	6	2	37.5%	
TOTAL	16			

Table 6.6| Numbers of mice from *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} x *Itga5*^{+/+} *Nisch*^{edsn/+} crosses including dead pups

Genotype	Observed	Expected	Percent	χ^2 P-value
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{+/+}	14	15	12.1%	0.8491
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{edsn/+}	29	29	25.0%	
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{+/+}	17	15	14.7%	0.8491
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/+}	25	29	21.6%	
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{edsn/edsn}	13	15	11.2%	0.8491
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/edsn}	18	15	15.5%	
TOTAL	116			

6.2.1.2.2 Weight

Analysis of weights across a longitudinal time course, from four to twenty weeks of age, demonstrated that *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} male mice were significantly smaller than their wild-type littermates throughout the longitudinal time course ($P < 0.001$) (Figure 6.7). *Itga5*^{+/+} *Nisch*^{edsn/edsn} male mice were on average 30% smaller compared with their wild-type littermates, with *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice 34% smaller. Female *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} animals were also significantly smaller compared to wild-type littermates throughout the time course ($P < 0.05$ and $P < 0.001$ respectively).

A significant difference was also observed between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} in female mice from 12wk and older ($P < 0.05$). *Itga5*^{+/+} *Nisch*^{edsn/edsn} female mice were on average 18% smaller compared with their wild-type littermates, with *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice 34% smaller.

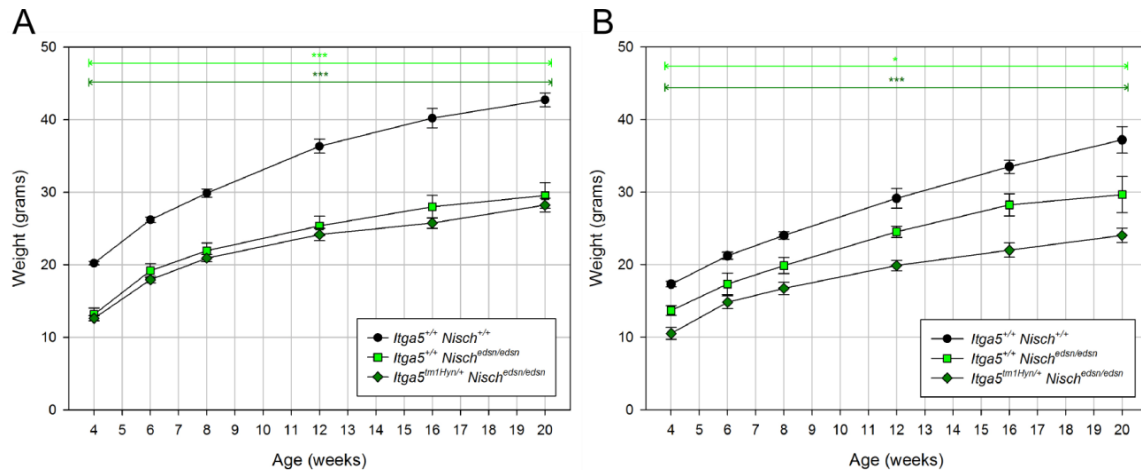


Figure 6.7| Weight data for *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} litters over a longitudinal time course.

(A) Male *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice are significantly smaller than wild-type littermates from 4wk. **(B)** Female *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice are also significantly smaller than wild-type littermates from 4wk. * $P < 0.05$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Itga5^{+/+} *Nisch*^{+/+} Male $n = 7$, Female $n = 4$; *Itga5*^{+/+} *Nisch*^{edsn/edsn} Male $n = 7$, Female $n = 9$; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} Male $n = 8$, Female $n = 5$.

6.2.1.2.3 ABR

Again, in order to assess whether an abnormal hearing phenotype was displayed by *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, ABR tests for auditory function were conducted across a time course with cohorts of age matched littermates (Figure 6.8). Hearing assessments were conducted as summarised in Section 6.2.1.3.

Analysis of ABR thresholds demonstrated that *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice both displayed significantly elevated auditory thresholds compared to their wild-type littermates throughout the longitudinal time course (Figure 6.8A). At 4wk, mice with the *Itga5*^{+/+} *Nisch*^{edsn/edsn} mutation were found to have significantly elevated auditory thresholds compared to wild-types, with a mean ABR threshold of 33.75 dB SPL ($\pm$ 3.113), just above the expected wild-type threshold range (P = 0.002). Hearing progressively deteriorated with age in *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice, displayed by increasingly elevated ABR thresholds. By 12wk, *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals were recorded with a mean ABR threshold of 42.19 dB SPL ($\pm$ 4.401). The ABR threshold appeared to plateau and persisted at this increased elevation, where at 20wk the mean ABR threshold was 40.31 dB SPL ($\pm$ 4.365). Similarly at 4wk, mice with the *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} alleles were found to have significantly elevated auditory thresholds compared to wild-types, with a mean ABR threshold of 45.83 dB SPL ($\pm$ 2.769). Hearing again progressively deteriorated with age in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, displayed by increasingly elevated ABR thresholds. At 20wk, *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} animals were recorded with a mean ABR threshold of 60.63 dB SPL ($\pm$ 3.161). Interestingly and more importantly, *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice exhibited significantly elevated auditory thresholds compared to *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice, throughout the time course (P < 0.01; Figure 6.8B). As previously mentioned with other genotypes, there was no obvious gender or left-right preference observed in ABR threshold data recorded from *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} animals (Appendix, Figure 10.11).

The ABR threshold range for each genotype was analysed using box and whisker plots as well. The ABR threshold range in *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice increased with age across the time course (Figure 6.8C). At 4wk, the threshold range was from 15 to 60 dB SPL and at 20wk, the threshold range was from 20 to 75 dB SPL. In *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 4wk, the threshold range was from 20 to 70 dB SPL and at 20wk, the threshold range increased to between 25 to 85 dB SPL. More

significantly, the median threshold also increased with age; from 30 dB SPL at 4wk to 35 dB SPL at 20wk in *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice; and from 45 dB SPL at 4wk to 65 dB SPL at 20wk in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice. This difference seen in threshold ranges indicated that the OM phenotype seen in *Nisch*^{edsn/edsn} mice showed greater penetrance with the addition of the *Itga5*^{tm1Hyn/+} allele.

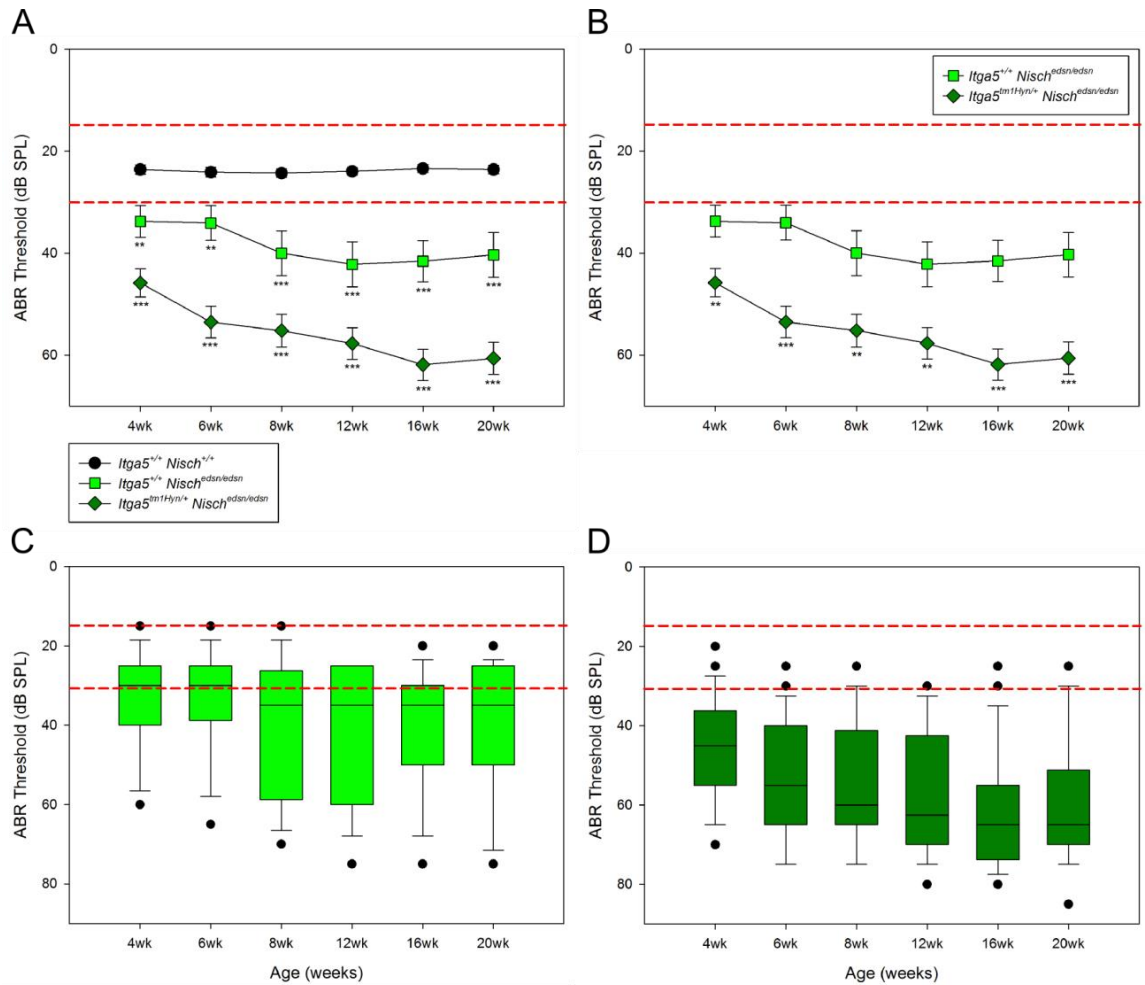


Figure 6.8| Progression of click-evoked ABR thresholds across a cohort time course for *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* litters.

(A) Both *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice display elevated hearing thresholds from 4wk, that progressively increase with age, due to OM. **(B)** Mice with the *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mutation have significantly elevated hearing thresholds throughout the entire time course compared to *Itga5^{+/+} Nisch^{edsn/edsn}* littermates. **(C & D)** Box plots show the ABR threshold ranges of (C) *Itga5^{+/+} Nisch^{edsn/edsn}* and (D) *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. ABR thresholds were measured for both ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and dots (•) show any outliers. ** $P < 0.01$; *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Itga5^{+/+} Nisch^{+/+} $n = 28$; *Itga5^{+/+} Nisch^{edsn/edsn}* $n = 16$; *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* $n = 24$.

6.2.1.2.4 Presence of OM

The incidence of OM was also assessed across a time course for each genotype (Figure 6.9A and B). In *Itga5^{+/+} Nisch^{edsn/edsn}* mice there was a clear trend in

increasing prevalence of OM from 4wk onwards. At 4wk, 13% of *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice had bilateral OM, 62% had unilateral OM and 25% had no OM phenotype. The prevalence of bilateral OM increased throughout the time course. A significant increase in prevalence of OM compared to wild-type littermates was observed at 20wk (z-test, $P = 0.005$), where 38% had bilateral OM, 25% unilateral OM and 37% showed no OM phenotype. In *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice there was a consistently high prevalence of OM throughout the time course. At 4wk, 67% of *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice had bilateral OM and 33% had unilateral OM. By the end of the time course, at 20wk, 83% had bilateral OM and 17% had unilateral OM. The level of OM prevalence seen in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 20wk compared to wild-type littermates was significantly different (z-test, $P < 0.001$). A notable difference in OM prevalence was observed between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 20wk, although the difference was not significant (z-test, $P = 0.097$).

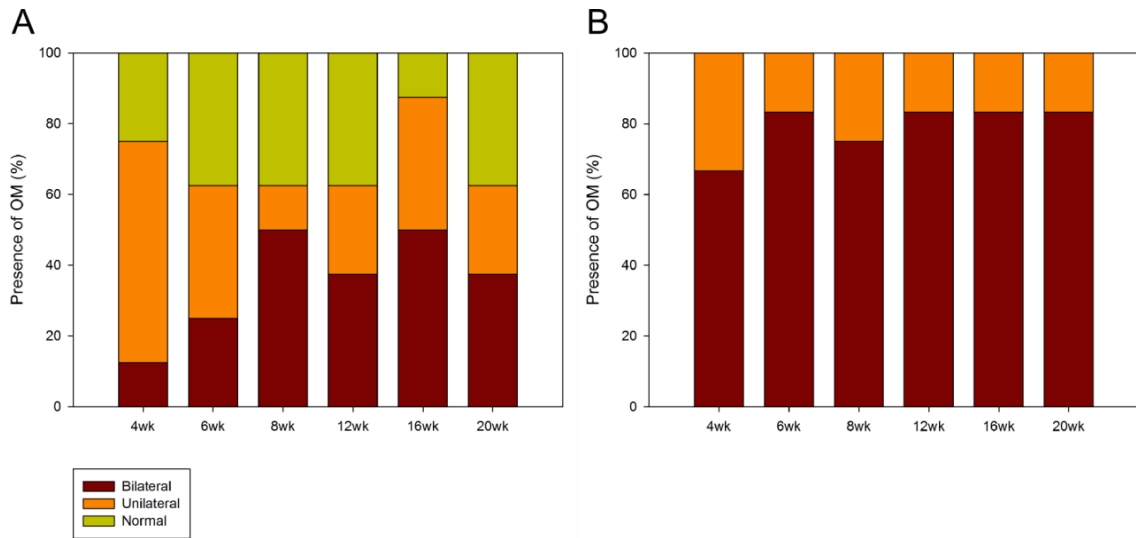


Figure 6.9| Incidence of OM in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} litters across a time course.

The presence or absence of OM for each mouse was recorded from visual inspection in **(A)** *Itga5*^{+/+} *Nisch*^{edsn/edsn} and **(B)** *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice. Prevalence of OM in *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals increases with age, whereas in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} animals the prevalence of OM is almost fully penetrant throughout. *Itga5*^{+/+} *Nisch*^{edsn/edsn} n = 8; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} n = 12.

As there was variation in the penetrance of OM between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, I proceeded to investigate whether there were any differences in ABR thresholds in 'OM' ears between genotypes. The ABR data recorded from individual ears was categorised into two groups; ears that had an OM phenotype, 'OM', and ears with a normal wild-type phenotype, 'Normal' (Figure 6.10). The criteria used to impartially categorise the ears into 'OM' and 'Normal' was from the subsequent visualisation of tympanic inflammation after dissection. Both *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} Normal ears displayed normal, wild-type hearing thresholds of between 15-30 dB SPL, with no significant differences compared to wild-type littermates. ABR thresholds from *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} OM ears both still showed a hearing loss that progressively deteriorates over the time course. At 4wk, *Itga5*^{+/+} *Nisch*^{edsn/edsn} OM ears had a mean ABR threshold of 45.00 dB SPL ($\pm$ 3.450) and by 20wk, the mean ABR threshold had increased to 54.38 dB SPL ($\pm$ 4.859). Similarly at 4wk,

Itga5^{tm1Hyn/+} *Nisch*^{edsn/edsn} OM ears had a mean ABR threshold of 49.75 dB SPL ($\pm$ 2.473) and by 20wk, the mean ABR threshold had increased to 63.86 dB SPL ($\pm$ 2.438). Analysis using a two-way ANOVA found there was a significant difference in ABR thresholds over the full time course between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} OM ears ($P < 0.001$). At individual time points, ABR thresholds of *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} OM ears were only significantly increased at 16wk compared to *Itga5*^{+/+} *Nisch*^{edsn/edsn} OM ears ($P = 0.003$).

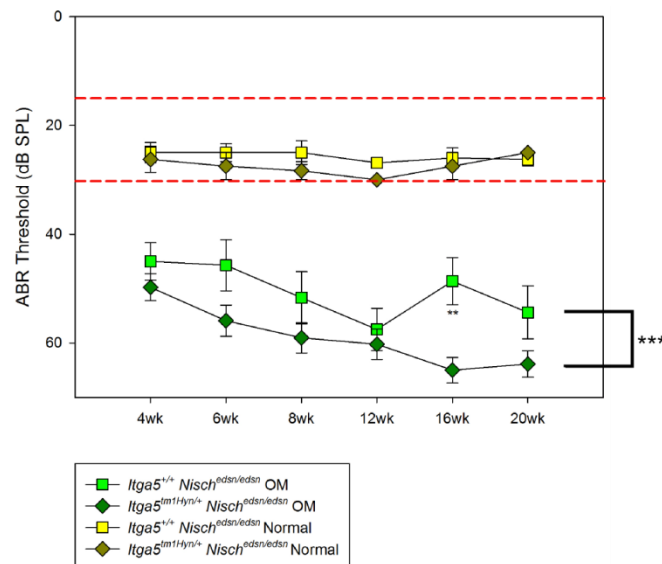


Figure 6.10| Comparison of click-evoked ABR thresholds, for OM and Normal ears, between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice.

Itga5^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} data used in Figure 6.8 was separated in to two groups for each genotype, 'OM' ears and 'Normal' ears, based on the presence/absence of inflammation of the tympanic membrane on visual inspection after dissection. 'OM' ears have elevated hearing thresholds, with a significant difference observed between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} 'OM' ears. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). ** $P < 0.01$; *** $P < 0.001$. Statistics were conducted using a two-way ANOVA and Holm-Sidak's multiple comparison tests for post-hoc analysis.

6.2.1.2.5 Histology

6.2.1.2.5.1 Middle ear mucosa

Histopathology was used to examine and compare the morphology of the ME in *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice. H&E sections at 20wk confirmed that *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice developed COM (Figure 6.11B and C). The mucosal inflammation in *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals was diffuse and of moderate severity, with the presence of a cellular ME effusion as seen previously (Figure 6.11E). *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice displayed a more severe mucosal inflammation, with increased polypoid exophytic growths and a thick cellular effusion (Figure 6.11F). Wild-type mice did not develop OM and had a ME free of effusion, with no thickening of the mucosal lining (Figure 6.11A and D).

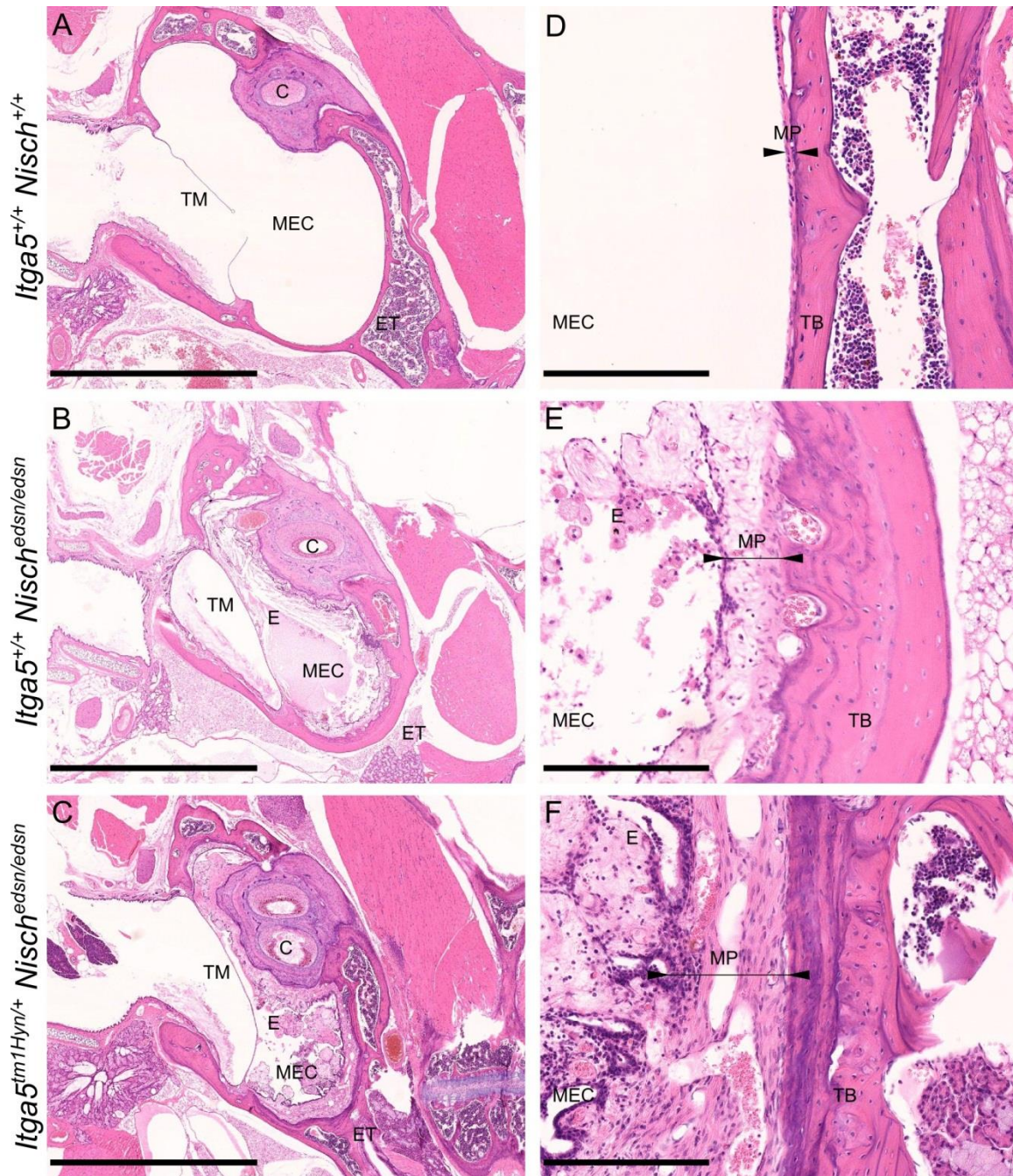


Figure 6.11| Characteristic ME histology in wild-type, *Itga5*^{+/+} *Nisch*^{edn/edn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} animals at 20wk.

(A-C) H&E stained transverse sections of the MEC of (A) *Itga5*^{+/+} *Nisch*^{+/+}, (B) *Itga5*^{+/+} *Nisch*^{edn/edn} and (C) *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} mice at 20wk. **(D-F)** H&E stained transverse sections of the ME mucoperiosteum of (D) *Itga5*^{+/+} *Nisch*^{+/+}, (E) *Itga5*^{+/+} *Nisch*^{edn/edn} and (F) *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} animals. Histopathology of the MEC from both *Itga5*^{+/+} *Nisch*^{edn/edn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} mice demonstrates chronic inflammation with an exudate. Inflammation of mucosa was more severe in sections from *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} ears. Wild-type animals have no inflammation or exudate in the MEC and a thin mucoperiosteum covers the temporal bone. C, cochlea; ET, eustachian tube; E, exudate; MEC, middle ear cavity; MP, mucoperiosteum (arrowheads); TB, temporal bone; TM, tympanic membrane. (A & B) Scale bar = 2 mm; (C & D) Scale bar = 200 μm.

From five *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice sacrificed at 20wk, there were eight ears that presented a chronic inflammation (80%, n = 10 ears), seven of which contained fluid. There was variability in the type of effusion found, where two ears had a thin watery effusion, and five ears displayed a cellular effusion with the presence of macrophages and PMNs. From five *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, there were nine ears that contained fluid in the MEC with a thickened epithelial lining (90%, n = 10 ears). A thick cellular effusion was present in all ears with chronic inflammation, with macrophages and PMNs. Cellular debris-like aggregates were present in the thick effusions and developing polyps were observed in the ME mucosa. No inflammatory phenotype was displayed in *Itga5*^{+/+} *Nisch*^{+/+} littermates (n = 12 ears).

To quantify and compare the chronic inflammation observed in *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, blinded assessment of the mucoperiosteum thickness was carried out (Figure 6.12A). Comparable regions of ME were measured for each ear assessed. As seen previously in Chapter 4, *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals had a significantly thicker ME mucosa compared to wild-type littermates ($P = 0.003$). The mean mucosal thickness was 80.73 μm (± 14.868) in *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals and 13.11 μm (± 0.687) for wild-types. A significant increase in mucosal thickness compared to wild-type was also observed in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice ($P < 0.001$), where the mean mucosal thickness was 125.70 μm (± 15.576). The difference in mucosal thickness observed between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice was almost significant ($P = 0.051$). As the penetrance of the OM phenotype differed between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, the data was categorised into ears that had an OM phenotype and ears that did not (Figure 6.12C). The presence of tympanic inflammation, previously recorded by visual assessment after dissection, was used to categorise ears with an OM phenotype. *Itga5*^{+/+} *Nisch*^{edsn/edsn} ears with no OM had a mean mucosal thickness

of 12.42 μm (± 1.659) and showed no significant difference compared to wild-type ($P = 0.710$). Whereas, *Itga5*^{+/+} *Nisch*^{edsn/edsn} ears with OM had a significantly thickened mucosa ($P < 0.001$), with an increased mean mucosal thickness of 97.81 μm (± 12.118). There was only one *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ear absent of tympanic inflammation and the mean mucosal thickness observed was similar to wild-type, at 16.08 μm . *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ears with OM had a significantly thickened mucosa ($P < 0.001$), with an increased mean mucosal thickness of 137.88 μm (± 10.854). When OM ears from *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice were compared (Figure 6.12B), there was now a significant increase in mucosal thickness observed in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice ($P = 0.026$).

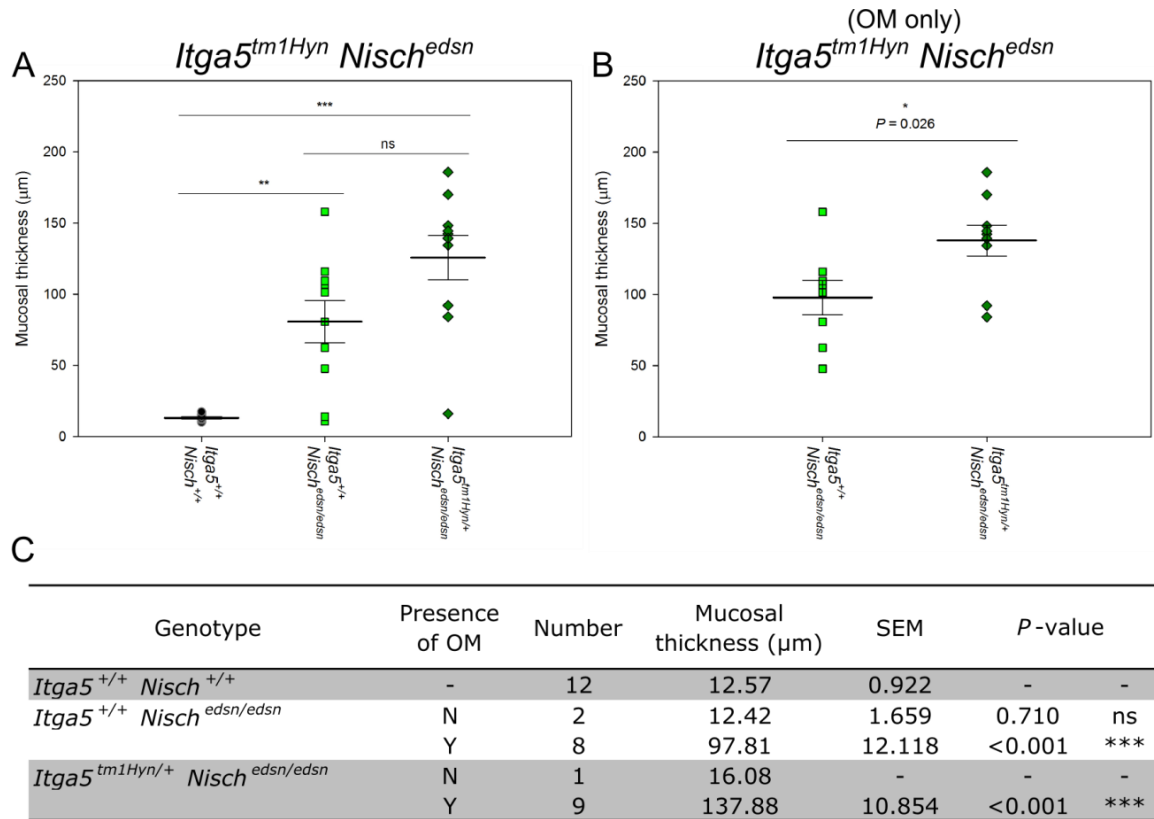


Figure 6.12| Analysis of mucosal thickness in the MEC of *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice.

H&E sections of ME were scanned into the Hamamatsu Pathology Imager and the mucosal thickness was averaged from 5 measurements of a standard 1000 μm length of ME mucosa (avoiding the cochlea and the region close to the ET). **(A)** Scatter plot showing mucosal thickness of the ME for *Itga5^{+/+} Nisch^{+/+}*, *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. Both *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice had significantly thicker mucosa compared to wild-type. No significant difference in mucosal thickness was observed between *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. **(B)** Scatter plot showing mucosal thickness of the ME for *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* 'OM' ears only. Mucosal thickness is significantly increased in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* 'OM' ears compared to *Itga5^{+/+} Nisch^{edsn/edsn}* 'OM' ears. **(C)** Table showing the mean mucosal thickness of *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* ears with or without OM, and corresponding *P*-values compared to wild-type littermates. In scatter plots each point represents a single ear and thick black line indicates mean mucosal thickness ± SEM. ns *P* > 0.05; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001. Data in panels A and C were analysed by Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc testing. Data in panel B was normally distributed and a Student's *t*-test was performed.

Itga5^{+/+} Nisch^{+/+} n = 12; *Itga5^{+/+} Nisch^{edsn/edsn}* n = 10; *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* n = 10.

6.2.1.2.5.2 Adult lungs

In order to quantify if there were significant genotypic differences in the lung morphology of *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, counts of the number of airways were conducted (Figure 6.13B) and the average airway widths were measured (Figure 6.13C).

Counts of the number of airways showed a significant decrease in the number of airways between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and wild-type mice ($P < 0.001$). The average number of airways in *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals was 132 (± 11.4) and for *Nisch*^{+/+} animals was 247 (± 9.0). A significant decrease in the number of airways compared to wild-type was also observed in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice ($P < 0.001$), where the mean number of airways was 124 (± 10.0). The difference in number of airways observed between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice was not significant ($P = 0.664$).

Itga5^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice also showed a difference in airway width, with a significant increase in the width compared to wild-type ($P < 0.001$ for both). The average airway width in *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals was 73.38 μm (± 6.274) and for *Itga5*^{+/+} *Nisch*^{+/+} animals was 35.21 μm (± 0.973). Again a significant increase was observed in airway width between wild-type and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, where the mean airway width was 76.52 μm (± 2.955). The difference in airway width observed between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice was again not significant ($P = 0.302$).

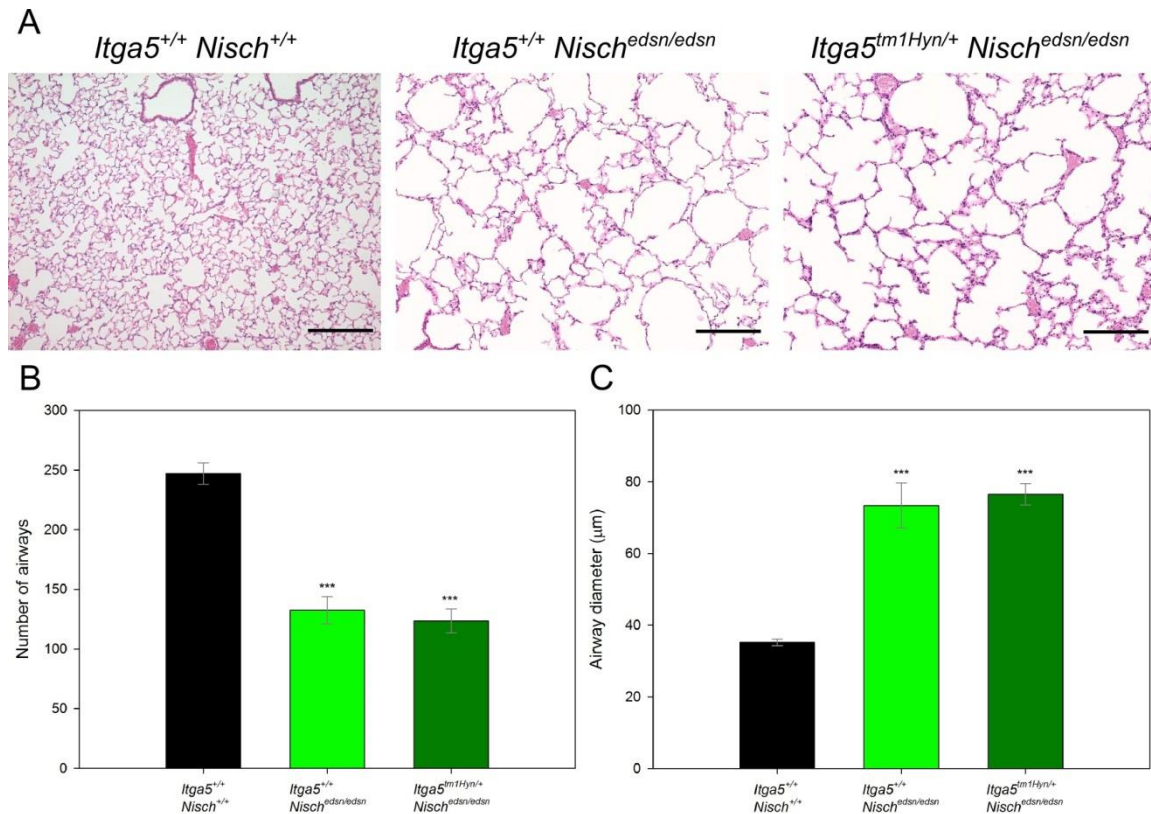


Figure 6.13| Analysis of adult lung histology in wild-type, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} animals at 20wk.

(A) Representative H&E stained lung sections from *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 20wk. Scale bars = 200 μm. **(B)** Bar chart showing counts of lung airways for each genotype. Airways were counted in three different 6.5 x 10⁵ μm² regions for all lungs. **(C)** Bar Chart showing airway diameters for each genotype. Airway diameters were measured in three different regions for all lungs. *** *P* < 0.001. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Itga5^{+/+} *Nisch*^{+/+} *n* = 12; *Itga5*^{+/+} *Nisch*^{edsn/edsn} *n* = 9; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} *n* = 9.

6.2.2 Downstream pathway analysis

As seen in the section 6.2.1 (Genetic cross-interaction between *Nisch* and *Itga5* in OM), an important functional interaction exists between NISCH and ITGA5. *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice display an increased severity and penetrance of COM compared to *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice. Immunohistochemistry (IHC) was utilised to analyse protein expression in ME and lung tissues, to further elucidate pathways involved in the genetic predisposition to OM in *edison* mice. Negative controls with

no primary antibody show no background staining (Appendix, Figure 10.12). Additionally, staining observed in *Itga5^{tm1Hyn/+} Nisch^{+/+}* mice was indistinguishable from wild-type littermates for each antibody analysed (data not shown).

6.2.2.1 NISCH

The causative missense mutation in the OM mutant *edison* was identified within *Nisch*. The resulting Leu972Pro amino acid was predicted to impact upon protein function. To determine the presence of the NISCH protein in ME and lung tissue, IHC was performed (Figure 6.14). NISCH was detected in the ME mucosa and lung tissue of wild-type mice. Similarly, in *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice NISCH was detected in the ME mucosa and lung tissue. NISCH was expressed in all airway cells and alveolar epithelial cells, with no difference in the localisation between genotypes. In the ME epithelial cells, similar expression of NISCH was displayed in each genotype. Additionally, a more intense staining was observed in alveolar macrophages of all genotypes and the macrophage-rich exudates in both *Itga5^{+/+} Nisch^{edsn/edsn}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* ears.

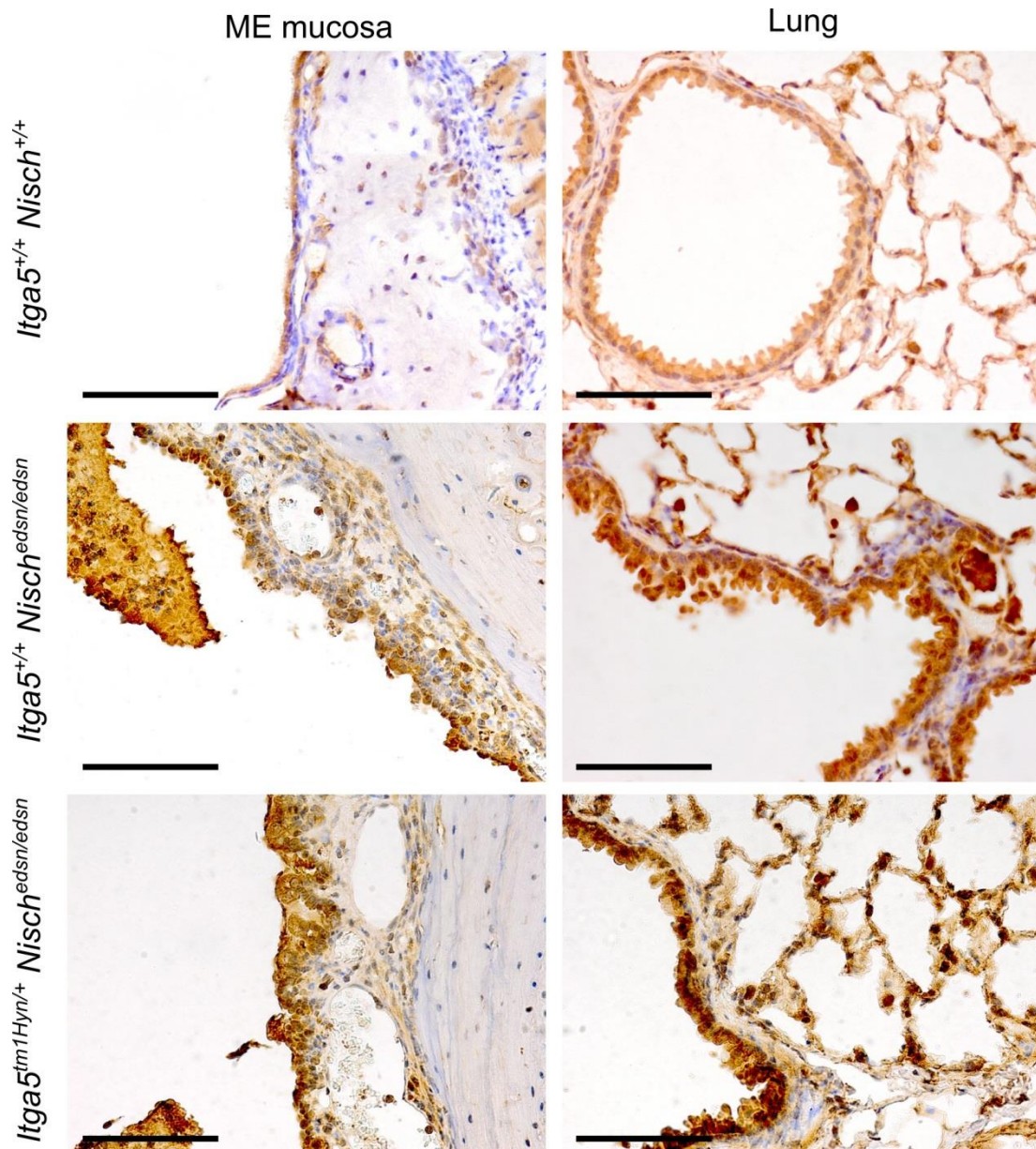


Figure 6.14| Immunohistochemistry (IHC) of ME and lung using a NISCH antibody.

ME and lung sections of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 4wk, stained with a NISCH antibody showing representative staining in the ME mucosa and lung. Scale bar = 100 μ m.

6.2.2.2 ITGA5

NISCH interacts with ITGA5 and overexpression of NISCH markedly reduces integrin $\alpha 5 \beta 1$ -dependent cell migration (Alahari, Lee et al. 2000). NISCH is thought to regulate ITGA5 expression through redistribution of ITGA5 from the cell membrane to intracellular endosomal structures (Lim and Hong 2004). I hypothesised the

edison mutation could result in increased expression of ITGA5 and therefore drive downstream regulators of angiogenesis and vascular permeability causing the OM phenotype. IHC was used to analyse the expression of ITGA5 in ME and lung tissue of *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice (Figure 6.15). In lung tissue, ITGA5 was expressed in airway epithelial cells and alveolar macrophages. No difference was observed between genotypes. Similarly, in ME sections there were no differences between genotypes for ITGA5 staining, with expression throughout the ME epithelial cells.

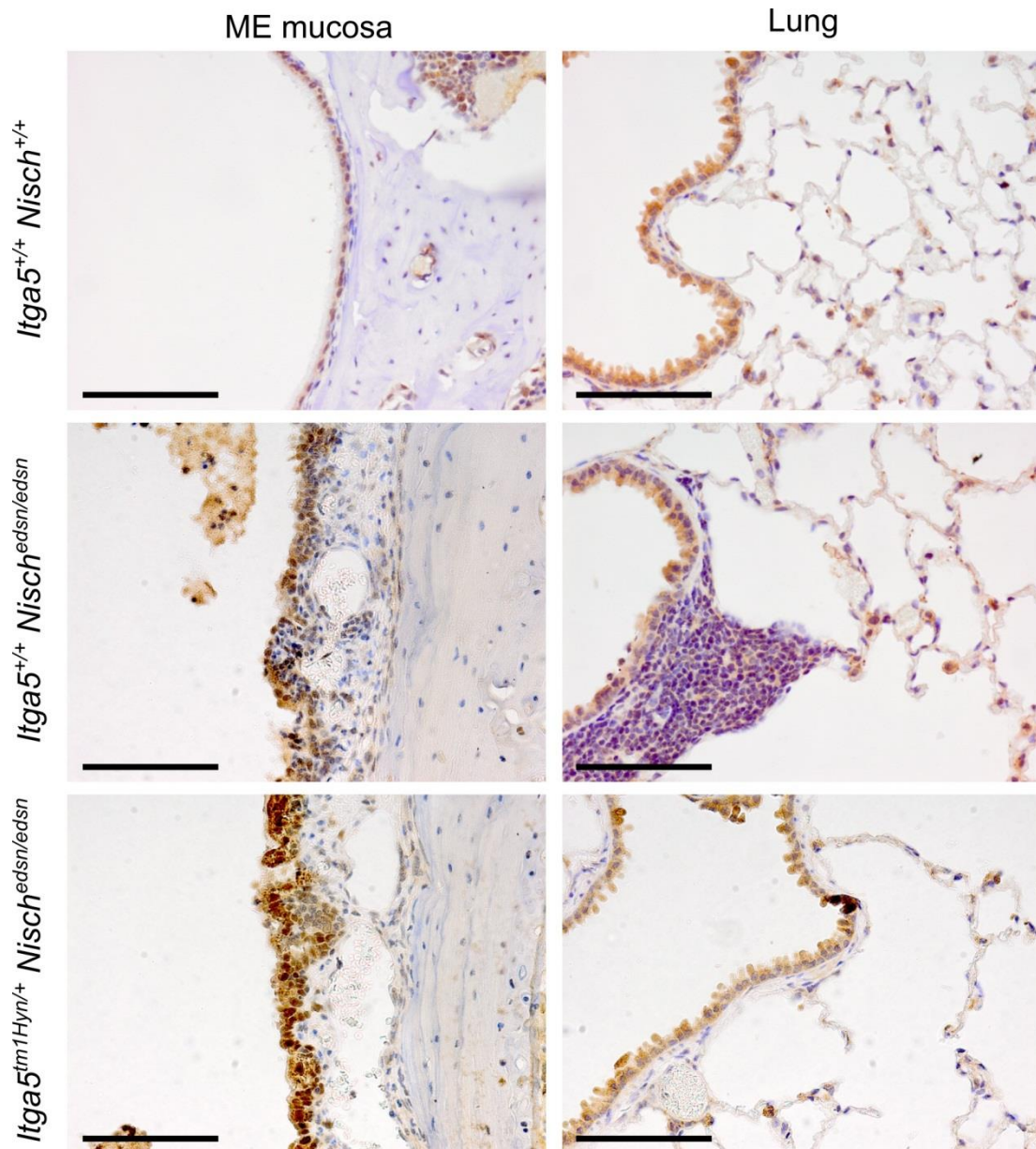


Figure 6.15| IHC of ME and lung using an ITGA5 antibody.

ME and lung sections of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 4wk, stained with an ITGA5 antibody showing representative staining in the ME mucosa and lung. Scale bar = 100 μ m.

6.2.2.3 SMAD2

Active TGF- β interacts with TGF- β Rs on the cell membrane and induces phosphorylation of SMAD proteins. Phospho-SMAD (p-SMAD) proteins translocate into the nucleus and act as transcription factors, regulating the expression of TGF- β pathway target genes. Therefore, phosphorylation and nuclear translocation of SMAD

proteins are considered good indicators of TGF- β activity (Shi and Massagué 2003). The TGF- β pathway signals through SMAD2/3. Immunohistochemical analysis of p-SMAD2 was performed on ME mucosa and lung tissues to evaluate activation of the TGF- β pathway during chronic ME disease in *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice (Figure 6.16). Staining for p-SMAD2 was detected in the both the ME mucosa and lung tissue of mice. No difference in the localisation between genotypes was observed in the lung tissue, with a few positive airway epithelial cells. In the ME epithelial cells, there was a greater proportion of nuclear localisation in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ears compared to wild-type. In contrast, this was not observed in the ME mucosa of *Itga5*^{+/+} *Nisch*^{edsn/edsn} ears.

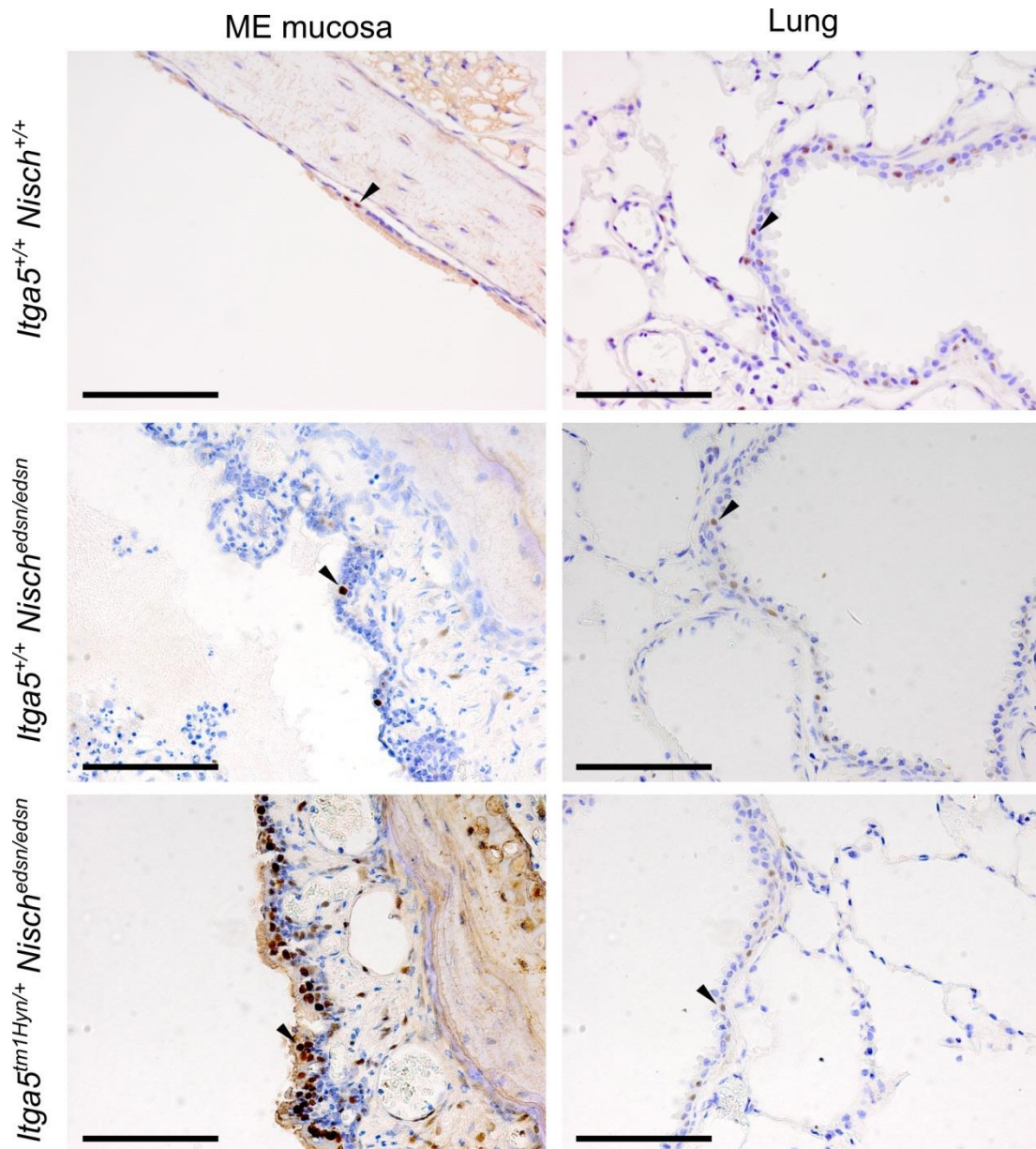


Figure 6.16| IHC of ME and lung using a phospho-SMAD2 antibody.

ME and lung sections of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 4wk, stained with p-SMAD2 antibody showing representative staining in the ME mucosa and lung. Arrows indicate nuclear localisation in the epithelial cells. Scale bar = 100 μ m.

In order to quantify if there were significant genotypic differences in the nuclear localisation of the p-SMAD2 staining in *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, counts of the number of p-SMAD2 positive epithelial cells were conducted in the ME mucosa and lung. The numbers of p-SMAD2 positive epithelial

cells with nuclear-localised signals were divided by the total numbers of epithelial cells to determine the proportion of p-SMAD2 positive staining in each section (Figure 6.17).

A clear difference was detected between the percentage of epithelial cells positive for the p-SMAD2 antibody in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mutant ME mucosa compared to wild-type ($P < 0.001$; Figure 6.17A). In contrast, the percentage of p-SMAD2 positive epithelial cells in *Itga5^{+/+} Nisch^{edsn/edsn}* ME mucosa showed no significant difference compared to wild type ears ($P = 0.680$). The mean percentage of p-SMAD2 positive epithelial cells in *Itga5^{+/+} Nisch^{edsn/edsn}* animals in the ME was 4.44 % (± 1.011) and for wild-types was 5.23 % (± 1.687). In the ME mucosa of *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* animals the mean percentage of p-SMAD2 positive epithelial cells increased to 49.27 % (± 1.286). In the lung sections, no significant difference was observed between genotypes for the mean percentage of p-SMAD2 positive epithelial cells ($P = 0.585$). The average percentage of p-SMAD2 positive epithelial cells in sections of lung epithelia was 9.98 % (± 1.227) in *Itga5^{+/+} Nisch^{+/+}* animals, 11.58 % (± 1.274) in *Itga5^{+/+} Nisch^{edsn/edsn}* mice and for *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* animals was 11.48 % (± 1.126).

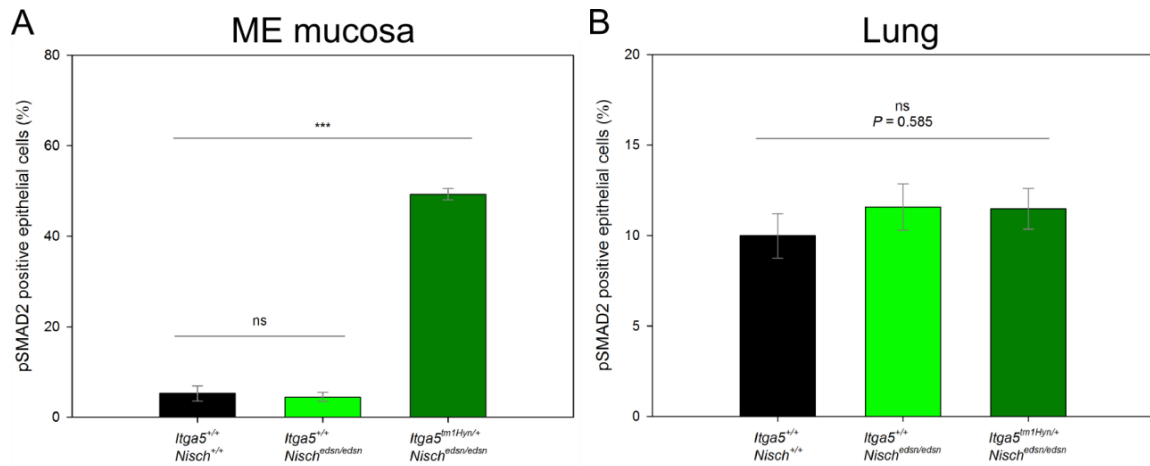


Figure 6.17| Analysis of phospho-SMAD2 IHC from *edison* animals.

(A) Graphic comparison of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice showing the percentage of epithelial cells in the ME positive for p-SMAD2.

(B) Graphic comparison of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice showing the percentage of epithelial cells in the lung positive for p-SMAD2. ns $P > 0.05$; *** $P < 0.001$. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Itga5^{+/+} *Nisch*^{+/+} ME $n = 4$, Lung $n = 12$; *Itga5*^{+/+} *Nisch*^{edsn/edsn} ME $n = 6$, Lung $n = 12$; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ME $n = 4$, Lung $n = 12$.

6.2.2.4 PAK1

NISCH has been shown to selectively inhibit RAC1 driven signalling cascades that affect migration through interactions with PAK1 (Alahari 2003). NISCH strongly inhibits the ability of PAK1 to phosphorylate its substrates (Alahari, Reddig et al. 2004). The expression of phospho-PAK1 (p-PAK1) was investigated by IHC to determine the presence of p-PAK1 and the possible role it plays in ME and lung tissue of *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice (Figure 6.18). Expression of p-PAK1 displayed a similar localisation as NISCH. No differences were observed in p-PAK1 expression between genotypes in lung tissue. Expression in lung tissue was seen in all airway cells, alveolar epithelial cells and alveolar macrophages. In the ME p-PAK1 was expressed in all epithelial cells, with weaker staining in the ME exudates of *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice. More staining was possibly observed in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} epithelial cells compared to *Itga5*^{+/+} *Nisch*^{edsn/edsn}, with some additional nuclear localisation of p-PAK1.

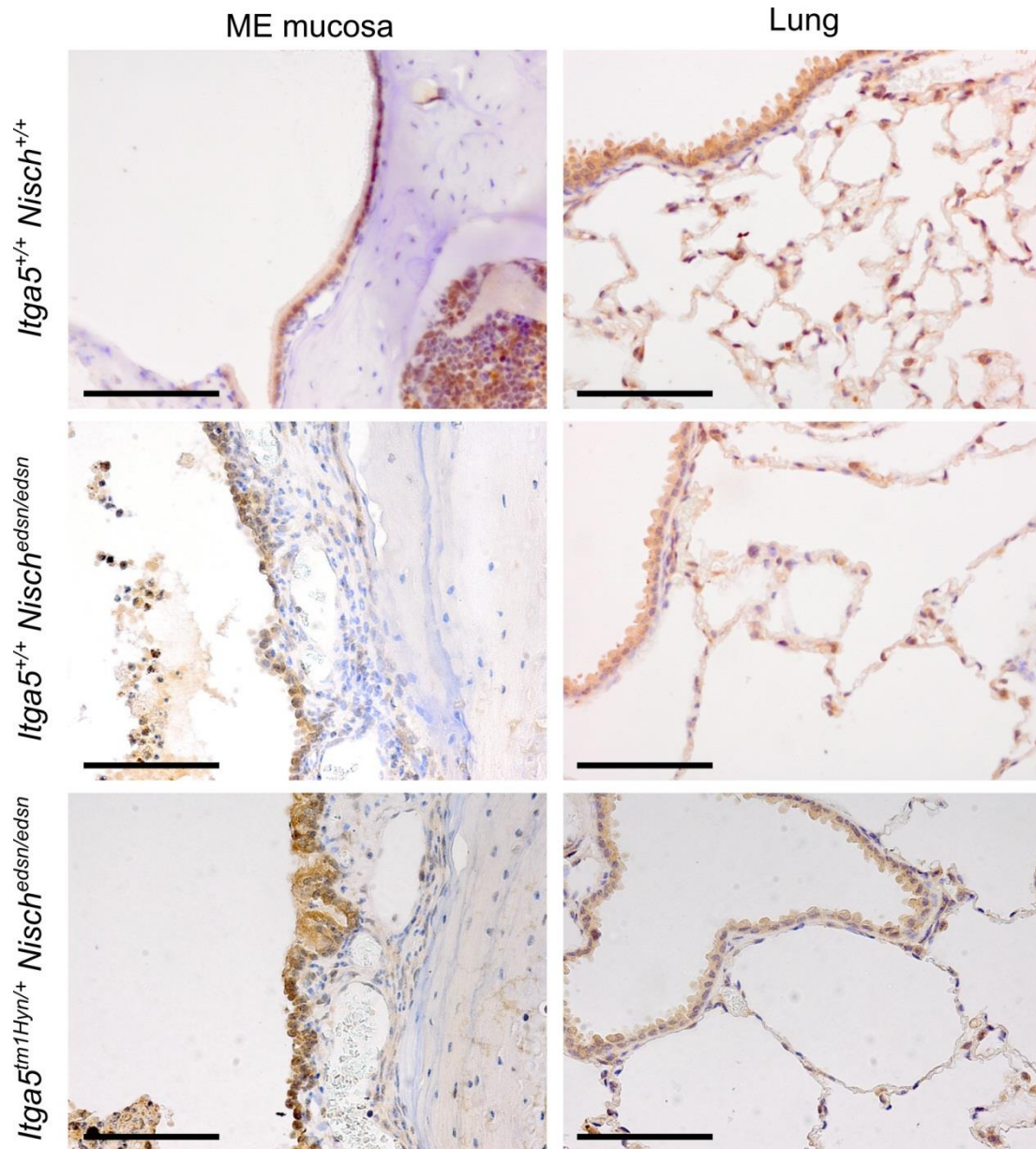


Figure 6.18 | IHC of ME and lung using a phospho-PAK1 antibody.

ME and lung sections of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 4wk, stained with a phospho-PAK1 antibody showing representative staining in the ME mucosa and lung. Scale bar = 100 μ m.

6.2.2.5 RAC1

In addition to regulating PAK1-dependent RAC1 signalling, NISCH can also regulate PAK1-independent RAC1 signal transduction through interactions directly with RAC1

(Reddig, Xu et al. 2005). An antibody for RAC1 was employed to examine expression in ME and lung tissue from *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice (Figure 6.19). Staining for RAC1 was detected in the both the ME mucosa and lung tissue of wild-type mice. In lung tissue, there was some weak staining localised in the smooth muscle surrounding the airways and more intense staining in alveolar macrophages. No expression was observed in epithelial cells in the lung tissue of wild-type mice. In ME tissue, RAC1 expression in wild-type mice was observed in ME epithelial cells. ME and lung tissue from *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice displayed similar RAC1 staining to wild-type, although the staining in the ME epithelial cells appeared weaker. Interestingly, a difference in RAC1 localisation was observed in both *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ears and lungs. In ME epithelial cells there was increased expression of RAC1, with more intense staining, and there appears to be some nuclear localisation. Similarly in lung tissue there was increased expression of RAC1 in epithelial cells compared to wild-type tissue.

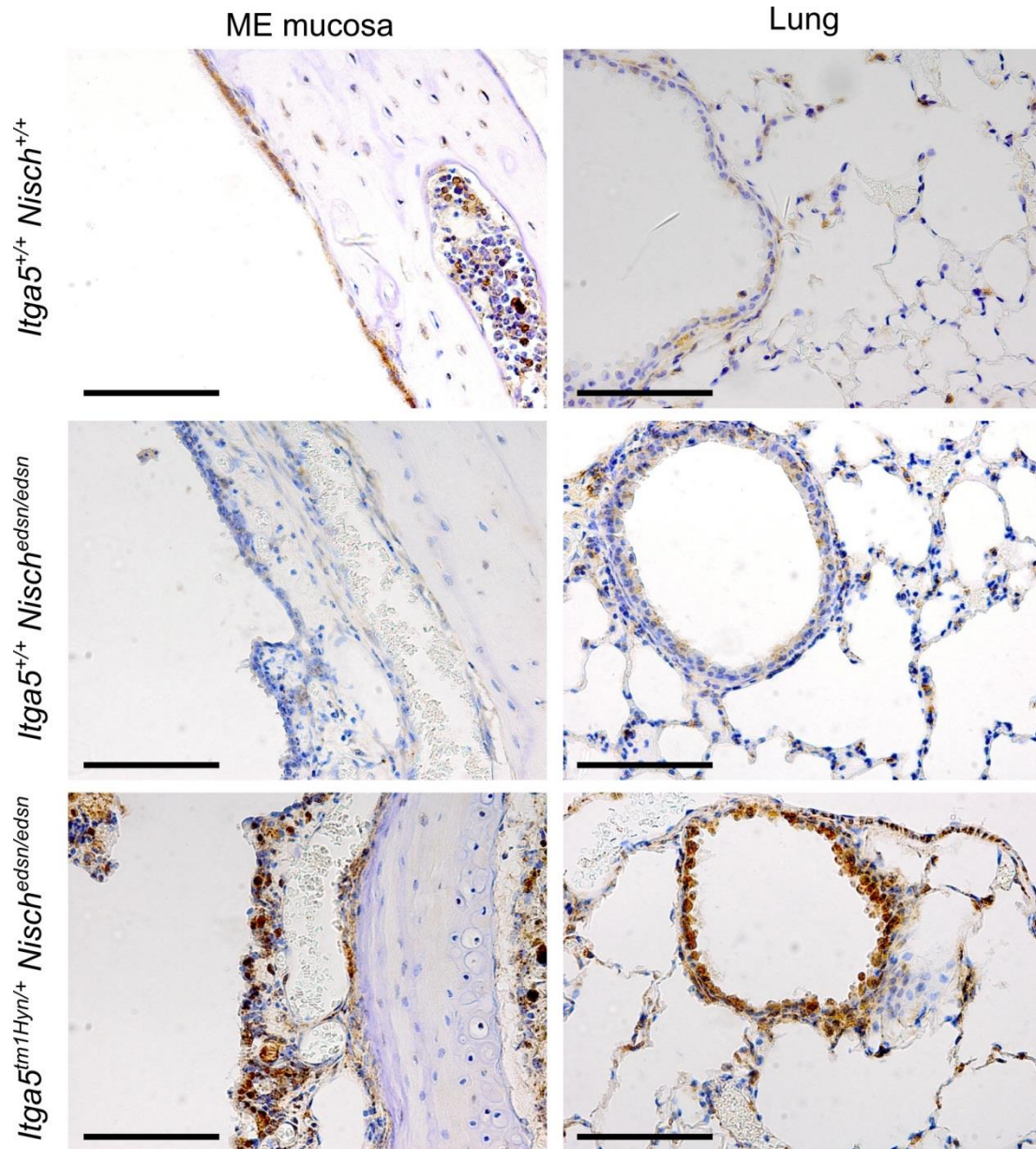


Figure 6.19| IHC of ME and lung using a RAC1 antibody.

ME and lung sections of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 4wk, stained with a RAC1 antibody showing representative staining in the ME mucosa and lung. Scale bar = 100 μ m.

In order to quantify if there were significant genotypic differences in the expression of the RAC1 staining in *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice, counts of the number of RAC1 positive epithelial cells were conducted in the ME mucosa and lung. The numbers of RAC1 positive epithelial cells were divided by the

total numbers of epithelial cells to determine the proportion of RAC1 positive staining in each section (Figure 6.20).

A clear difference was detected between the percentage of epithelial cells positive for the RAC1 antibody in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mutant ME mucosa compared to wild-type ($P < 0.001$; Figure 6.20A). In contrast, the percentage of RAC1 positive epithelial cells in *Itga5*^{+/+} *Nisch*^{edsn/edsn} ME mucosa showed no significant difference compared to wild type ears ($P = 0.776$). The mean percentage of RAC1 positive epithelial cells in *Itga5*^{+/+} *Nisch*^{edsn/edsn} animals in the ME was 9.43 % (± 1.169) and for wild-types was 10.98 % (± 1.230). In the ME mucosa of *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} animals the mean percentage of RAC1 positive epithelial cells increased to 64.27 % (± 8.435). Similarly, in lung sections a significant difference was observed in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ME mucosa compared to wild-type ($P < 0.001$), with no significant difference between *Itga5*^{+/+} *Nisch*^{edsn/edsn} and wild type ME mucosa ($P = 0.882$; Figure 6.20B). The average percentage of RAC1 positive epithelial cells in sections of lung epithelia was 3.93 % (± 1.413) in *Itga5*^{+/+} *Nisch*^{+/+} animals and for *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice was 4.38 % (± 1.087). In the lung epithelia of *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} animals the mean percentage of RAC1 positive epithelial cells increased to 76.46 % (± 3.284).

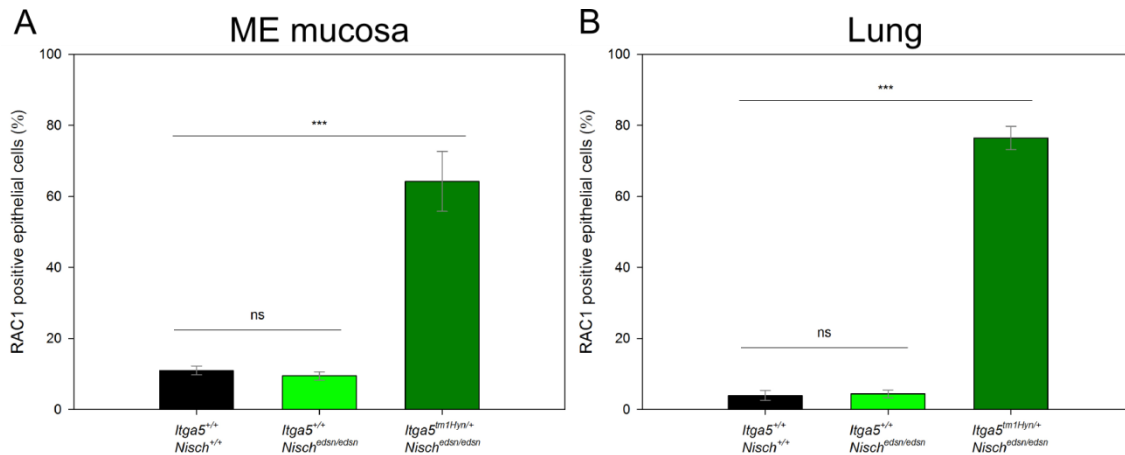


Figure 6.20 | Analysis of RAC1 IHC from *edison* animals.

(A) Graphic comparison of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edn/edn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} mice showing the percentage of epithelial cells in the ME positive for RAC1. **(B)** Graphic comparison of *Itga5*^{+/+} *Nisch*^{+/+}, *Itga5*^{+/+} *Nisch*^{edn/edn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} mice showing the percentage of epithelial cells in the lung positive for RAC1. ns P > 0.05; ** P < 0.01. Statistics were conducted using one-way ANOVA's and Holm-Sidak's multiple comparison tests for post-hoc analysis.

Itga5^{+/+} *Nisch*^{+/+} ME n = 4, Lung n = 10; *Itga5*^{+/+} *Nisch*^{edn/edn} ME n = 4, Lung n = 10; *Itga5*^{tm1Hyn/+} *Nisch*^{edn/edn} ME n = 4, Lung n = 10.

6.3 Discussion

6.3.1 Summary

The genetic interaction data presented in this report demonstrated a strong interaction between *Nisch* and *Itga5*. In this study I have presented data that showed a cross between *edison* and *Itga5^{tm1Hyn}* alleles, resulted in an increased penetrance of COM displayed in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. Additionally, preliminary analysis of downstream pathways suggests that the *edison* allele is impacting upon both RAC1 and TGF- β /SMAD signalling.

6.3.2 Addition of the *Itga5^{tm1Hyn}* allele to the *edison* mouse exacerbates the severity of COM

Genetic-cross interaction between *Nisch* and *Itga5* was attempted in an effort to identify the role that NISCH and ITGA5 interaction plays in COM. Addition of the *Itga5^{tm1Hyn}* allele to *edison* was hypothesised to moderate OM. Interestingly, the opposite was observed and the OM phenotype was exacerbated in both *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* and *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice as seen through ABR testing and histological analysis.

The OM phenotype displayed in *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice is a mild form of chronic ME disease with a late onset elevation in hearing thresholds. The hearing loss is observed from 12wk and is marginally progressive compared to littermates. The incidence of OM in *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice is similarly late onset and progressive. However, even at 20wk the majority of mice displayed no incidence of OM (Table 6.7). Typical changes due to COM occur in the MEC of *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice, however histological analysis demonstrated that in ears with an OM phenotype *Itga5^{tm1Hyn/+} Nisch^{edsn/+}* mice have a reduced mucosal thickening compared to *Itga5^{+/+} Nisch^{edsn/edsn}* mice (Table 6.7). There also appears to be a

smaller volume of fluid present in the MEC and, along with the reduced inflammation, may lead to the better auditory function observed.

Table 6.7| Summary of OM phenotypes in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice at 20wk.

Table summarising the incidence of OM for *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice; and the mean mucosal thickness for *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+}, *Itga5*^{+/+} *Nisch*^{edsn/edsn} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ears with an OM phenotype.

Incidence of OM: *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} n = 13; *Itga5*^{+/+} *Nisch*^{edsn/edsn} n = 8; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} n = 12.

Mucosal thickness: *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/+} n = 3; *Itga5*^{+/+} *Nisch*^{edsn/edsn} n = 8; *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} n = 9.

OM mouse mutant	Bilateral OM	Unilateral OM	None	Mucosal thickness (µm)
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/+}	0%	31%	69%	63.44 (± 5.368)
<i>Itga5</i> ^{+/+} <i>Nisch</i> ^{edsn/edsn}	38%	25%	37%	97.81 (± 12.118)
<i>Itga5</i> ^{tm1Hyn/+} <i>Nisch</i> ^{edsn/edsn}	83%	17%	0%	137.88 (± 10.854)

An early onset COM develops in mice with the *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} alleles and is associated with an elevation in hearing thresholds. Spontaneous onset of the reduced hearing deficit is observed at 4wk and is progressive. Similarly a progressive hearing deficit is also observed from 4wk in the control *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice, however the ABR thresholds recorded are significantly elevated in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice. The incidence of OM in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice was assessed and was significantly more prevalent than in *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice, with a very high incidence of bilateral OM from 4wk. Furthermore, in the *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice assessed there were no instances of no affected ears, with each mouse displaying either a unilateral or bilateral disease. Comparable to data in chapter 4, the incidence of OM in *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice was progressive, with an increased prevalence with age. However, instances where *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice displayed no affected ears were recorded throughout the time course. This difference

in OM incidence indicates that the OM phenotype is more penetrant in *Itga5^{tm1Hyn/+}* *Nisch^{edns/edns}* mice (Table 6.7). Even when this difference in OM penetrance was compensated for by analysing the ABR thresholds in OM ears only, *Itga5^{tm1Hyn/+}* *Nisch^{edns/edns}* mice still had significantly elevated hearing thresholds compared to *Itga5^{+/+}* *Nisch^{edns/edns}* mice. This increased hearing deficit is indicative of a more severe OM phenotype, and was further confirmed through histological analysis. Both *Itga5^{+/+}* *Nisch^{edns/edns}* and *Itga5^{tm1Hyn/+}* *Nisch^{edns/edns}* mice displayed changes in the MEC associated with COM, including mucosal inflammation, edematous polyps and a dense granulocyte-rich effusion. However, increased severity was observed in *Itga5^{tm1Hyn/+}* *Nisch^{edns/edns}* mice, with the presence of a greater number of polypoid exophytic growths and increased mucosal inflammation. In ears with an OM phenotype *Itga5^{tm1Hyn/+}* *Nisch^{edns/edns}* mice have significantly increased mucosal thickening compared to *Itga5^{+/+}* *Nisch^{edns/edns}* mice (Table 6.7). Addition of the *Itga5^{tm1Hyn}* allele clearly exacerbates the severity and penetrance of OM in *edison* mice.

Histological analysis of adult lungs displayed the same emphysema-like phenotype found in *edison*, in both *Itga5^{+/+}* *Nisch^{edns/edns}* and *Itga5^{tm1Hyn/+}* *Nisch^{edns/edns}* mice, however the severity of the phenotype was not impacted upon by addition of the *Itga5^{tm1Hyn}* allele. Proper lung development during the neonatal period is crucial for the formation of fully functional adult lungs (Shi, Bellusci et al. 2007), suggesting that investigation of embryonic lungs may be more valuable. No histology was carried out on embryonic lungs, although in chapter 4 the reduced numbers of *Nisch^{edns/edns}* progeny was a result of an embryonic lung phenotype. Both *Itga5^{+/+}* *Nisch^{edns/edns}* and *Itga5^{tm1Hyn/+}* *Nisch^{edns/edns}* pups were being found dead in increased proportions before weaning age; however in similar numbers, indicating no significant difference in the embryonic lung phenotype that causes the reduced progeny. Integrins have overlapping substrate specificity and cell-type-specific expression patterns (Hynes 2002; Humphries, Byron et al. 2006), and the same

integrin molecule can have different ligand-binding specificities in different cell types (Alberts 2002). Therefore, it is possible that addition of the *Itga5*^{tm1Hyn} allele only impacts upon the OM phenotype in *edison* mice, however further investigation will be required to confirm this.

6.3.3 Downstream analysis highlights RAC1 and TGF- β signalling in the development of COM

NISCH preferentially binds the cytoplasmic domain of ITGA5 and is thought to negatively regulate ITGA5 expression, shuttling it from membrane to endosome (Alahari, Lee et al. 2000; Lim and Hong 2004). Highlighted earlier, ITGA5 is known to have extensive cross-talk with VEGF and TGF- β signalling pathways. My initial hypothesis was that loss of function of NISCH would lead to upregulation of ITGA5 in the ME and, as discussed above, signalling pathways leading to vascular leak and OM. However, my finding that *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mutants exhibit an exacerbation of the OM phenotype seemed to contradict my hypothesis.

IHC was utilised to analyse protein expression, in ME and lung tissues, to identify downstream pathways involved in the development of COM in *edison* mice. Analysis revealed significantly increased expression of p-SMAD2 in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ME epithelial cells relative to ME sections from wild-type and *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice. NISCH is also known to regulate RAC1 signalling through both PAK1 and PAK1-independent pathways (Alahari 2003; Reddig, Xu et al. 2005). RAC1 expression was significantly increased in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ME epithelial cells when compared to sections from both wild-type and *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice. Similarly, it appears that p-PAK1 expression may also be increased in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ME epithelial cells, although further investigation is required. Interestingly, in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} lung tissue increased staining was only observed when

analysing RAC1 expression, with no differences in p-SMAD2 or p-PAK1 expression. In contrast, expression of p-SMAD2, p-PAK1 and RAC1 in both ME and lung tissue of *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice was comparable to wild-type expression. Analysis of NISCH and ITGA5 showed expression was present in both ME mucosa and lung tissue, with no observable differences exhibited between genotypes. The cumulative effect of deficits in both NISCH and ITGA5 is enough to significantly increase expression of RAC1 and p-SMAD2 in *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} ME epithelial cells, highlighting RAC1 and TGF- β signalling in the development of COM.

RAC1 signalling regulates numerous and disparate cellular functions, mediating these effects through interactions with a variety of effector proteins. PAK1 is one of the key downstream effectors of RAC1 (Kumar and Vadlamudi 2002; Bokoch 2003). Binding of RAC1 is an important step in the activation of PAK1 (Buchwald, Hostinova et al. 2001; Parrini, Lei et al. 2002). NISCH has been shown to block RAC1 induced cell migration through PAK1 (Alahari 2003). Interaction with NISCH strongly inhibits the kinase activity of PAK1, repressing RAC1 signal transduction pathways (Alahari, Reddig et al. 2004). RAC1 activation of PAK1 enhances the interaction between NISCH and PAK1, with the three proteins forming a complex (Alahari, Reddig et al. 2004). Expression of integrin $\alpha 5\beta 1$ also increases this association between NISCH and PAK1 (Alahari, Reddig et al. 2004). LIMK1 is a downstream effector of PAK1 (Edwards, Sanders et al. 1999). Similarly, NISCH has been shown to be an interacting partner with LIMK, regulating cell invasion through negative modulation of the LIMK-Cofilin pathway (Ding, Milosavljevic et al. 2008). Enhanced pulmonary vascular permeability is associated with acute lung injury (ALI); in LIMK1 knockout mice reduced edema formation was observed during endotoxin-induced ALI, suggesting a key role for LIMK1 in vascular permeability (Gorovoy, Han et al. 2009). Downregulation of LIMK1 with siRNAs was also shown to suppress ocular inflammation and LIMK1 has been further suggested to mediate inflammation through TGF- β signalling (Gorovoy, Koga et al. 2008).

NISCH can also regulate PAK1-independent RAC1 signal transduction through a direct interaction with RAC1 (Reddig, Xu et al. 2005). In addition to activation of PAK1, RAC1 can stimulate the phosphorylation of I κ B, an inhibitor of NF- κ B function, leading to its degradation and NF- κ B activation (Perona, Montaner et al. 1997; Bonizzi and Karin 2004). RAC1 activation of NF- κ B inducing kinase (NIK) results in the subsequent activation of I κ B kinase (IKK), further mediating the phosphorylation and degradation of I κ B. The NF- κ B family of transcription factors are involved in stress-induced, immune, and inflammatory responses (Baldwin 1996). Overexpression of NISCH has been shown to suppress the ability of RAC1 to stimulate the function of NF- κ B, and siRNA knockdown of NISCH results in activation of NF- κ B (Reddig, Xu et al. 2005). Activated PAK1 can also stimulate NF- κ B function (Frost, Swantek et al. 2000; Balasenthil, Sahin et al. 2004), however the ability of NISCH to disrupt RAC1 stimulation of NF- κ B does not require PAK1 (Reddig, Xu et al. 2005). Increased RAC1 activity and PAK1 overexpression have been associated with lymphovascular invasion and lymph node metastasis of upper urinary tract cancer (Kamai, Shirataki et al. 2010). In human breast cancer cell lines, NISCH was found to regulate expression of pro-oncogenic factors through a feedback loop mechanism by suppressing NF- κ B phosphorylation (Jin, Wessely et al. 2013). The *Junbo* mutant carries a mutation in *Evi1* and develops spontaneous COM (Parkinson, Hardisty-Hughes et al. 2006). Recent work has suggested that EVI1 acts as an inducible negative-feedback regulator of NF- κ B and the mutation in *Junbo* leads to enhanced activation of NF- κ B and inappropriate regulation of the inflammatory response (Xu, Woo et al. 2012).

6.3.4 Proposed mechanism of action in *edison* mice

Activation of NF- κ B plays a central role in inflammation through its ability to induce transcription of pro-inflammatory genes (Baldwin 1996). Additionally, LIMK1 is a

downstream effector of PAK1 (Edwards, Sanders et al. 1999); with a key role in vascular permeability (Gorovoy, Han et al. 2009), and inflammation possibly through TGF- β signalling (Gorovoy, Koga et al. 2008). As mentioned above, NISCH regulates RAC1 signalling through both PAK1 and PAK1-independent pathways (Alahari, Reddig et al. 2004; Reddig, Xu et al. 2005). In the latter case, interaction between RAC1 and NISCH leads to repression of NF- κ B activity, and knockdown of NISCH leads to activation of NF- κ B (Reddig, Xu et al. 2005). It is noteworthy that the mutation in *Junbo* mice leads to enhanced NF- κ B activity and inappropriate regulation of the inflammatory response (Xu, Woo et al. 2012). Moreover, interactions between NISCH, PAK1 and RAC1 are enhanced by expression of integrin α 5 β 1 (Alahari, Reddig et al. 2004). Deficits in both NISCH and ITGA5 enhances pathway activation in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* leading to significantly increased RAC1 and TGF- β signalling, with exacerbation of the COM phenotype (proposed mechanisms summarised in Figure 6.21 – 6.23).

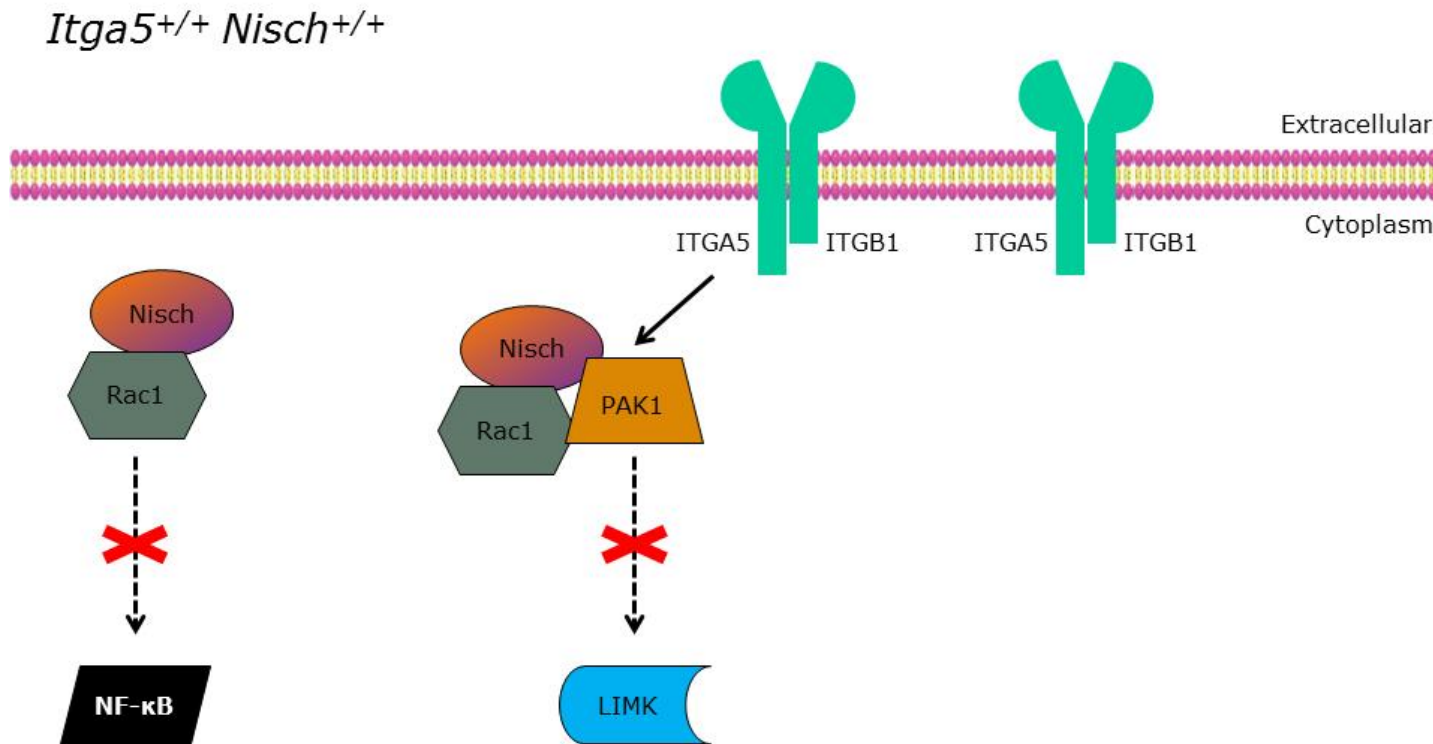


Figure 6.21 | Proposed mechanism of action in *Itga5^{+/+} Nisch^{+/+}* mice.

Schematic representation of the protein interactions showing how NISCH regulates downstream effectors in wild-type mice. NISCH interacts with PAK1 inhibiting the kinase activity of PAK1 and repressing RAC1 signal transduction pathways. RAC1 activation of PAK1 enhances the interaction between NISCH and PAK1, with the three proteins forming a complex. Additionally, expression of integrin α5β1 also increases this association between NISCH and PAK1. LIMK1 is a downstream effector of PAK1. NISCH can also regulate PAK1-independent RAC1 signal transduction through a direct interaction with RAC1, leading to suppression of NF-κB activity. See the text for a more detailed description of pathways.

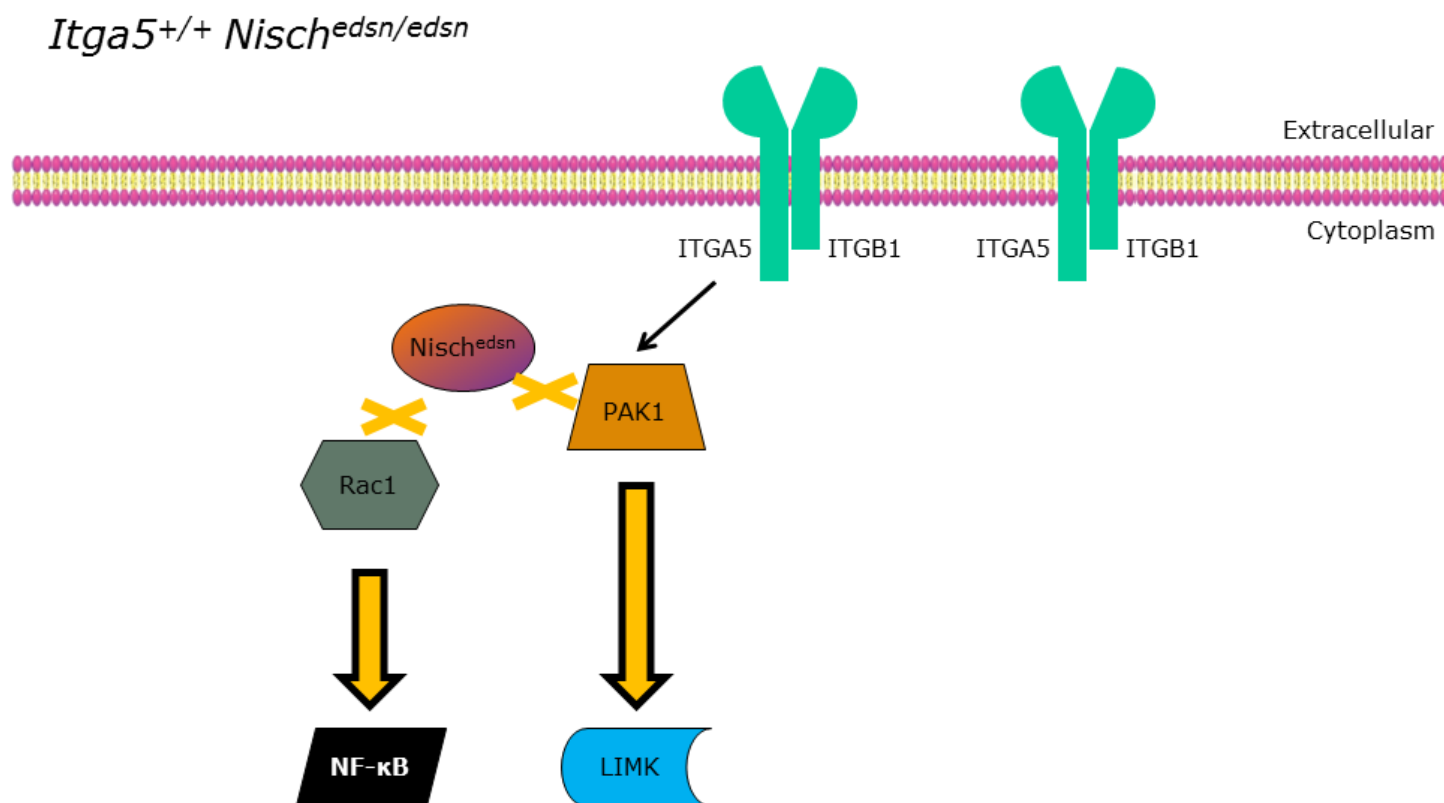


Figure 6.22| Proposed mechanism of action in *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice.

Schematic representation of the protein interactions showing how deficits in NISCH impact upon downstream effectors in *Itga5*^{+/+} *Nisch*^{edsn/edsn} mice. In *edison* mice the Leu972Pro causative mutation identified in NISCH was predicted to impact upon the biological function. NISCH interactions with PAK1 and RAC1 are impaired leading to inappropriate regulation of RAC1 signal transduction pathways and development of chronic OM. Expression of integrin α5β1 may still increase the NISCH/PAK1 association. See the text for a more detailed description of pathways.

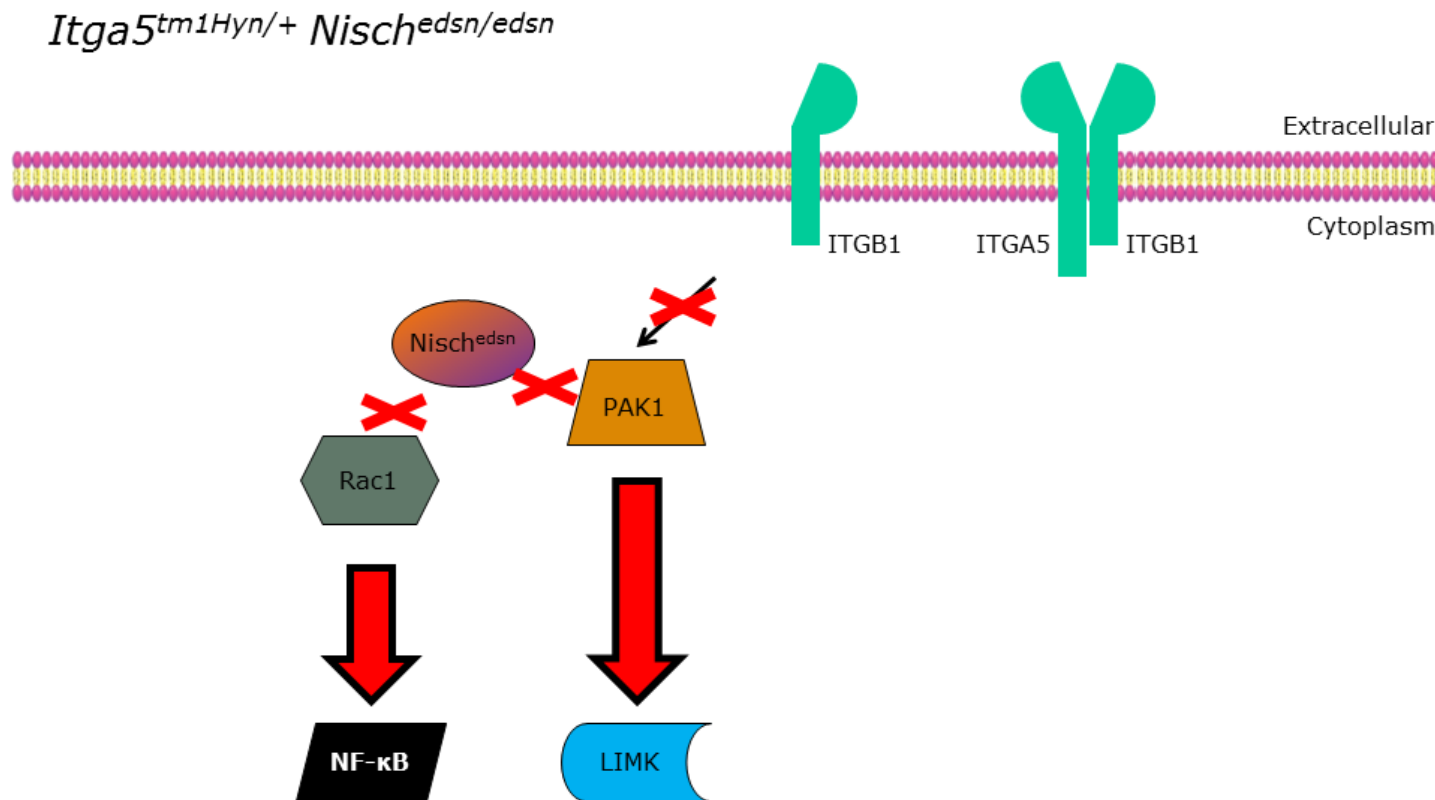


Figure 6.23 | Proposed mechanism of action in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice.

Schematic representation of the protein interactions showing how deficits in NISCH and ITGA5 impact upon downstream effectors in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. The *edison* mutation leads to impaired interactions between NISCH, PAK1 and RAC1. ITGA5 deficits further impair the association between NISCH and PAK1. The cumulative deficits in NISCH and ITGA5 leads to enhanced RAC1 signal transduction, with increased LIMK1 and NF-κB activity. Enhancements in pathway activation cause the inappropriate regulation of the inflammatory response resulting in exacerbation of the chronic OM phenotype. See the text for a more detailed description of pathways.

6.3.5 Conclusion

A genetic cross-interaction between *Nisch* and *Itga5* mutants revealed that cumulative deficits in both NISCH and ITGA5 further exacerbated the OM phenotype seen in *edison*, indicating a significant functional interaction exists. Analysis of downstream pathways identified increases in expression of RAC1 and p-SMAD2 in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* ME epithelial cells, highlighting RAC1 and TGF- β signalling in the development of COM. In sum, the *edison* mutant has the potential to throw significant further light on inflammatory responses in the development of chronic ME disease.

CHAPTER 7: *edison* as a model for *NTHi* middle ear infection

7.1 Introduction

The identification of bacterial species such as *Moraxella catarrhalis*, *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* (NTHi) in cultured ear fluids from human patients with OM, in conjunction with evidence indicating the importance of the mucosal immune system in ME inflammatory disease, has led to the investigation and implementation of vaccination as a valid prevention strategy for AOM. The use of pneumococcal vaccines in recent years has meant that NTHi has become the most prevalent isolated pathogen in AOM (Block, Hedrick et al. 2004; Casey and Pichichero 2004), however there is currently no effective vaccine. This chapter concentrates on validating *edison* as a robust model for experimental NTHi inoculation alongside the *Junbo* mouse mutant.

7.1.1 Vaccination to prevent OM

7.1.1.1 Pneumococcal polysaccharide vaccines

There are over 90 serotypes of pneumococcus, although relatively few contribute to AOM (Bluestone and Klein 2007). In 1978 a 14-serotype pneumococcal polysaccharide vaccine was licensed in the USA, later replaced by a 23-valent vaccine in 1983. Clinical trials of the vaccine failed to decrease the number of episodes of AOM in children younger than two years, possibly due to low immunogenicity in this age group (Karma, Luotonen et al. 1980; Mäkelä, Sibakov et al. 1980; Mäkelä, Leinonen et al. 1981; Sloyer, Ploussard et al. 1981; Teele, Klein et al. 1981).

To circumvent this problem of immunogenicity the conjugate pneumococcal polysaccharide vaccine was developed. The current licensed vaccine, Prevnar, was approved for use in the USA in 2000 and is a heptavalent conjugate vaccine employing a diphtheria mutant toxin protein (CRM197) as a protein carrier. This

vaccine is immunogenic in children as young as 2 months of age and has demonstrated efficacy in preventing pneumococcal AOM (Black, Shinefield et al. 2000). Two clinical trials of the vaccine showed a reduction in the number of episodes of OM, with a 7.8% reduction in California and 6% reduction in Finland (Eskola, Kilpi et al. 2001; Fireman, Black et al. 2003). Despite this reduction, it has been reported that the number of episodes of OM due to all other serotypes, not included within the vaccine, increased by 33% and the number of OM episodes owing to NTHi increased by 11% since the introduction of the vaccine (Eskola, Kilpi et al. 2001).

7.1.1.2 The conjugate *H. influenzae* type b vaccine and NTHi

The current licensed vaccine for *H. influenzae* is against the capsular type b strain, which only causes around 2% of AOM cases (Bluestone and Klein 2007). Therefore, the vaccine has limited value in preventing OM. NTHi are unencapsulated strains deficient of capsular serotypes, therefore have a distinct lack of target antigens for vaccine development. Investigations into proteins associated with NTHi outer membrane vesicles (OMVs) as candidate antigens have shown some success, with a number of outer membrane proteins identified as antigenically conserved among NTHi strains and targets for human bactericidal antibody (Murphy, Bartos et al. 1986; Murphy and Bartos 1988). However, an investigation into the antibody response in otitis-prone children to the NTHi outer membrane protein, P6, revealed uncertainties for its use as an adequate vaccine candidate (Yamanaka and Faden 1993). In contrast, a recent study in mice has suggested that intranasal immunisation with NTHi OMVs induces cross-protective immunity (Roier, Leitner et al. 2012).

7.1.2 *Junbo* as a model for NTHi ME infection

Animal models offer a critical opportunity to investigate the mechanisms that underlie human disease. Using the *Junbo* mouse, a robust model of bacterial NTHi infection has been established at MRC Harwell (Hood, Cheeseman and Brown, data not published). A single intranasal inoculation with NTHi produces high rates of ME infection (up to 95%) and bacterial titers in bulla fluids with clearance from 28-56 days post-inoculation. In contrast, wild-type mice cannot be infected by the same NTHi strain, even in conjunction with barotrauma. Pre-existing bulla fluids are a critical requirement for efficient NTHi infection of the ME. *Junbo* as an infection model has enabled studies of host-microbial interactions influencing ME disease and shows potential for a model of efficient NTHi vaccine screening (Hood, Cheeseman and Brown, data not published). *Tgif*-null and *Jeff* mutant mice are two further OM models assessed for NTHi infection, however significantly reduced infection rates compared to *Junbo* were observed (~15%). It is important to replicate the findings from *Junbo* infection with NTHi in another robust OM model to confirm the findings are not unique to the *Junbo* host immune response.

7.2 Results

7.2.1 Intranasal inoculation of *edison* mice using the human otopathogen NTHi

7.2.1.1 Pilot study using NTHi isolate #162

The human otopathogen NTHi is one of three bacterial species commonly associated with AOM in humans and is used in AOM research worldwide. I proceeded to explore the effects that inoculation with NTHi would have on the colonisation of the ME in *edison* mice. The first pilot experiment utilised *Nisch*^{edsn/edsn} mice at 6wk and mice were inoculated with bacterial NTHi isolate #162 and sampled at 7 days post inoculation (Figure 7.1). It was found that *Nisch*^{edsn/edsn} ears without a ME effusion did not produce culturable NTHi from the ME after culture of ME washes with PBS.

The proportion of *Nisch*^{edsn/edsn} ears with samplable ME exudates at 6wk was low, at 39% (Figure 7.1A). For those ears where ME fluid was collected there was evidence of infection of the ME fluid with NTHi, with two ME fluid samples from *Nisch*^{edsn/edsn} mice producing culturable NTHi at an infection rate of 0.286 (Figure 7.1B). In contrast, for ears where no ME fluid was collected there was no evidence of infection of the ME fluid with NTHi (data not shown).

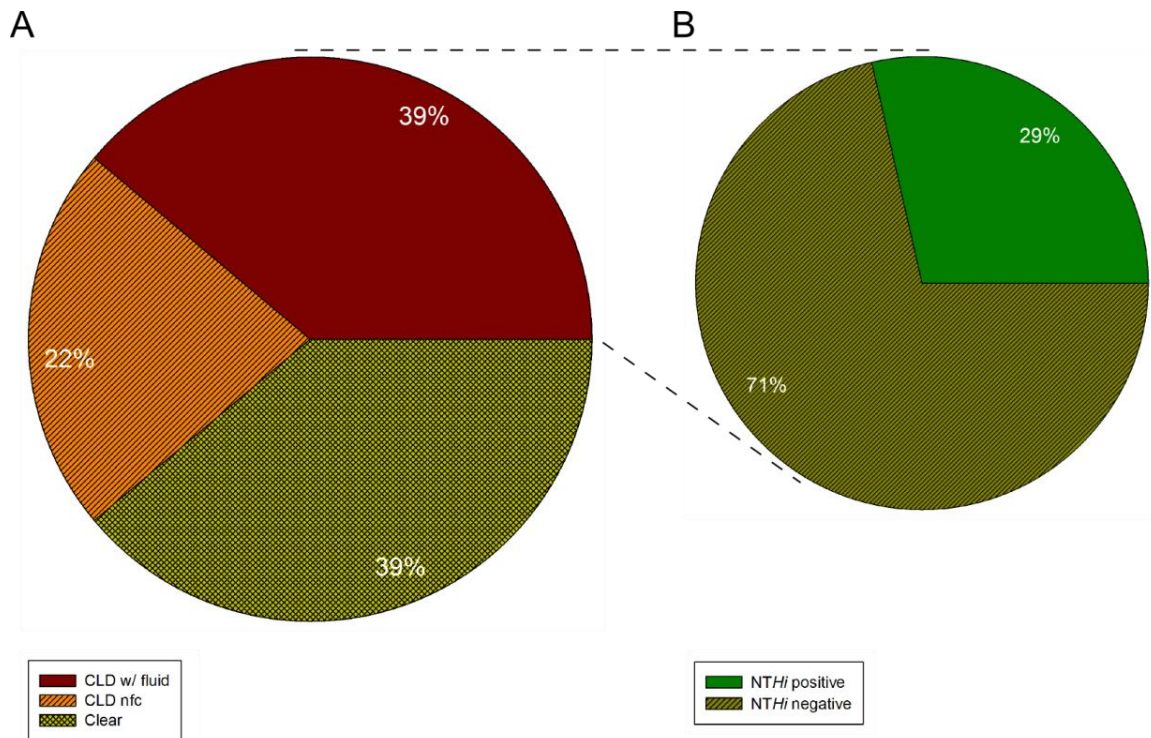


Figure 7.1| Inoculation of *Nisch^{edsn/edsn}* mice with NTHi isolate #162 at 6wk.

(A) The presence or absence of samplable fluid, due to OM, in *Nisch^{edsn/edsn}* ears was differentiated by three groups; cloudy ear with samplable fluid (CLD w/ fluid), cloudy ear with no fluid collected (CLD nfc) and clear ear with no fluid present (Clear). **(B)** Percentage of NTHi positive or negative cultures from sampled ME fluid (CLD w/ fluid).

(A) n = 18; (B) n = 7.

Due to the low infection rate observed when inoculated at 6wk and that infection with NTHi seems dependant on the collection of samplable ME exudates, I decided to investigate the onset and development of ME fluid in *Nisch^{edsn/edsn}* mice. To find the optimum age to inoculate *edison* mice with NTHi, the presence of samplable fluid in the ME and the mean amount of fluid collected across a time course was recorded in *Nisch^{edsn/edsn}* mice (Figure 7.2).

In *Nisch^{edsn/edsn}* mice there was a clear trend with increasing presence of samplable ME fluid from 3wk onwards (Figure 7.2A). The trend seen in ME fluid mirrored that seen in section 4.2.4.2, Figure 4.13, for the incidence of OM in *Nisch^{edsn/edsn}* mice across a time course. At 6wk, only 25% of *Nisch^{edsn/edsn}* ears had samplable ME fluid. The presence of ME fluid increased throughout the time course, where at 12wk 58%

of ears had a samplable amount of fluid and by >20wk it was at 66%. The mean amount of fluid collected from *Nisch^{edsn/edsn}* ears showed no significant difference across the time course ($P = 0.725$; Figure 7.2B).

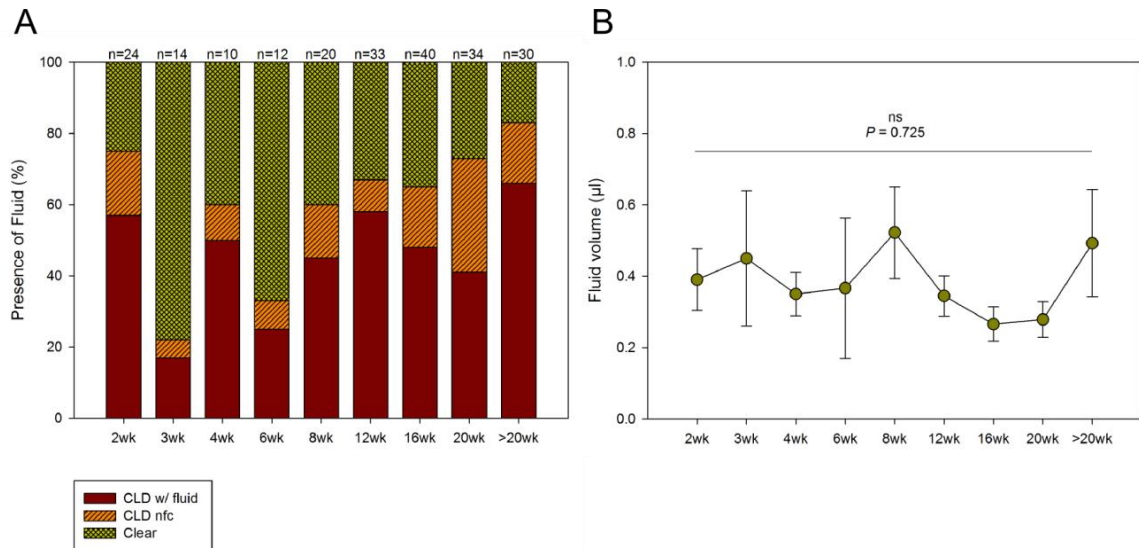


Figure 7.2| Presence of samplable ME exudate in *Nisch^{edsn/edsn}* ears across a time course.

(A) The presence or absence of samplable fluid, due to OM, in *Nisch^{edsn/edsn}* ears was differentiated by three groups; cloudy ear with samplable fluid (CLD w/ fluid), cloudy ear with no fluid collected (CLD nfc) and clear ear with no fluid present (Clear). **(B)** There was no significant change at any time point in the mean fluid volumes sampled from 'CLD w/ fluid' *Nisch^{edsn/edsn}* ears. ns $P > 0.05$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis. 'CLD w/ fluid' 2wk n = 16, 3wk n = 3, 4wk n = 5, 6wk n = 3, 8wk n = 9, 12wk n = 19, 16wk n = 19, 20wk n = 14, >20wk n = 20.

To increase the proportion of samplable ME exudates from *Nisch^{edsn/edsn}* ears and in order to avoid the more complex microbial communities of the ME in older mice, the same pilot infection experiment was repeated using mice at 12wk. This later time point resulted in an increase in the presence of samplable exudate in the ME, up to 71% (Figure 7.3A). However, the presence of the bacterial species *Proteus* (a nasopharyngeal commensal in mice) was confounding the reading of plates through

its mass overgrowth (swarm) in a large number of cases (Figure 7.3D). For ears where ME fluid was collected there was a slight increase in infection with NTHi, where twelve ME fluid samples from *Nisch^{edsn/edsn}* mice produced culturable NTHi at an infection rate of 0.400. *Proteus* contamination confounded the results from ten ME fluid samples collected (33%).

The number of NTHi colonies cultured from infected ME fluids was counted to measure the bacterial titers. Of the ME fluids with culturable NTHi, there was no significant difference in the bacterial titers between 6wk and 12wk data, where mice inoculated at 6wk had a median of 66,000 colony forming units per μl (CFU/ μl) and mice inoculated with NTHi at 12wk had a median of 88,000 CFU/ μl ($P = 0.927$; Figure 7.3E).

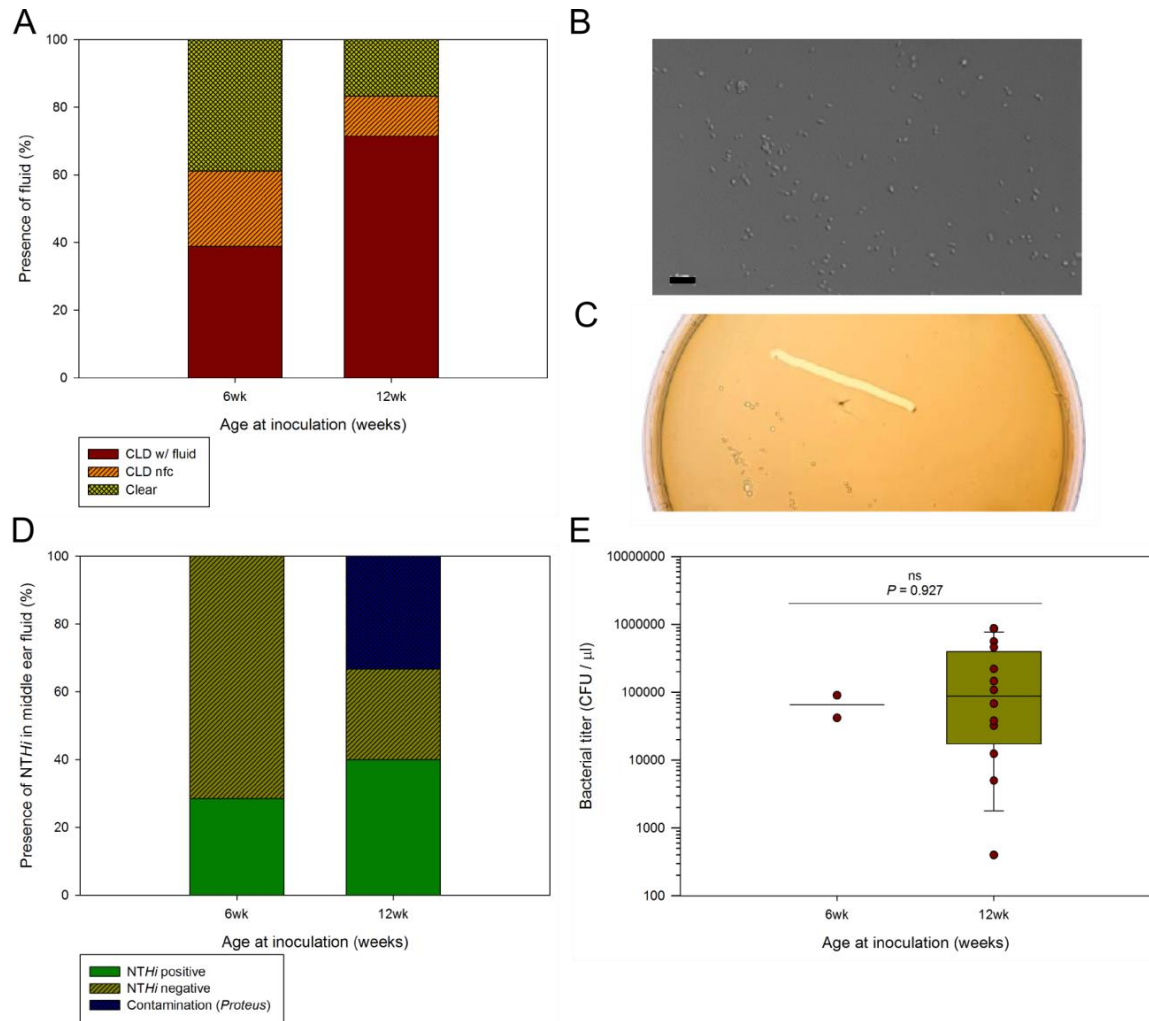


Figure 7.3| Inoculation of *Nisch*^{edsn/edsn} mice with NTHi isolate #162 at 6 and 12wk.

(A) The presence or absence of samplable fluid, due to OM, in *Nisch*^{edsn/edsn} ears. **(B)** DIC image of NTHi. Scale bar = 5 μ m. **(C)** BHI-LEV agar plate contaminated with a *Proteus* swarm. The line shows where *Proteus* has been removed. **(D)** Histogram showing the presence of NTHi from sampled ME fluid (CLD w/ fluid). The percentage of NTHi positive or negative cultures and those with the presence of the swarming bacteria species *Proteus*. **(E)** Bacterial titers from NTHi positive cultures show no significant difference between 6 and 12wk. Boxes show the median, 25th and 75th percentiles. Error bars show the 10th and 90th percentiles, and circles show individual data points. ns $P > 0.05$. Statistics were conducted using a Mann Whitney Rank Sum test.

(A) 6wk n = 18; 12wk n = 42. (D) 6wk n = 7; 12wk n = 30. (E) 6wk n = 2; 12wk n = 12.

7.2.1.2 Inoculation experiments using NTHi Streptomycin resistant isolate #162 (#162^{SR})

In order to assess the onset and progression of NTHi infection in *edison* animals, mice were inoculated via intranasal inoculation with NTHi #162^{SR} at 12wk. Ear fluids

obtained from all mice were spread onto agar plates and colonies of NTHi counted. Any ears without ME fluid were washed with PBS and cultured for detection of NTHi. As the mice were intranasally inoculated, the NP was also washed with PBS and cultured on agar plates to assess the presence of NTHi. Samples were taken across a time course with cohorts of age matched littermates at 1, 7 and 21 days post inoculation (D1, D7 and D21 respectively). To prevent the loss of data due to overgrowth on plates from *Proteus*, it was decided that NTHi #162^{SR} would be used in all future experiments as antibiotic additives in agar plates should reduce undesirable contamination. The presence of streptomycin in the plates eliminated all *Proteus* contamination.

As seen in the previous section, no wild-type or *Nisch*^{edsn/+} animals had culturable NTHi from the ME, which was replicated at each time point analysed. Interestingly, the nasal washes from *Nisch*^{+/+} and *Nisch*^{edsn/+} mice showed evidence of NTHi infection at D1, although at a significantly reduced infection rate compared to nasal washes from *Nisch*^{edsn/edsn} ($P = 0.033$; Figure 7.4A). Nasal washes from *Nisch*^{+/+} animals at D1 were infected by NTHi at a rate of 0.667, with an infection rate of 0.966 seen in *Nisch*^{edsn/edsn}. By D7 and D21, no wild-type or *Nisch*^{edsn/+} animals had culturable NTHi from nasal washes. However, NTHi infection persisted in nasal washes from *Nisch*^{edsn/edsn} animals at D7 and D21, with infection rates of 0.821 and 0.267 respectively (D7, $P < 0.001$ and D21, $P = 0.190$ compared to wild-type).

Of the nasal washes with culturable NTHi at D1, there was a significant difference in the bacterial titers between *Nisch*^{edsn/edsn} and wild-type littermates, where *Nisch*^{+/+} mice inoculated with NTHi had a mean of 85 (± 24.7) CFU/ml and *Nisch*^{edsn/edsn} mice had a mean of 1,323 (± 407.2) CFU/ml ($P < 0.001$; Figure 7.4B). There was no significant difference in bacterial titer observed between *Nisch*^{edsn/+} and wild-type mice. At D7, the mean bacterial titer found in *Nisch*^{edsn/edsn} nasal washes increased to 2,289 (± 893.8) CFU/ml and at D21, this had decreased to 215 (± 149.7) CFU/ml.

However, there was a large decrease in the mean bacterial titer found in *Nisch^{edsn/edsn}* nasal washes at D21, there was no significant difference in the bacterial titers in *Nisch^{edsn/edsn}* nasal washes across the time course ($P = 0.150$).

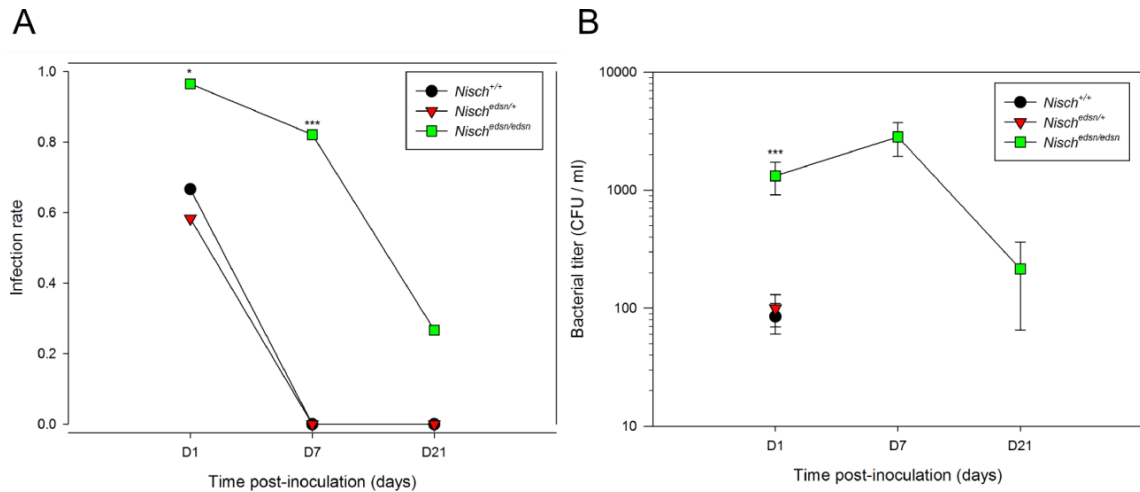


Figure 7.4| Nasal wash of *edison* mice inoculated with NTHi isolate #162^{SR} across a time course.

(A) NTHi infection rate of nasal wash, with PBS, shows there was a significantly higher infection rate of NTHi in nasal washes from *Nisch^{edsn/edsn}* NP, which persists to 21 days post inoculation. **(B)** Bacterial titers from NTHi positive cultures from *Nisch^{edsn/edsn}* nasal washes are significantly higher than what is observed in *Nisch^{+/+}* and *Nisch^{edsn/+}* littermates. * $P < 0.05$; *** $P < 0.001$. Statistics were performed using Kruskal-Wallis tests and Dunn's multiple comparison tests for post-hoc analysis.

Nisch^{+/+} D1 n = 12, D7 n = 12, D21 n = 11; *Nisch^{edsn/+}* D1 n = 12, D7 n = 12, D21 n = 12; *Nisch^{edsn/edsn}* D1 n = 29, D7 n = 28, D21 n = 15.

At all three time points, wild-type and *Nisch^{edsn/+}* animals never produced culturable NTHi from the ME after culture of ME washes with PBS. The ME exudate collected from *Nisch^{edsn/edsn}* mice showed evidence of NTHi infection at D1, although at a significantly reduced infection rate compared to nasal washes from *Nisch^{edsn/edsn}* ($P < 0.001$; Figure 7.5A). ME fluids from *Nisch^{edsn/edsn}* animals at D1 were infected by NTHi at a rate of 0.483. NTHi infection persisted in ME fluids sampled from *Nisch^{edsn/edsn}*

animals at D7 and D21, with infection rates of 0.533 and 0.333 respectively (D7, $P = 0.040$ and D21, $P = 1.000$ compared to nasal washes).

Of the ME fluids sampled with culturable NTHi at D1, the bacterial titers from *Nisch^{edsn/edsn}* mice had a mean of 61,193 ($\pm 23,798.7$) CFU/ μ l (Figure 7.4B). At D7, the mean bacterial titer found in *Nisch^{edsn/edsn}* ME exudates increased to 126,817 ($\pm 54,002.5$) CFU/ μ l and at D21, this had decreased to 15,360 ($\pm 9,828.0$) CFU/ μ l. However, there was a large decrease in the mean bacterial titer found in *Nisch^{edsn/edsn}* ME fluids at D21, there was no significant difference in the bacterial titers in *Nisch^{edsn/edsn}* ME fluids across the time course ($P = 0.534$). The bacterial titers recorded from *Nisch^{edsn/edsn}* ME fluids were, however, significantly larger at each time point compared to those cultured from *Nisch^{edsn/edsn}* nasal washes ($P < 0.001$).

In *Nisch^{edsn/edsn}* mice inoculated with NTHi #162^{SR} the presence of samplable ME fluid was consistently replicated at each time point (Figure 7.5C) At D1, 50% of *Nisch^{edsn/edsn}* ears had samplable ME fluid, at D7 54% of ears had a samplable amount of fluid and at D21 it was 50% of *Nisch^{edsn/edsn}* ears. The mean amount of fluid collected from *Nisch^{edsn/edsn}* ears showed no significant difference across the time course ($P = 0.389$; Figure 7.5D).

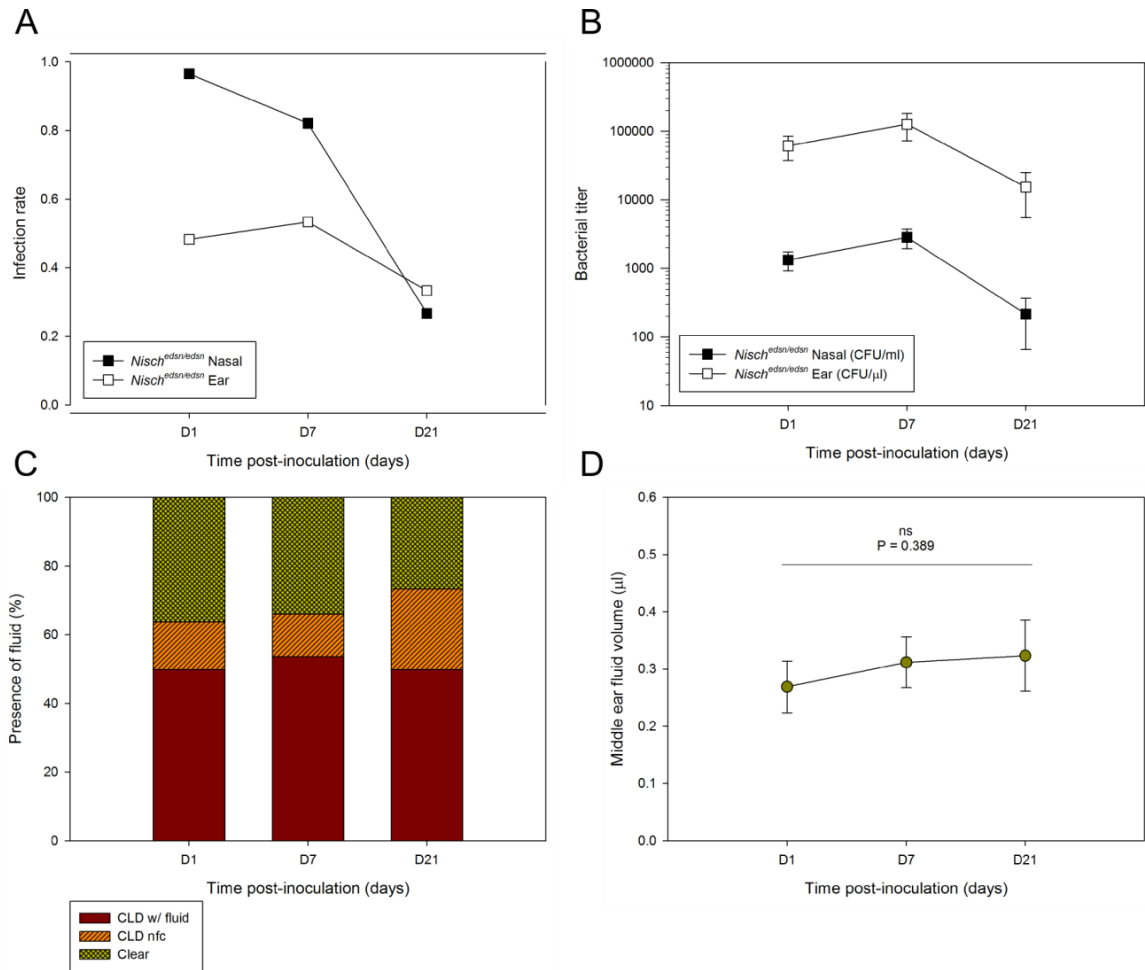


Figure 7.5| Inoculation of *Nisch^{edsn/edsn}* mice with NTHi isolate #162^{SR} across a time course.

(A) NTHi infection rate of sampled *Nisch^{edsn/edsn}* ME fluid and nasal wash. **(B)** Bacterial titers from NTHi positive cultures from *Nisch^{edsn/edsn}* ME fluid and nasal wash. **(C)** The presence or absence of samplable fluid, due to OM, in *Nisch^{edsn/edsn}* ears. **(D)** There was no significant difference in the mean fluid volumes sampled from *Nisch^{edsn/edsn}* ears across the time course. (C) D1 n = 58; D7 n = 56; D21 n = 30. Statistics were performed using a Kruskal-Wallis test.

I explored whether the NTHi infection seen in the ME exudates was dependent on total fluid volume sampled. The infection rate was calculated for each time point post-inoculation for sampled ME fluid volumes of 0.1, 0.25, 0.5, 0.75, 1 and 1.25 μ l (Figure 7.6A). There was a strong trend to show that the ME infection rate increases when a greater volume of exudate was sampled, which was almost significant ($P = 0.061$). Only 8/101 (7.9%) ME exudates sampled were greater than 0.5 μ l in volume; therefore I decided to analyse the mean infection rate for sampled ME fluid

volumes of 0.1, 0.25 and 0.5 μ l (Figure 7.6B). ME fluid volumes of 0.5 μ l showed a significant increase in NTHi infection rate compared to both 0.25 and 0.1 μ l samples ($P = 0.019$ and $P < 0.001$ respectively). A significant difference was also observed between ME fluid samples of 0.1 and 0.25 μ l ($P = 0.032$). The average infection rate in 0.1 μ l samples of ME fluid was 0.287 (± 0.0271). In ME fluid volumes of 0.25 μ l the mean infection rate was 0.469 (± 0.0556), and with 0.5 μ l samples the mean was 0.674 (± 0.0625).

To show the effect the changes in NTHi infection rate with increasing ME fluid volume had on the post-inoculation time course, I analysed the data again firstly omitting all data from 0.1 μ l samples and then analysing after omitting data from 0.1 and 0.25 μ l samples (Figure 7.6C). There was a strong trend showing increased infection rates across the post-inoculation time course when low volume ME fluids were excluded from the analysis, however there was no significant difference observed.

Interestingly, even though the NTHi infection rate seen in ME exudates was dependent on total fluid volume sampled, there was no significant difference in the NTHi bacterial titers from ME exudates when compared between amounts of fluid sampled ($P = 0.339$; Figure 7.6D).

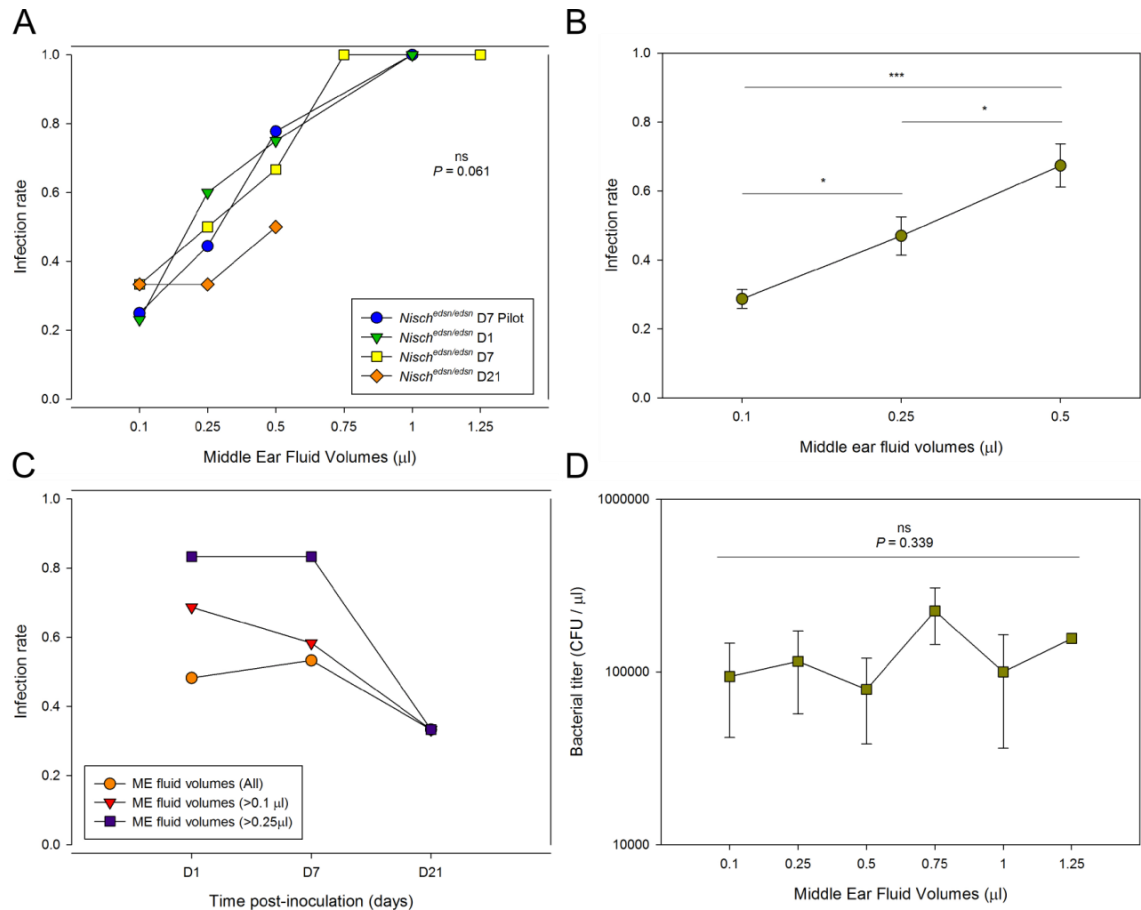


Figure 7.6| Analysis of ME fluid volume sampled on infection rate and bacterial titer in *Nisch^{edsn/edsn}* mice inoculated with NTHi isolate #162^{SR}.

(A) NTHi infection rate of *Nisch^{edsn/edsn}* ME fluid from all time points shows a strong trend where increased volume sampled, increases the infection rate observed. **(B)** Mean infection rate of NTHi shows significant differences between 0.1 μ l, 0.25 μ l and 0.5 μ l ME fluid volumes. **(C)** Change seen in NTHi infection rate of *Nisch^{edsn/edsn}* ME fluid when low ME volumes omitted from analysis. **(D)** Bacterial titers from NTHi positive cultures show no significant difference between volumes of ME fluid sampled. ns $P > 0.05$; * $P < 0.05$; *** $P < 0.001$. Data in panels A and D were analysed by Kruskal-Wallis tests. Data in panel B was normally distributed and a one-way ANOVA was performed followed by Holm-Sidak's multiple comparison test for post-hoc analysis.

7.3 Discussion

It is well documented in humans that NTHi is a common bacterial strain isolated from children with OM, and use of pneumococcal vaccines in recent years has led to an increase in cases of AOM owing to NTHi from 41% to 56% (Block, Hedrick et al. 2004). In mice, NTHi challenge is commonly used to generate mouse models with AOM (Sabirov and Metzger 2008). Direct inoculation of bacteria into the ME, either by transbullar or transtympanic injection, can cause OM; although direct ME inoculation is an artificial route of infection as it bypasses colonisation of the NP. Using intranasal inoculation, initial bacterial colonisation occurs in the NP, with the infection ascending the ET, leading to colonisation in the MEC and thus resembling the disease process in humans. However, with intranasal inoculation in mice development of ME infection is often sporadic (Ryan, Ebmeyer et al. 2006). The *Junbo* NTHi infection model provides a robust model with high infection rates and bacterial titers (Hood, Cheeseman and Brown, data not published). In this chapter I have shown that *edison*, a mouse model of OM that spontaneously develops chronic ME disease, has significant potential for ME infection studies in addition to the *Junbo* NTHi infection model. Here, intranasal inoculation of *Nisch*^{edsn/edsn} mutants resulted in efficient ascension of NTHi to the ME.

Initial experiments inoculating 6 week old mice with NTHi #162 were confounded by a reduced incidence of OM in *Nisch*^{edsn/edsn} mice. This was improved by using older mice at 12wk, however results were still confounded by the presence of the bacterium *Proteus* which swarmed over plates. *Proteus* is a commensal bacterium found in the NP of mice and is present in around 21% of mice in our animal facility. As there were concerns that the older age of the mice used here for infection challenge perhaps meant that they had a more complex bacterial ecology, I decided to use an NTHi Streptomycin resistant strain for inoculation and Streptomycin selective plates for bacterial growth. This resulted in elimination of the presence of this swarming bacterium, *Proteus*.

A single intranasal inoculation of *Nisch*^{edsn/edsn} mutants with NTHi resulted in bacterial colonisation of the NP, with the infection rapidly ascending the ET, leading to colonisation in the MEC. NTHi infection was detected in bulla fluids at D1, with more than 30% of individual ME infected up to D21. Infection of the NP by NTHi also persisted up to D21, where more than 25% of nasal washes were NTHi culture positive. Bacterial titers from *Nisch*^{edsn/edsn} bulla fluids peaked at D7, between 10⁴ and 10⁵ cfu/μl, and were comparable to those observed in the *Junbo* infection model (Hood, Cheeseman and Brown, data not published). In contrast, intranasal inoculation with NTHi did not result in bacterial ascension to the ET and colonisation of the ME in wild-type or *Nisch*^{edsn/+} mice. The presence of a rich suppurative inflammatory fluid was a critical requirement for NTHi infection in the ME space. Additionally, the NTHi infection rates in *edison* ME exudates were dependent on total fluid volume sampled. Higher infection rates were observed with increasing volumes of ME fluid sampled, further supporting the importance of pre-existing ME bulla inflammatory fluid for NTHi infection. Perturbation of signalling pathways that contribute to the inflammatory OM phenotype in the *edison* mouse, via impaired NISCH, may also play a role in the susceptibility to NTHi infection.

Bacterial infection of the ME by intranasal inoculation of the *edison* mouse has been validated as a robust and reproducible alternative to the *Junbo* infection model identified at MRC Harwell (Hood, Cheeseman and Brown, data not published). These novel infection models will help facilitate further investigation of host-microbial interactions that influence the carriage and infection of human otopathogens. Additionally, with no effective vaccine against NTHi currently licensed, these novel infection models could be used to identify improved means to treat or prevent OM through screening of candidate NTHi vaccine antigens.

CHAPTER 8: Discussion

8.1 Overview

8.1.1 The importance of OM research

The ability to hear properly as a child is a vital means by which to learn speech and language. Children susceptible to recurrent ear infections, with prolonged periods of time with ME effusion, can suffer serious delays in development due to interference with language acquisition (Teele, Klein et al. 1984). Conductive hearing loss as a consequence of OM can therefore be distressing and have huge implications for healthcare systems globally, with an associated social and medical cost (Monasta, Ronfani et al. 2012). OM also has associated morbidity levels in developing countries that are comparable with conditions such as polio and trachoma (WHO 1993). Together, there is a requirement for a greater level of understanding regarding the molecular pathways contributing to OM pathology so that new therapeutics can be developed. The use of appropriate animal models, in particular mouse models, to aid in the understanding of OM pathogenesis is fundamental for future therapeutic development. This thesis outlines experiments undertaken to comprehensively characterise the novel OM mouse mutant *edison*; identify and validate *Nisch* as the causative gene; investigate the genetic and molecular roles of NISCH, in particular its association with ITGA5; and explore the potential use of *edison* as a model for bacterial challenge with human otopathogens.

8.1.2 Summary of results

The results presented in this thesis demonstrate that the *edison* mutant spontaneously develops COM, often with a dense granulocyte-rich effusion, resulting in an associated conductive hearing loss. This novel mutant shares important features with COM found in human disease, and hence provides an important genetic disease model of COM. Additionally, emphysematous lungs characterised by an

increased alveolar airspace are observed in *edison* mice, causing a reduced ventilatory response under hypoxic-hypercapnic conditions. Data presented here replicates previously published data showing upregulation of genes associated with inflammation and hypoxia in ME fluids from *edison* mice, comparable to *Junbo* and *Jeff* mouse models (Cheeseman, Tyrer et al. 2011). I have demonstrated here the identification of *Nisch* as the causative gene in *edison* mice. The characterisation of additional *Nisch* alleles re-derived from the ENU-DNA archive at MRC Harwell revealed the identification of an additional mouse model of COM. *Nisch*^{V33A/V33A} animals develop a milder form of the ME inflammatory disease compared to *Nisch*^{edsn/edsn} mice, resulting in better auditory function and limited disease progression. Complementation testing between the *Nisch*^{V33A} and *Nisch*^{edsn} alleles further validated *Nisch* as the causative gene in *edison* mice. The generation of compound mutants with the *Nisch*^{edsn} mutation and the *Itga5* targeted null mutation revealed that cumulative deficits in both NISCH and ITGA5 further exacerbated the OM phenotype seen in *edison*, indicating a significant functional interaction. Analysis of downstream pathways identified increases in expression of RAC1 and p-SMAD2, highlighting RAC1 and TGF- β signalling in the development of COM. Finally, *NTHi* infection of the ME by intranasal inoculation of *Nisch*^{edsn/edsn} mice has been validated as a robust and reproducible infection model that has the potential to facilitate further investigation into developing new therapeutics for OM.

8.2 Contribution to the field

Rodents have long been used in OM research, traditionally using larger mammals such as chinchilla because the large bulla affords easy access to the ME and serial sampling of the ME fluid during the lifetime of the animal (Bakaletz 2009). In recent years the mouse has become the preferred model for OM research (Ryan, Ebmeyer et al. 2006; Zheng, Hardisty-Hughes et al. 2006; Rye, Bhutta et al. 2011), due to its

utility in exploring the genetic basis of human disease (Rosenthal and Brown 2007). The mouse is the current preferred genetic model organism owing to several unique advantages compared to other animal models. Firstly, determination of the mouse genome sequence indicates that 99% of mouse genes have human homologues (Brown and Hancock 2006); second, the mouse shares many developmental, biochemical and physiological processes with human; thirdly, the ease of genetic manipulation due to a large repertoire of techniques available and relatively short generation times (Brown, Wurst et al. 2009); and finally, the functional and anatomical similarities between human and mouse ears make the mouse an ideal model for hearing research and OM (Brown, Hardisty-Hughes et al. 2008).

8.2.1 Nischarin as novel gene in OM susceptibility

The development and persistence of COM is multi-factorial with a significant genetic component. Despite the importance of the disease, many of the genes involved in OM susceptibility have still yet to be identified. At present the use of mouse models seems the most promising method to identify candidate loci underlying susceptibility to COM (Rye, Bhutta et al. 2011). The ENU-induced mouse model, *edison*, was discovered in a recessive G₃ mutagenesis screen at MRC Harwell and displayed spontaneous COM. Next-generation sequencing was utilised and a putative functional mutation was identified in a relatively unknown gene, *Nisch*. The mutation in exon 16 of *Nisch* gives rise to a non-synonymous Leu972Pro substitution and is predicted to have a biological impact on the protein function. DNA and sperm archives derived from ENU mutagenesis programmes (Quwailid, Hugill et al. 2004) were utilised to identify additional alleles at the *Nisch* locus. Preliminary characterisation of the *Nisch*^{V33A} allele and generation of compound mutants with the *Nisch*^{edsn} and *Nisch*^{V33A} mutations revealed that both *Nisch*^{V33A/V33A} and *Nisch*^{V33A/edsn} mice display an OM

phenotype that shows a good resemblance to the *edison* mouse, validating *Nisch* as the causative gene.

NISCH is a highly conserved protein across mammalian species, consisting of an N-terminal phox homology (PX) domain, five putative leucine-rich repeats (LRRs), a predicted coiled-coil domain, a proline-rich region (PRR) and a long C-terminal region. NISCH has a multitude of interacting partners, including ITGA5 (Alahari, Lee et al. 2000), PAK1 (Alahari, Reddig et al. 2004), Rac1 (Reddig, Xu et al. 2005; Kuijl, Pilli et al. 2013), LIMK1 (Ding, Milosavljevic et al. 2008), Rab14 (Kuijl, Pilli et al. 2013) and LKB1 (Jain, Baranwal et al. 2013). Data presented in this thesis implicates *Nisch* as a potential candidate loci underlying susceptibility to COM. Given the interaction of NISCH with its many interacting proteins, polymorphisms at some of these genes could also conceivably be relevant.

8.2.2 The *edison* mouse as a model for chronic OM

A major motivation for studying murine models of disease is to understand the pathogenesis of human disease. Models of COM have been identified from mouse mutagenesis programs, including gene-driven knockout approaches (Chen, Ingham et al. 2013; Tateossian, Morse et al. 2013) and phenotype-driven ENU mutagenesis screens (Hardisty, Erven et al. 2003; Parkinson, Hardisty-Hughes et al. 2006). Most of these models are syndromic and display a multitude of anatomical and functional deficits as well as ME inflammation, and mirror the phenotypes of some human syndromes. Mouse models of chronic spontaneous OM that do not have an association to a known human syndrome could be considered of greater importance in implicating potential roles for pathways involved in driving chronic inflammation. The number of mouse models of non-syndromic OM is currently small. My data

shows that *edison* mice do not show the wide-ranging pleiotropic effects often associated with ME inflammatory disease in other models.

In humans, the histopathological features of COM are the presence of inflammatory granulation tissue, newly formed capillaries and ME metaplasia, sometimes with an active suppuration and polyps (Meyerhoff, Kim et al. 1978; Salvinelli, Trivelli et al. 1999). A mild to moderate conductive hearing loss usually accompanies COM (WHO 2004). The *edison* mouse develops spontaneous COM with an associated moderate hearing impairment. Typical changes due to COM occur in the MEC mucoperiosteum of *edison* mice; including edematous polyps, stromal edema, and increased lymphatics with variable numbers of infiltrating neutrophil leukocytes. Another common feature of OM is mucus hyper-secretion, with studies finding that MUC5B is the predominant mucin glycoprotein in OME and COM (Elsheikh and Mahfouz 2006; Preciado, Goyal et al. 2010). COM in *Nisch^{edsn/edsn}* mice is also associated with increased mucin-secreting goblet cell populations in the ET. The incidence of OM in *Nisch^{edsn/edsn}* mice is also progressive, becoming more prevalent in older mice and is comparable to other non-syndromic mouse models of COM.

A characteristic sequela of OM is the accumulation of fluid and inflammatory cells in the bulla and mucosal inflammation. This is observed in *edison* mice with large populations of foamy macrophages present within the *Nisch^{edsn/edsn}* ME exudate. Sampling of ME fluids revealed upregulation of genes associated with inflammation and hypoxia in *edison* mice. VEGF facilitates angiogenesis leading to increased vascular permeability and recruitment of neutrophils and macrophages (Angelo and Kurzrock 2007; Nagy, Benjamin et al. 2008). Similar to the observed upregulation of *Vegfa* in *edison* ME exudates, VEGF mRNA and protein are detectable in bulla fluids of patients with OME (Jung, Kim et al. 1999; Sekiyama, Ohori et al. 2011). Upregulation of *Tnfa* was observed in *edison* ME exudates, and notably candidate gene studies identified that TNF α polymorphisms are associated with increased risk

for OM susceptibility in humans (Patel, Nair et al. 2006; Emonts, Veenhoven et al. 2007). Additionally, the Low Oxygen in Otitis Media (LOOM) study was set up to collect glue ear samples from children undergoing grommet insertion for COME. Gene expression profiles, cytology and protein content within these samples were examined, focusing specifically on hypoxia signalling pathways and their similarities to mouse models of COM discovered at MRC Harwell (Tyrer et al. data not published). Gene expression analysis of ME fluids revealed a similar pattern of gene expression between humans and the mouse models studied at MRC Harwell, including *Jeff*, *Junbo* and *edison*. This further supports *edison* as an excellent disease model for COM.

OM is a condition that comprises a continuum of related ME diseases, as simplified by Rye *et al.* in a phenotype map (Figure 8.1A) (Rye, Bhutta et al. 2011). In reality, the presence of one form of OM in an individual is associated with a coincident or subsequent risk of presentation of another form of OM (Rosito, da Costa et al. 2007; Selaimen da Costa, Rosito et al. 2008). Clinically defined OM phenotypes display differing histological features, although OM actually occurs on a histological continuum (Paparella, Schachern et al. 1990; Yoon, Paparella et al. 1990). Histopathological analysis of the onset and progression of OM in *edison* mice displayed a similar histological continuum, which can be applied to a phenotype map (Figure 8.1B). The phenotype map presented in this thesis creates a framework for the progression of OM in *edison* mice. This revised map may allow a better understanding of the spectrum of ME inflammation and the interrelationship of its various phenotypes.

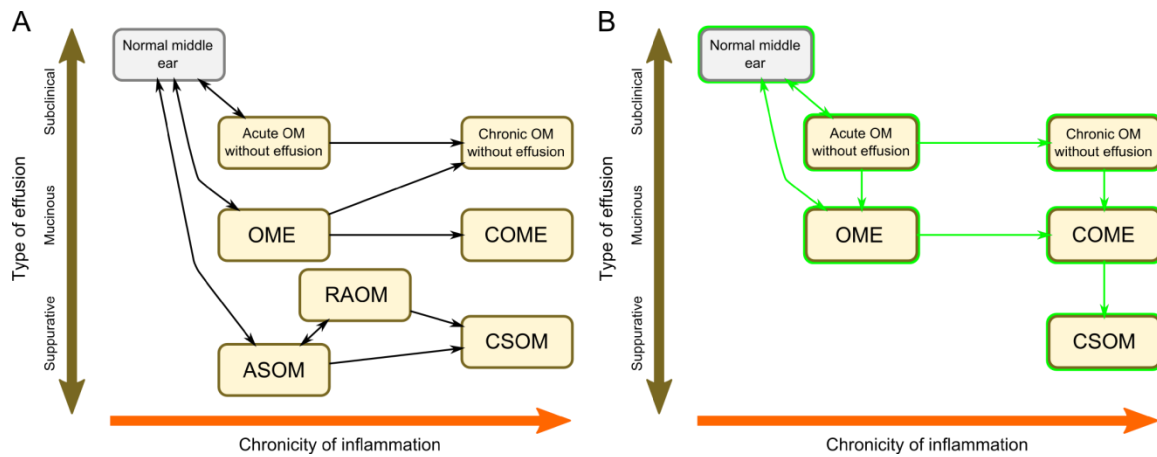


Figure 8.1| A phenotype map of OM in *edison* mice.

(A) OM is a continuum of related ME conditions as shown in this phenotype map taken from Rye *et al.*, 2011. **(B)** Revised phenotype map of OM from observations in the *edison* mouse (green). OME, OM with effusion; COME, chronic OM with effusion; RAOM, recurrent acute OM; ASOM, acute suppurative OM; CSOM, chronic suppurative OM.

8.2.3 Proposed mechanism for inflammation in *edison* mice

In SPF conditions the *edison* mouse develops a spontaneous COM, however it is not clear what the initial trigger is that leads to OM. It is probable that there is an initial ascension of a bacterial species, or a respiratory irritant, to the ME from the ET that causes an acute inflammatory event that develops into COM. Similarly, the *Junbo* mouse develops a spontaneous COM under SPF conditions, however *Evi1*^{Jbo/+} mice still develop COM under germ-free conditions (Cheeseman et al. data not published). This suggests that bacterial load is not the only contributing factor in the development of COM in the *Junbo* mouse. This is likely also true of the *edison* mouse.

It can be postulated that in response to a bacterial pathogen or some environmental insult the immune system recruits leukocytes to the site of infection. Additionally, thickening of the ME mucosa and alterations in the adhesive properties of the endothelium occurs, leading to enhancements in local blood flow and recruitment of additional leukocytes. Leukocytes produce large numbers of cytokines that further

contribute to inflammation, such as TNF α . In normal circumstances, elimination of the pathogen would lead to resolution of the acute inflammation. However, it seems possible that the *Nisch* mutation in *edison* mice may prevent this from occurring.

In this thesis I have shown that the cumulative effect of deficits in both NISCH and ITGA5 is enough to significantly increase expression of RAC1 and p-SMAD2 in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* ME epithelial cells, highlighting RAC1 and TGF- β /SMAD signalling in the development of OM. NISCH regulates RAC1 signaling through both PAK1 and PAK1-independent pathways (Alahari, Reddig et al. 2004; Reddig, Xu et al. 2005). RAC1 can stimulate NF- κ B activation (Perona, Montaner et al. 1997; Bonizzi and Karin 2004), which plays a central role in inflammation through its ability to induce transcription of pro-inflammatory genes (Baldwin 1996). Interaction between RAC1 and NISCH leads to repression of NF- κ B activity, and knockdown of NISCH leads to activation of NF- κ B (Reddig, Xu et al. 2005). This perturbation in RAC1 signalling possibly results in enhanced NF- κ B activity and an excessive inflammatory phenotype as a consequence of inappropriate regulation of the inflammatory response.

Furthermore, activity of macrophages and neutrophils at the inflammatory site likely contribute to hypoxia in the ME and activation of the HIF signalling cascade. The cumulative effects of NF- κ B, HIF and TGF- β signalling pathways drive VEGF-induced angiogenesis and vascular leak, resulting in accumulation of fluid into the MEC and contributing to OM pathogenesis. The acute inflammatory event then progresses into COM with continual recruitment of leukocytes from the blood stream to the ME and the progressive thickening of the ME mucosa.

Other common causes of the inflammation that contributes to the development of OM, such as defects in ET morphology and the mucociliary clearing apparatus, cannot be fully excluded. Allometric comparisons against skull length showed that *Nisch^{edsn/edsn}* mice display a mild craniofacial abnormality, resulting in a shortened

snout. However, the orientation of the ET in *edison* mice appeared normal. Further investigation of the ET morphology, using micro computed tomography (Miller, Burghard et al. 2014) or 3D reconstruction (Spauwen, Hillen et al. 1991), would be required to exclude ET dysmorphology as an alternative mechanism of inflammation. The *edison* mouse does not display any phenotypes normally associated with immotile cilia, such as left-right asymmetry or infertility due to sperm dysmotility, indicating normal cilia function. However, SEM analysis of cilia morphology and distribution in the ME of adult *Nisch^{edsn/edsn}* mice would help exclude cilia defects as an alternative cause of inflammation.

8.2.4 *NTHi* and colonisation of the ME in the *Nisch^{edsn/edsn}* mouse

NTHi is a commensal of the respiratory tract in humans and is a commonly isolated strain in human OM, accounting for the majority of cases of AOM (Block, Hedrick et al. 2004; Casey and Pichichero 2004). Colonisation of the ME with *NTHi* usually occurs after an initial URTI, as a consequence of damage to the ME mucosa which aids consequent bacterial ascension and persistence in the ME (Bakaletz 2010). In mice, *NTHi* challenge is commonly used to generate mouse models with AOM (Sabirov and Metzger 2008). It was found here that wild-type mice cannot be infected through intranasal route after a single inoculation. This supports work conducted by others where ME colonisation only occurs with multiple inoculations or direct inoculation into the ME space, either by transbullar or transtympanic injection (Sabirov and Metzger 2008). The inability here to infect wild-type mice through intranasal challenge alone led to bacterial challenge studies being conducted on *Nisch^{edsn/edsn}* mice. This mutant was a permissive host for bacterial colonisation of the ME. Culture of ME fluid and NP washes after challenge with *NTHi* demonstrated that *Nisch^{edsn/edsn}* mice had *NTHi* present in their ME and NP after challenge. The presence of a rich suppurative inflammatory fluid was a critical requirement for *NTHi* infection

in the ME space. The strong association between the incidence of ME and NP infection and the high number of *NTHi* in the ME relative to NP suggests that the ME can act as a reservoir for NP re-infection. Intranasal inoculation of *Nisch*^{edsn/edsn} mutants resulted in efficient ascension of *NTHi* to the ME, with high infection rates and bacterial titers comparable to those observed in the *Junbo* infection model (Hood, Cheeseman and Brown, data not published). Using intranasal inoculation, initial bacterial colonisation occurs in the NP, with the infection ascending the ET, leading to colonisation in the MEC and thus resembling the disease process in humans.

8.3 Future experiments

8.3.1 Elucidating the molecular mechanisms of Nischarin in *edison*

NISCH is a highly conserved protein across mammalian species, however it lacks significant homology with any known protein with a well-described function. Data from this thesis helped highlight RAC1 signalling in the development of COM. Comprehensive expression analysis of RAC signalling pathway members, including PAK, LIMK1 and NF-κB, will aid the further understanding of the molecular pathways leading to COM. It would be useful to examine this protein expression in ME epithelium from 3 week old *edison* mice, before the chronic inflammation develops. Furthermore, expression analysis in ME epithelium from *Nisch*^{V33A} and *Itga5*^{tm1Hyn/+} *Nisch*^{edsn/edsn} mice would enhance our understandings.

NISCH associates with a multitude of interacting partners, expression analysis of all its interacting partners would provide useful information regarding the biological function of NISCH in *edison* mice. Analysis of the effects that the *Nisch*^{edsn} and *Nisch*^{V33A} mutations have on NISCH binding to these interacting partners, especially RAC1 and PAK1, would be highly important in understanding the molecular

mechanism of NISCH. Additionally, direct protein–protein interactions involving PX domains have been reported, where the PX domains of SNX1, SNX2, SNX4 and SNX6 can associate with members of the TGF- β receptor family, where overexpression of SNX6 was found to downregulate TGF- β signalling (Parks, Frank et al. 2001). It would be interesting to assess whether the PX domain of NISCH is involved in any direct protein–protein interactions, particularly TGF- β signalling.

The International Knockout Mouse Consortium (IKMC) is systematically generating null mutant ES cells that provide both complete null and conditional null alleles for every protein coding gene in the mouse genome (Skarnes, Rosen et al. 2011). It would be worth exploiting this knockout mouse resource to determine the phenotype of *Nisch*-null and conditional models and compare to the *edison* phenotype. Recently, the generation and preliminary characterisation of a *Nisch*-null mutant has been published (Zhang, Zhao et al. 2013). No hearing phenotype was observed, however the mutant is not well characterised.

8.3.2 Development of emphysema in the *edison* mouse

Adult lungs in *Nisch*^{*edsn/edsn*} mice displayed an emphysematous phenotype with increased airspace. Interestingly, the severity of the adult lung phenotype mirrored the severity of the OM phenotype, whereby mice with a bilateral OM had the most severe lung phenotype and mice with no OM phenotype had an almost normal lung phenotype. Work on lung morphology was limited to a single terminal time point in this study. By conducting further experiments analysing the development of the emphysema-like phenotype over an earlier time point range it would help inform us as to the onset and progression of the phenotype. Upregulation of inflammatory genes has been observed in the ME fluid of *Nisch*^{*edsn/edsn*} mice. It would be useful to examine respiratory tract lavage fluid to assess gene expression and compare to

results observed in the ME fluids of *Nisch*^{edsn/edsn} mice, to help understand the significant association between OM and emphysema.

Lung development is completed during the neonatal period and proper development during this period is crucial for the formation of fully functional adult lungs (Shi, Bellusci et al. 2007). Similar to *edison* embryos, *Fbxo11*^{Jf/Jf} and *Evi1*^{Jbo/+} mice display defects in embryonic lung development (Tateossian, Hardisty-Hughes et al. 2009) (Tyrer et al. data not published). Abnormal lung development leads to an increased propensity towards adult lung diseases (Shi, Bellusci et al. 2007). Full characterisation of adult lungs in the other OM models at MRC Harwell would reveal whether the emphysema-like phenotype was unique to *edison* mice and similarly help elucidate the molecular mechanism involved.

Whole body plethysmography is a frequently used method due to its ease of use and non-invasive collection of data from conscious, unrestrained animals. This method was utilised to assess lung function and indicated that the lung function of *edison* mice was considerably impacted by the emphysema-like phenotype. However, controversy remains on the validity of using Penh to measure lung function (Enhorning, van Schaik et al. 1998; Petak, Habre et al. 2001; Albertine, Wang et al. 2002; Lundblad, Irvin et al. 2002; Bates and Irvin 2003; Irvin and Bates 2003; Adler, Cieslewicz et al. 2004). To further validate the findings made using Penh in this thesis, experiments should be replicated using other direct and more widely-acknowledged methods; for instance a Resistance and Compliance (RC) system (Glaab, Taube et al. 2007) or a Non-invasive Airway Mechanics (NAM) system (Pennock, Cox et al. 1979).

8.3.3 Pathway exploration

In this thesis I examined the cumulative effect that deficits in NISCH and ITGA5 had on the OM phenotype, which highlighted RAC1 and TGF- β signalling in the development of OM in *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. Interaction between RAC1 and NISCH leads to repression of NF- κ B activity, and knockdown of NISCH leads to activation of NF- κ B (Reddig, Xu et al. 2005). It would perhaps be informative to use a RAC1 or NF- κ B knockout mouse to assess the effect these deletions have on the OM phenotype in *edison* mice.

VEGF causes angiogenesis and likely causes the increased ME mucosal thickness observed in the *Nisch^{edsn/edsn}* model of OM. There are several signalling cascades that are known to induce the production of VEGF, including NF- κ B, HIF and TGF- β signalling pathways. Thus, it may be relevant to investigate these signalling cascades that stimulate VEGF, as well as VEGF signalling itself. It is also worth noting that there are other positive regulators of angiogenesis in addition to the VEGF family, including TGF- β , TGF- α , acidic fibroblast growth factors (aFGF), basic FGF (bFGF), TNF α , IL-8 and angiopoietins (Carmeliet and Collen 1998), which would also be worth investigating.

In analysing the effect of the *Nisch* mutation in *Nisch^{edsn/edsn}* I examined gene expression of ME fluids using TaqMan probes. This method only offers a glimpse into some of the genes upregulated in the *Nisch^{edsn/edsn}* mouse. It would be useful to use RNA-Seq (Cloonan and Grimmond 2008) to try and build a more comprehensive picture of the signalling cascades involved in OM in the *Nisch^{edsn/edsn}* mouse. This would potentially highlight other important pathways and molecules in this OM model and provide us with additional targets for future OM treatments.

8.3.4 Nischarin as a new candidate for OM susceptibility in humans

Causative genes identified from mouse models are beginning to be identified in the human population; such as *Fbxo11*, the causative gene in the *Jeff* mouse (Rye, Wiertsema et al. 2011). Identification of *Nisch* as the gene underlying the OM phenotype in the *edison* mouse provides a novel candidate loci that may also play a role in human susceptibility to OM. Genetic association studies in humans, using the various OM cohort studies from around the world, would confirm or reject an association of *NISCH* with susceptibility to OM.

With collaboration between MRC Harwell and NDS (Nuffield Department of Surgical Sciences, University of Oxford) the Genetics of Otitis Media Study was created as a resource for human genetic association testing. Over the past five years DNA has been collected from children under 10 years of age affected by COM, as evidenced by the need to undergo grommet surgery. To date, the Genetics of Otitis Media Study has recruited over 1,800 families, or over 7,000 individuals, and is the largest collection of DNA for OM in the world. An interim analysis of 500 families showed modest levels of association with SNPs in the loci *FBXO11* and *TGIF1* (Bhutta et al. data not published). Around 1,200 families have now been analysed, including at the *NISCH* locus, and the results are currently being examined.

Mouse models are often useful in modelling human disease and trialling therapeutics which later enter human clinical trials. However, they do not always replicate disease to a sufficient extent and can cause problems in the drug discovery pipeline. With knowledge gained from the *Nisch*^{*edsn/edsn*} mouse and understanding of the stimulated signalling pathways downstream of *NISCH* in COM, RT-qPCR could be utilised to analyse human ME fluids to see if the same signalling pathways were also present in human OM. These findings from the human population would hopefully add support to the use of the *Nisch*^{*edsn/edsn*} mouse as an excellent disease model for OM research. Additionally, results from human OM samples would help feed back into the types of

experiments conducted in the *Nisch*^{*edsn/edsn*} model and vice versa, which would greatly help improve our understanding of OM pathology and therapeutic development.

8.4 Concluding Remarks

The limited understanding regarding the precise molecular biology of AOM and COM underlies the difficulties posed when trying to develop new therapeutics for treatment. While antibiotics have helped stem the incidence of AOM in developed countries, the whole spectrum of OM ranging from acute to chronic is still a huge problem in developing countries and in certain ethnic groups worldwide. Even in the developed world, a significant number of children develop chronic forms of OM which are usually treatable only by surgery. In this thesis I have identified *edison* as an excellent model of COM and provided information regarding the role of Nischarin in the development of OM. The identification and analysis of such OM mouse models will continue to be of crucial importance to aid our understanding of the underlying molecular mechanisms and elaborate further the genetic aetiology of human ME inflammatory disease. Ultimately, our understanding of these pathways can be applied in a pharmacological and medical setting, facilitating the identification and testing of potential therapeutic targets, with the aim of treating OM.

CHAPTER 9: References

- Abida, W. M., A. Nikolaev, et al. (2007). "FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity." The Journal of biological chemistry **282**(3): 1797-1804.
- Acevedo-Arozena, A., S. Wells, et al. (2008). "ENU mutagenesis, a way forward to understand gene function." Annual review of genomics and human genetics **9**: 49-69.
- Adachi, T., H. Ogawa, et al. (2006). "Mice with blunted hypoxic ventilatory response are susceptible to respiratory disturbance during hypoxia." Tohoku J Exp Med **209**(2): 125-134.
- Adler, A., G. Cieslewicz, et al. (2004). "Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice." J Appl Physiol (1985) **97**(1): 286-292.
- Aggarwal, V. S. and B. E. Morrow (2008). "Genetic modifiers of the physical malformations in velo-cardio-facial syndrome/DiGeorge syndrome." Developmental disabilities research reviews **14**(1): 19-25.
- Agius, A. M., M. Wake, et al. (1995). "Nasal and middle ear ciliary beat frequency in chronic suppurative otitis media." Clinical otolaryngology and allied sciences **20**(5): 470-474.
- Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nature reviews. Immunology **4**(7): 499-511.
- Akira, S., S. Uematsu, et al. (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.
- Alahari, S. K. (2003). "Nischarin inhibits Rac induced migration and invasion of epithelial cells by affecting signaling cascades involving PAK." Experimental cell research **288**(2): 415-424.
- Alahari, S. K., J. W. Lee, et al. (2000). "Nischarin, a novel protein that interacts with the integrin alpha5 subunit and inhibits cell migration." The Journal of cell biology **151**(6): 1141-1154.
- Alahari, S. K., P. J. Reddig, et al. (2004). "The integrin-binding protein Nischarin regulates cell migration by inhibiting PAK." The EMBO journal **23**(14): 2777-2788.
- Albertine, K. H., L. Wang, et al. (2002). "Temporal correlation of measurements of airway hyperresponsiveness in ovalbumin-sensitized mice." Am J Physiol Lung Cell Mol Physiol **283**(1): L219-233.
- Alberts, B. (2002). Integrins. Molecular biology of the cell. New York, Garland Science.
- Alitalo, K. (2011). "The lymphatic vasculature in disease." Nature medicine **17**(11): 1371-1380.
- Allen, E. K., W.-M. M. Chen, et al. (2013). "A genome-wide association study of chronic otitis media with effusion and recurrent otitis media identifies a novel susceptibility locus on chromosome 2." Journal of the Association for Research in Otolaryngology : JARO **14**(6): 791-800.
- Allen, E. K., A. Manichaikul, et al. (2014). "Evaluation of replication of variants associated with genetic risk of otitis media." PloS one **9**(8).
- Alliston, T., T. C. Ko, et al. (2005). "Repression of bone morphogenetic protein and activin-inducible transcription by Evi-1." The Journal of biological chemistry **280**(25): 24227-24237.

- Alpay, H. C., E. O. Etem, et al. (2010). "Evaluation of the polymorphism in the Toll-like receptor 4 (TLR4) genes of tympanosclerosis patients." Auris, nasus, larynx **37**(1): 29-32.
- Alper, C. M., B. Winther, et al. (2009). "Cytokine polymorphisms predict the frequency of otitis media as a complication of rhinovirus and RSV infections in children." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery **266**(2): 199-205.
- Angelo, L. S. and R. Kurzrock (2007). "Vascular endothelial growth factor and its relationship to inflammatory mediators." Clinical cancer research : an official journal of the American Association for Cancer Research **13**(10): 2825-2830.
- Ar, A., P. Herman, et al. (2007). "Middle ear gas loss in inflammatory conditions: the role of mucosa thickness and blood flow." Respiratory physiology & neurobiology **155**(2): 167-176.
- Astrof, S. and R. O. Hynes (2009). "Fibronectins in vascular morphogenesis." Angiogenesis **12**(2): 165-175.
- Atef, A. and E. E. Ayad (2004). "Ciliary count in chronic suppurative otitis media: comparative quantitative study between mucosal and squamous types using scanning electron microscopy and image analysis." The Journal of laryngology and otology **118**(5): 343-347.
- Austin, C. P., J. F. Battey, et al. (2004). "The knockout mouse project." Nature genetics **36**(9): 921-924.
- Avraamides, C. J., B. Garmy-Susini, et al. (2008). "Integrins in angiogenesis and lymphangiogenesis." Nature reviews. Cancer **8**(8): 604-617.
- Bakaletz, L. O. (2009). "Chinchilla as a robust, reproducible and polymicrobial model of otitis media and its prevention." Expert Rev Vaccines **8**(8): 1063-1082.
- Bakaletz, L. O. (2010). "Immunopathogenesis of polymicrobial otitis media." Journal of leukocyte biology **87**(2): 213-222.
- Balasenthil, S., A. A. Sahin, et al. (2004). "p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells." The Journal of biological chemistry **279**(2): 1422-1428.
- Baldwin, A. S. (1996). "The NF-kappa B and I kappa B proteins: new discoveries and insights." Annual review of immunology **14**: 649-683.
- Baranwal, S., Y. Wang, et al. (2011). "Molecular characterization of the tumor-suppressive function of nisharin in breast cancer." Journal of the National Cancer Institute **103**(20): 1513-1528.
- Barnes, P. J. (2000). "Chronic obstructive pulmonary disease." The New England journal of medicine **343**(4): 269-280.
- Barnes, P. J. (2003). "New concepts in chronic obstructive pulmonary disease." Annual review of medicine **54**: 113-129.
- Bates, J. H. and C. G. Irvin (2003). "Measuring lung function in mice: the phenotyping uncertainty principle." J Appl Physiol (1985) **94**(4): 1297-1306.
- Beck, J. A., S. Lloyd, et al. (2000). "Genealogies of mouse inbred strains." Nature genetics **24**(1): 23-25.
- Beery, Q. C., W. J. Doyle, et al. (1980). "Eustachian tube function in an American Indian population." The Annals of otology, rhinology & laryngology. Supplement **89**(3 Pt 2): 28-33.

- Berger, G., M. Hawke, et al. (1984). "Mast cells in human middle ear mucosa in health and in disease." The Journal of otolaryngology **13**(6): 370-374.
- Bhutta, M. and M. Burton (2013). "The Genetics of Chronic Otitis Media." The Otorhinolaryngologist **6**(3): 150-159.
- Bhutta, M. F. (2011). "Chronic otitis media in mucopolysaccharidosis may not be due to Eustachian tube dysfunction." International journal of pediatric otorhinolaryngology **75**(1): 140-141.
- Bhutta, M. F. (2014). "Epidemiology and pathogenesis of otitis media: construction of a phenotype landscape." Audiology & neuro-otology **19**(3): 210-223.
- Bhutta, M. F., M. T. Cheeseman, et al. (2013). "Surveying the Down syndrome mouse model resource identifies critical regions responsible for chronic otitis media." Mammalian genome : official journal of the International Mammalian Genome Society **24**(11-12): 439-445.
- Black, S., H. Shinefield, et al. (2000). "Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group." The Pediatric infectious disease journal **19**(3): 187-195.
- Block, S. L., J. Hedrick, et al. (2004). "Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media." The Pediatric infectious disease journal **23**(9): 829-833.
- Bluestone, C. D. (1996). "Pathogenesis of otitis media: role of eustachian tube." The Pediatric infectious disease journal **15**(4): 281-291.
- Bluestone, C. D. (2003). Definitions, terminology, and classification. Evidence-based otitis media. R. M. Rosenfeld and C. D. Bluestone. Hamilton ; London, BC Decker: 120-135.
- Bluestone, C. D. and J. O. Klein (2007). Otitis media in infants and children. Hamilton, Ontario, BC Decker ; Oxford : Elsevier Science [distributor].
- Bogaert, D., C. M. Thompson, et al. (2010). "The role of complement in innate and adaptive immunity to pneumococcal colonization and sepsis in a murine model." Vaccine **28**(3): 681-685.
- Bokoch, G. M. (2003). "Biology of the p21-activated kinases." Annual review of biochemistry **72**: 743-781.
- Bonizzi, G. and M. Karin (2004). "The two NF-kappaB activation pathways and their role in innate and adaptive immunity." Trends in immunology **25**(6): 280-288.
- Bonora, M., J. F. Bernaudin, et al. (2004). "Ventilatory responses to hypercapnia and hypoxia in conscious cystic fibrosis knockout mice Cftr." Pediatr Res **55**(5): 738-746.
- Borsani, G., A. DeGrandi, et al. (1999). "EYA4, a novel vertebrate gene related to Drosophila eyes absent." Hum Mol Genet **8**(1): 11-23.
- Boswell, J. B. and T. G. Nienhuys (1996). "Patterns of persistent otitis media in the first year of life in aboriginal and non-aboriginal infants." The Annals of otology.
- Brault, V., V. Besson, et al. (2007). "Cre/loxP-mediated chromosome engineering of the mouse genome." Handbook of experimental pharmacology(178): 29-48.
- Bromberg, Y. and B. Rost (2007). "SNAP: predict effect of non-synonymous polymorphisms on function." Nucleic acids research **35**(11): 3823-3835.

- Brooks, P. C., R. A. Clark, et al. (1994). "Requirement of vascular integrin alpha v beta 3 for angiogenesis." Science (New York, N.Y.) **264**(5158): 569-571.
- Brown, S. D., P. Chambon, et al. (2005). "EMPreSS: standardized phenotype screens for functional annotation of the mouse genome." Nature genetics **37**(11): 1155.
- Brown, S. D. and J. M. Hancock (2006). "The mouse genome." Genome Dyn **2**: 33-45.
- Brown, S. D., R. E. Hardisty-Hughes, et al. (2008). "Quiet as a mouse: dissecting the molecular and genetic basis of hearing." Nature reviews. Genetics **9**(4): 277-290.
- Brown, S. D. and M. W. Moore (2012). "Towards an encyclopaedia of mammalian gene function: the International Mouse Phenotyping Consortium." Disease models & mechanisms **5**(3): 289-292.
- Brown, S. D., W. Wurst, et al. (2009). "The functional annotation of mammalian genomes: the challenge of phenotyping." Annual review of genetics **43**: 305-333.
- Bryant, J., R. J. Goodyear, et al. (2002). "Sensory organ development in the inner ear: molecular and cellular mechanisms." British medical bulletin **63**: 39-57.
- Bryson, K., D. Cozzetto, et al. (2007). "Computer-assisted protein domain boundary prediction using the DomPred server." Current protein & peptide science **8**(2): 181-188.
- Buchwald, G., E. Hostinova, et al. (2001). "Conformational switch and role of phosphorylation in PAK activation." Molecular and cellular biology **21**(15): 5179-5189.
- Burkhard, P., J. Stetefeld, et al. (2001). "Coiled coils: a highly versatile protein folding motif." Trends in cell biology **11**(2): 82-88.
- Calvert, J. A., S. G. Dedos, et al. (2011). "A missense mutation in Fgfr1 causes ear and skull defects in hush puppy mice." Mammalian genome : official journal of the International Mammalian Genome Society **22**(5-6): 290-305.
- Campbell, R. G., C. S. Birman, et al. (2009). "Management of otitis media with effusion in children with primary ciliary dyskinesia: a literature review." International journal of pediatric otorhinolaryngology **73**(12): 1630-1638.
- Capecci, M. R. (1989). "The new mouse genetics: altering the genome by gene targeting." Trends in genetics : TIG **5**(3): 70-76.
- Carmeliet, P. and D. Collen (1998). "Vascular development and disorders: molecular analysis and pathogenic insights." Kidney Int **53**(6): 1519-1549.
- Caruana, G., P. G. Farlie, et al. (2013). "Genome-wide ENU mutagenesis in combination with high density SNP analysis and exome sequencing provides rapid identification of novel mouse models of developmental disease." PLoS one **8**(3).
- Casey, J. R. and M. E. Pichichero (2004). "Changes in frequency and pathogens causing acute otitis media in 1995-2003." The Pediatric infectious disease journal **23**(9): 824-828.
- Casselbrant, M. L., E. M. Mandel, et al. (1999). "The heritability of otitis media: a twin and triplet study." JAMA : the journal of the American Medical Association **282**(22): 2125-2130.
- Casselbrant, M. L., E. M. Mandel, et al. (2009). "Otitis media: a genome-wide linkage scan with evidence of susceptibility loci within the 17q12 and 10q22.3 regions." BMC medical genetics **10**: 85.

- Casselbrant, M. L., E. M. Mandel, et al. (2004). "The genetic component of middle ear disease in the first 5 years of life." Archives of otolaryngology--head & neck surgery **130**(3): 273-278.
- Castagno, L. A. and L. Lavinsky (2002). "Otitis media in children: seasonal changes and socioeconomic level." International journal of pediatric ...
- Cayé-Thomasen, P., A. Hermansson, et al. (1995). "Changes in goblet cell density in rat middle ear mucosa in acute otitis media." The American journal of otology **16**(1): 75-82.
- Cayé-Thomasen, P., A. Hermansson, et al. (1999). "Bone modeling dynamics in acute otitis media." The Laryngoscope **109**(5): 723-729.
- Cayé-Thomasen, P. and M. Tos (2003). "Eustachian tube goblet cell density during and after acute otitis media caused by Streptococcus pneumoniae: a morphometric analysis." Otology & neurotology : official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology **24**(3): 365-370.
- Chantry, C. J., C. R. Howard, et al. (2006). "Full breastfeeding duration and associated decrease in respiratory tract infection in US children." Pediatrics **117**(2): 425-432.
- Cheeseman, M. T., H. E. Tyrer, et al. (2011). "HIF-VEGF pathways are critical for chronic otitis media in Junbo and Jeff mouse mutants." PLoS genetics **7**(10).
- Chen, J., J. S. Alexander, et al. (2012). "Integrins and their extracellular matrix ligands in lymphangiogenesis and lymph node metastasis." International journal of cell biology **2012**: 853703.
- Chen, J., N. Ingham, et al. (2013). "McpH1-deficient mice reveal a role for MCPH1 in otitis media." PloS one **8**(3).
- Chen, W.-M. M., E. K. Allen, et al. (2011). "Significant linkage at chromosome 19q for otitis media with effusion and/or recurrent otitis media (COME/ROM)." BMC medical genetics **12**: 124.
- Chen, X. L., J.-O. O. Nam, et al. (2012). "VEGF-induced vascular permeability is mediated by FAK." Developmental cell **22**(1): 146-157.
- Chen, Y.-G. G., M. H. Forsberg, et al. (2014). "Gene targeting in NOD mouse embryos using zinc-finger nucleases." Diabetes **63**(1): 68-74.
- Chen, Y., D. Yee, et al. (2000). "Genotype-based screen for ENU-induced mutations in mouse embryonic stem cells." Nature genetics **24**(3): 314-317.
- Chittka, L. and A. Brockmann (2005). "Perception space--the final frontier." PLoS biology **3**(4).
- Choi, Y., G. E. Sims, et al. (2012). "Predicting the functional effect of amino acid substitutions and indels." PloS one **7**(10).
- Churg, A., R. D. Wang, et al. (2004). "Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse." American journal of respiratory and critical care medicine **170**(5): 492-498.
- Cloonan, N. and S. M. Grimmond (2008). "Transcriptome content and dynamics at single-nucleotide resolution." Genome Biol **9**(9): 234.
- Coghill, E. L., A. Hugill, et al. (2002). "A gene-driven approach to the identification of ENU mutants in the mouse." Nature genetics **30**(3): 255-256.
- Cong, L., F. A. Ran, et al. (2013). "Multiplex genome engineering using CRISPR/Cas systems." Science (New York, N.Y.) **339**(6121): 819-823.

- Cordenonsi, M., S. Dupont, et al. (2003). "Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads." Cell **113**(3): 301-314.
- Cripps, A. W., D. C. Otczyk, et al. (2005). "Bacterial otitis media: a vaccine preventable disease?" Vaccine **23**(17-18): 2304-2310.
- Cruikshanks, K. J., T. L. Wiley, et al. (1998). "Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin the epidemiology of hearing loss study." American Journal of Epidemiology **148**(9): 879-886.
- Cureoglu, S., P. A. Schachern, et al. (2004). "Cochlear changes in chronic otitis media." The Laryngoscope **114**(4): 622-626.
- Daly, K. A., W. M. Brown, et al. (2004). "Chronic and recurrent otitis media: a genome scan for susceptibility loci." American journal of human genetics **75**(6): 988-997.
- Daly, K. A., H. J. Hoffman, et al. (2010). "Epidemiology, natural history, and risk factors: panel report from the Ninth International Research Conference on Otitis Media." International journal of pediatric otorhinolaryngology **74**(3): 231-240.
- Daly, K. A., S. S. Rich, et al. (1996). "The family study of otitis media: design and disease and risk factor profiles." Genetic epidemiology **13**(5): 451-468.
- Darrow, D. H., N. Dash, et al. (2003). "Otitis media: concepts and controversies." Current opinion in otolaryngology & head and neck surgery **11**(6): 416-423.
- Davidson, J., M. L. Hyde, et al. (1989). "Epidemiologic patterns in childhood hearing loss: a review." International journal of pediatric otorhinolaryngology **17**(3): 239-266.
- Davies, B., G. Davies, et al. (2013). "Site specific mutation of the Zic2 locus by microinjection of TALEN mRNA in mouse CD1, C3H and C57BL/6J oocytes." PloS one **8**(3).
- De, S., O. Razorenova, et al. (2005). "VEGF-integrin interplay controls tumor growth and vascularization." Proceedings of the National Academy of Sciences of the United States of America **102**(21): 7589-7594.
- de Visser, K. E., A. Eichten, et al. (2006). "Paradoxical roles of the immune system during cancer development." Nature reviews. Cancer **6**(1): 24-37.
- Dehne, N. and B. Brüne (2009). "HIF-1 in the inflammatory microenvironment." Experimental cell research **315**(11): 1791-1797.
- Depreux, F. F., K. Darrow, et al. (2008). "Eya4-deficient mice are a model for heritable otitis media." The Journal of clinical investigation **118**(2): 651-658.
- Derynck, R. and L. Rhee (1987). "Sequence of the porcine transforming growth factor-beta precursor." Nucleic acids research **15**(7): 3187.
- Dietrich, T., J. Onderka, et al. (2007). "Inhibition of inflammatory lymphangiogenesis by integrin alpha5 blockade." The American journal of pathology **171**(1): 361-372.
- Ding, Y., T. Milosavljevic, et al. (2008). "Nischarin inhibits LIM kinase to regulate cofilin phosphorylation and cell invasion." Molecular and cellular biology **28**(11): 3742-3756.
- Ding, Y., R. Zhang, et al. (2013). "Nischarin is differentially expressed in rat brain and regulates neuronal migration." PloS one **8**(1): e54563.
- Easton, D. F. and R. A. Eeles (2008). "Genome-wide association studies in cancer." Human molecular genetics **17**(R2): 15.

- Ebmeyer, J., A. Leichtle, et al. (2011). "TNFA deletion alters apoptosis as well as caspase 3 and 4 expression during otitis media." BMC immunology **12**: 12.
- Edwards, D. C., L. C. Sanders, et al. (1999). "Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics." Nature cell biology **1**(5): 253-259.
- Elsheikh, M. N. and M. E. Mahfouz (2006). "Up-regulation of MUC5AC and MUC5B mucin genes in nasopharyngeal respiratory mucosa and selective up-regulation of MUC5B in middle ear in pediatric otitis media with effusion." The Laryngoscope **116**(3): 365-369.
- Emanuel, D. C., S. Maroonroge, et al. (2009). Auditory Function: Physiology and function of the hearing system. Helmet-Mounted Displays: Sensation, Perception and Cognition Issues, U.S. Army Aeromedical Research Laboratory: 307-308.
- Emonts, M., R. H. Veenhoven, et al. (2007). "Genetic polymorphisms in immunoresponse genes TNFA, IL6, IL10, and TLR4 are associated with recurrent acute otitis media." Pediatrics **120**(4): 814-823.
- Emonts, M., S. P. Wiertsema, et al. (2007). "The 4G/4G plasminogen activator inhibitor-1 genotype is associated with frequent recurrence of acute otitis media." Pediatrics **120**(2): 23.
- Engel, J., L. Anteunis, et al. (1999). "Prevalence rates of otitis media with effusion from 0 to 2 years of age: healthy-born versus high-risk-born infants." International journal of
- Enhorning, G., S. van Schaik, et al. (1998). "Whole-body plethysmography, does it measure tidal volume of small animals?" Can J Physiol Pharmacol **76**(10-11): 945-951.
- Eriksson, P.-O. O., J. Li, et al. (2006). "Spontaneous development of otitis media in plasminogen-deficient mice." International journal of medical microbiology : IJMM **296**(7): 501-509.
- Eskola, J., T. Kilpi, et al. (2001). "Efficacy of a pneumococcal conjugate vaccine against acute otitis media." The New England journal of medicine **344**(6): 403-409.
- Faden, H. (2001). "The microbiologic and immunologic basis for recurrent otitis media in children." European journal of pediatrics **160**(7): 407-413.
- Faden, H., L. Duffy, et al. (1997). "Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics." The Journal of infectious diseases **175**(6): 1440-1445.
- Faden, H., L. Duffy, et al. (1996). "Epidemiology of nasopharyngeal colonization with nontypeable Haemophilus influenzae in the first two years of life." Acta otolaryngologica. Supplementum **523**: 128-129.
- Felding, J. U., J. B. Rasmussen, et al. (1987). "Gas composition of the normal and the ventilated middle ear cavity." Scandinavian journal of clinical and laboratory investigation. Supplementum **186**: 31-41.
- Ferrara, N. and W. J. Henzel (1989). "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells." Biochemical and biophysical research communications **161**(2): 851-858.
- Fireman, B., S. B. Black, et al. (2003). "Impact of the pneumococcal conjugate vaccine on otitis media." The Pediatric infectious disease journal **22**(1): 10-16.

- Frede, S., U. Berchner-Pfannschmidt, et al. (2007). "Regulation of hypoxia-inducible factors during inflammation." Methods in enzymology **435**: 405-419.
- Fritz, J. H. and S. E. Girardin (2005). "How Toll-like receptors and Nod-like receptors contribute to innate immunity in mammals." Journal of endotoxin research **11**(6): 390-394.
- Frost, J. A., J. L. Swantek, et al. (2000). "Stimulation of NFkappa B activity by multiple signaling pathways requires PAK1." The Journal of biological chemistry **275**(26): 19693-19699.
- Fuchs, J. C., F. A. Zinnamon, et al. (2013). "Hearing loss in a mouse model of 22q11.2 Deletion Syndrome." PloS one **8**(11).
- Gaj, T., C. A. Gersbach, et al. (2013). "ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering." Trends in biotechnology **31**(7): 397-405.
- Gates, G. A., J. O. Klein, et al. (2002). "Recent advances in otitis media. 1. Definitions, terminology, and classification of otitis media." The Annals of otology
- Gates, H., A.-M. M. Mallon, et al. (2011). "High-throughput mouse phenotyping." Methods (San Diego, Calif.) **53**(4): 394-404.
- Gentile, D. A., W. J. Doyle, et al. (2003). "Cytokine gene polymorphisms moderate illness severity in infants with respiratory syncytial virus infection." Human immunology **64**(3): 338-344.
- Giovannini, M., E. Robanus-Maandag, et al. (2000). "Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2." Genes & development **14**(13): 1617-1630.
- Glaab, T., C. Taube, et al. (2007). "Invasive and noninvasive methods for studying pulmonary function in mice." Respir Res **8**: 63.
- Gorovoy, M., J. Han, et al. (2009). "LIM kinase 1 promotes endothelial barrier disruption and neutrophil infiltration in mouse lungs." Circulation research **105**(6): 549-556.
- Gorovoy, M., T. Koga, et al. (2008). "Downregulation of LIM kinase 1 suppresses ocular inflammation and fibrosis." Molecular vision **14**: 1951-1959.
- Gurr, A., T. Stark, et al. (2009). "The ciliary beat frequency of middle ear mucosa in children with chronic secretory otitis media." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery **266**(12): 1865-1870.
- Han, F., H. Yu, et al. (2012). "Mutation in Phex gene predisposes BALB/c-Phex(Hyp-Duk)/Y mice to otitis media." PloS one **7**(9).
- Han, F., H. Yu, et al. (2009). "Otitis media in a mouse model for Down syndrome." International journal of experimental pathology **90**(5): 480-488.
- Harabuchi, Y., H. Faden, et al. (1994). "Nasopharyngeal colonization with nontypeable Haemophilus influenzae and recurrent otitis media. Tonawanda/Williamsville Pediatrics." The Journal of infectious diseases **170**(4): 862-866.
- Hardisty-Hughes, R. E., A. Parker, et al. (2010). "A hearing and vestibular phenotyping pipeline to identify mouse mutants with hearing impairment." Nature protocols **5**(1): 177-190.
- Hardisty-Hughes, R. E., H. Tateossian, et al. (2006). "A mutation in the F-box gene, Fbxo11, causes otitis media in the Jeff mouse." Human molecular genetics **15**(22): 3273-3279.

- Hardisty, R. E., A. Erven, et al. (2003). "The deaf mouse mutant Jeff (Jf) is a single gene model of otitis media." Journal of the Association for Research in Otolaryngology : JARO **4**(2): 130-138.
- He, Y., M. A. Scholes, et al. (2013). "Complement activation in pediatric patients with recurrent acute otitis media." International journal of pediatric otorhinolaryngology **77**(6): 911-917.
- Heldermon, C. D., A. K. Hennig, et al. (2007). "Development of sensory, motor and behavioral deficits in the murine model of Sanfilippo syndrome type B." PloS one **2**(8).
- Heldin, C. H., K. Miyazono, et al. (1997). "TGF-beta signalling from cell membrane to nucleus through SMAD proteins." Nature **390**(6659): 465-471.
- Hernandez, M., A. Leichtle, et al. (2008). "Myeloid differentiation primary response gene 88 is required for the resolution of otitis media." The Journal of infectious diseases **198**(12): 1862-1869.
- Hildebrand, M. S., D. Coman, et al. (2007). "A novel splice site mutation in EYA4 causes DFNA10 hearing loss." American journal of medical genetics. Part A **143A**(14): 1599-1604.
- Hilton, J. M., M. A. Lewis, et al. (2011). "Exome sequencing identifies a missense mutation in Isl1 associated with low penetrance otitis media in dearisch mice." Genome biology **12**(9).
- Hindley, C. J., G. S. McDowell, et al. (2011). "Regulation of cell fate determination by Skp1-Cullin1-F-box (SCF) E3 ubiquitin ligases." The International journal of developmental biology **55**(3): 249-260.
- Hirano, T., S. Kodama, et al. (2007). "Role of Toll-like receptor 4 in innate immune responses in a mouse model of acute otitis media." FEMS immunology and medical microbiology **49**(1): 75-83.
- Hoffman, J. D., K. L. Cipraro, et al. (2005). "Immune abnormalities are a frequent manifestation of Kabuki syndrome." American journal of medical genetics. Part A **135**(3): 278-281.
- Hogan, S. C., K. J. Stratford, et al. (1997). "Duration and recurrence of otitis media with effusion in children from birth to 3 years: prospective study using monthly otoscopy and tympanometry." BMJ **314**(7077): 350-353.
- Homøe, P. (2001). "Otitis media in Greenland. Studies on historical, epidemiological, microbiological, and immunological aspects." International journal of circumpolar health **60 Suppl 2**: 1-54.
- Hong, S. H., H. Chu, et al. (2012). "Auditory characteristics and therapeutic effects of enzyme replacement in mouse model of the mucopolysaccharidosis (MPS) II." American journal of medical genetics. Part A **158A**(9): 2131-2138.
- Hong, W., D. Peng, et al. (2010). "Protection against nontypeable Haemophilus influenzae challenges by mucosal vaccination with a detoxified lipooligosaccharide conjugate in two chinchilla models." Microbes and infection / Institut Pasteur **12**(1): 11-18.
- Howie, V. M., J. H. Ploussard, et al. (1975). "The "otitis-prone" condition." American journal of diseases of children (1960) **129**(6): 676-678.
- Huang, M., D. Dulon, et al. (1990). "Outer hair cells as potential targets of inflammatory mediators." The Annals of otology, rhinology & laryngology. Supplement **148**: 35-38.
- Humbert, P. O., C. Rogers, et al. (2000). "E2F4 is essential for normal erythrocyte maturation and neonatal viability." Molecular cell **6**(2): 281-291.

- Humphries, J. D., A. Byron, et al. (2006). "Integrin ligands at a glance." Journal of cell science **119**(Pt 19): 3901-3903.
- Hynes, R. O. (2002). "Integrins: bidirectional, allosteric signaling machines." Cell **110**(6): 673-687.
- Hynes, R. O. (2007). "Cell-matrix adhesion in vascular development." Journal of thrombosis and haemostasis : JTH **5 Suppl 1**: 32-40.
- Ibañez-Tallon, I., S. Gorokhova, et al. (2002). "Loss of function of axonemal dynein Mdnah5 causes primary ciliary dyskinesia and hydrocephalus." Human molecular genetics **11**(6): 715-721.
- Irvin, C. G. and J. H. Bates (2003). "Measuring the lung function in the mouse: the challenge of size." Respir Res **4**: 4.
- Izutsu, K., M. Kurokawa, et al. (2001). "The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling." Blood **97**(9): 2815-2822.
- Jacoby, P. A., H. L. Coates, et al. (2008). "The effect of passive smoking on the risk of otitis media in Aboriginal and non-Aboriginal children in the Kalgoorlie? Boulder region of Western Australia." Medical Journal of
- Jain, P., S. Baranwal, et al. (2013). "Integrin-binding protein nischarin interacts with tumor suppressor liver kinase B1 (LKB1) to regulate cell migration of breast epithelial cells." The Journal of biological chemistry **288**(22): 15495-15509.
- Jaskoll, T. F. and P. F. Maderson (1978). "A histological study of the development of the avian middle ear and tympanum." The Anatomical record **190**(2): 177-199.
- Jensen, R. G., P. Homøe, et al. (2011). "Long-term follow-up of chronic suppurative otitis media in a high-risk children cohort." International journal of pediatric otorhinolaryngology **75**(7): 948-954.
- Jero, J., D. E. Coling, et al. (2001). "The use of Preyer's reflex in evaluation of hearing in mice." Acta oto-laryngologica **121**(5): 585-589.
- Jin, L., O. Wessely, et al. (2013). "Prooncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF- α in breast cancer." Cancer research **73**(9): 2884-2896.
- Joki-Erkkilä, V.-P. P., H. Puhakka, et al. (2002). "Cytokine gene polymorphism in recurrent acute otitis media." Archives of otolaryngology--head & neck surgery **128**(1): 17-20.
- Jono, H., T. Shuto, et al. (2002). "Transforming growth factor-beta -Smad signaling pathway cooperates with NF-kappa B to mediate nontypeable Haemophilus influenzae-induced MUC2 mucin transcription." The Journal of biological chemistry **277**(47): 45547-45557.
- Jono, H., H. Xu, et al. (2003). "Transforming growth factor-beta-Smad signaling pathway negatively regulates nontypeable Haemophilus influenzae-induced MUC5AC mucin transcription via mitogen-activated protein kinase (MAPK) phosphatase-1-dependent inhibition of p38 MAPK." The Journal of biological chemistry **278**(30): 27811-27819.
- Jung, H. H., M. W. Kim, et al. (1999). "Expression of vascular endothelial growth factor in otitis media." Acta oto-laryngologica **119**(7): 801-808.
- Jung, S.-C. C., E.-S. S. Park, et al. (2010). "Characterization of a novel mucopolysaccharidosis type II mouse model and recombinant AAV2/8 vector-mediated gene therapy." Molecules and cells **30**(1): 13-18.
- Justice, M. J., J. K. Noveroske, et al. (1999). "Mouse ENU mutagenesis." Human molecular genetics **8**(10): 1955-1963.

- Jyonouchi, S., D. M. McDonald-McGinn, et al. (2009). "CHARGE (coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness) syndrome and chromosome 22q11.2 deletion syndrome: a comparison of immunologic and nonimmunologic phenotypic features." *Pediatrics* **123**(5): 7.
- Kalm, O., U. Johnson, et al. (1991). "HLA frequency in patients with recurrent acute otitis media." ... -Head & Neck Surgery.
- Kamai, T., H. Shirataki, et al. (2010). "Increased Rac1 activity and Pak1 overexpression are associated with lymphovascular invasion and lymph node metastasis of upper urinary tract cancer." *BMC cancer* **10**: 164.
- Karjalainen, H., M. Koskela, et al. (1991). "Occurrences of antibodies against *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Branhamella catarrhalis* in middle ear effusion and serum during the course of acute otitis media." *Acta oto-laryngologica* **111**(1): 112-119.
- Karma, P., J. Luotonen, et al. (1980). "Efficacy of pneumococcal vaccination against recurrent otitis media. Preliminary results of a field trial in Finland." *The Annals of otology, rhinology & laryngology. Supplement* **89**(3 Pt 2): 357-362.
- Kawai, T. and S. Akira (2010). "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." *Nature immunology* **11**(5): 373-384.
- Kennedy, C. L. and M. K. O'Bryan (2006). "N-ethyl-N-nitrosourea (ENU) mutagenesis and male fertility research." *Human reproduction update* **12**(3): 293-301.
- Kerschner, J. E., W. Hong, et al. (2013). "A novel model of spontaneous otitis media with effusion (OME) in the *Oxgr1* knock-out mouse." *International journal of pediatric otorhinolaryngology* **77**(1): 79-84.
- Khanna, S., S. Biswas, et al. (2010). "Macrophage dysfunction impairs resolution of inflammation in the wounds of diabetic mice." *PloS one* **5**(3).
- Kile, B. T. and D. J. Hilton (2005). "The art and design of genetic screens: mouse." *Nature reviews. Genetics* **6**(7): 557-567.
- Kim, S., K. Bell, et al. (2000). "Regulation of angiogenesis in vivo by ligation of integrin $\alpha 5 \beta 1$ with the central cell-binding domain of fibronectin." *The American journal of pathology* **156**(4): 1345-1362.
- Kleemola, M., J. Nokso-Koivisto, et al. (2006). "Is there any specific association between respiratory viruses and bacteria in acute otitis media of young children?" *The Journal of infection* **52**(3): 181-187.
- Kobe, B. and A. V. Kajava (2001). "The leucine-rich repeat as a protein recognition motif." *Current opinion in structural biology* **11**(6): 725-732.
- Kong, K. and H. L. Coates (2009). "Natural history, definitions, risk factors and burden of otitis media." *The Medical journal of Australia* **191**(9 Suppl): 43.
- Kubba, H., J. P. Pearson, et al. (2000). "The aetiology of otitis media with effusion: a review." *Clinical otolaryngology and allied sciences* **25**(3): 181-194.
- Kuijl, C., M. Pilli, et al. (2013). "Rac and Rab GTPases dual effector Nischarin regulates vesicle maturation to facilitate survival of intracellular bacteria." *The EMBO journal* **32**(5): 713-727.
- Kumar, R. and R. K. Vadlamudi (2002). "Emerging functions of p21-activated kinases in human cancer cells." *Journal of cellular physiology* **193**(2): 133-144.
- Kunimoto, K., Y. Yamazaki, et al. (2012). "Coordinated ciliary beating requires Odf2-mediated polarization of basal bodies via basal feet." *Cell* **148**(1-2): 189-200.

- Kurokawa, M., K. Mitani, et al. (1998). "The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3." Nature **394**(6688): 92-96.
- Kurokawa, M., K. Mitani, et al. (2000). "The evi-1 oncoprotein inhibits c-Jun N-terminal kinase and prevents stress-induced cell death." The EMBO journal **19**(12): 2958-2968.
- Kusters, M. A. A., R. H. Versteegen, et al. (2009). "Intrinsic defect of the immune system in children with Down syndrome: a review." Clinical and experimental immunology **156**(2): 189-193.
- Kvaerner, K. J., K. Tambs, et al. (1997). "Distribution and heritability of recurrent ear infections." The Annals of otology.
- Lander, E. S., L. M. Linton, et al. (2001). "Initial sequencing and analysis of the human genome." Nature **409**(6822): 860-921.
- Lappalainen, U., J. A. Whitsett, et al. (2005). "Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung." American journal of respiratory cell and molecular biology **32**(4): 311-318.
- Lee, C. G., H. Link, et al. (2004). "Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung." Nature medicine **10**(10): 1095-1103.
- Leichtle, A., M. Hernandez, et al. (2010). "CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF-/- mice." Journal of immunology (Baltimore, Md. : 1950) **184**(6): 3087-3097.
- Leichtle, A., M. Hernandez, et al. (2012). "The role of DNA sensing and innate immune receptor TLR9 in otitis media." Innate immunity **18**(1): 3-13.
- Leichtle, A., M. Hernandez, et al. (2009). "The toll-Like receptor adaptor TRIF contributes to otitis media pathogenesis and recovery." BMC immunology **10**: 45.
- Leichtle, A., M. Hernandez, et al. (2009). "TLR4-mediated induction of TLR2 signaling is critical in the pathogenesis and resolution of otitis media." Innate immunity **15**(4): 205-215.
- Li, D., Z. Qiu, et al. (2013). "Heritable gene targeting in the mouse and rat using a CRISPR-Cas system." Nature biotechnology **31**(8): 681-683.
- Li, M. O., Y. Y. Wan, et al. (2006). "Transforming growth factor-beta regulation of immune responses." Annual review of immunology **24**: 99-146.
- Lim, J. H., H. Jono, et al. (2007). "Tumor suppressor CYLD acts as a negative regulator for non-typeable Haemophilus influenza-induced inflammation in the middle ear and lung of mice." PloS one **2**(10).
- Lim, K.-P. P. and W. Hong (2004). "Human Nischarin/imidazoline receptor antisera-selected protein is targeted to the endosomes by a combined action of a PX domain and a coiled-coil region." The Journal of biological chemistry **279**(52): 54770-54782.
- Liu, Z., K. Kobayashi, et al. (2009). "VEGF and inhibitors of TGFbeta type-I receptor kinase synergistically promote blood-vessel formation by inducing alpha5-integrin expression." Journal of cell science **122**(Pt 18): 3294-3302.
- Lous, J., M. J. Burton, et al. (2005). "Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children." The Cochrane database of systematic reviews(1).
- Lu, M., J. S. Munger, et al. (2002). "Integrin alpha8beta1 mediates adhesion to LAP-TGFbeta1." Journal of cell science **115**(Pt 23): 4641-4648.

- Lucas, J. S., E. C. Adam, et al. (2012). "Static respiratory cilia associated with mutations in Dnahc11/DNAH11: a mouse model of PCD." Human mutation **33**(3): 495-503.
- Ludbrook, S. B., S. T. Barry, et al. (2003). "The integrin α v β 3 is a receptor for the latency-associated peptides of transforming growth factors β 1 and β 3." The Biochemical journal **369**(Pt 2): 311-318.
- Luk, C. K. and M. J. Dulfano (1983). "Effect of pH, viscosity and ionic-strength changes on ciliary beating frequency of human bronchial explants." Clinical science (London, England : 1979) **64**(4): 449-451.
- Lundblad, L. K., C. G. Irvin, et al. (2002). "A reevaluation of the validity of unrestrained plethysmography in mice." J Appl Physiol (1985) **93**(4): 1198-1207.
- Lundblad, L. K., J. Thompson-Figueroa, et al. (2005). "Tumor necrosis factor- α overexpression in lung disease: a single cause behind a complex phenotype." American journal of respiratory and critical care medicine **171**(12): 1363-1370.
- MacArthur, C. J., S. H. Hefeneider, et al. (2006). "C3H/HeJ mouse model for spontaneous chronic otitis media." The Laryngoscope **116**(7): 1071-1079.
- MacArthur, C. J., D.-A. M. A. Pillers, et al. (2011). "Altered expression of middle and inner ear cytokines in mouse otitis media." The Laryngoscope **121**(2): 365-371.
- MacArthur, C. J., B. Wilmot, et al. (2014). "Genetic susceptibility to chronic otitis media with effusion: candidate gene single nucleotide polymorphisms." The Laryngoscope **124**(5): 1229-1235.
- Maguire, S., J. Estabel, et al. (2014). "Targeting of Slc25a21 is associated with orofacial defects and otitis media due to disrupted expression of a neighbouring gene." PloS one **9**(3).
- Maison, S. F., M. Le, et al. (2010). "Mice lacking adrenergic signaling have normal cochlear responses and normal resistance to acoustic injury but enhanced susceptibility to middle-ear infection." Journal of the Association for Research in Otolaryngology : JARO **11**(3): 449-461.
- Mäkelä, P. H., M. Leinonen, et al. (1981). "A study of the pneumococcal vaccine in prevention of clinically acute attacks of recurrent otitis media." Reviews of infectious diseases **3 Suppl**: 32.
- Mäkelä, P. H., M. Sibakov, et al. (1980). "Pneumococcal vaccine and otitis media." Lancet **2**(8194): 547-551.
- Mao, M., D. R. Thedens, et al. (2009). "The podosomal-adaptor protein SH3PXD2B is essential for normal postnatal development." Mammalian genome : official journal of the International Mammalian Genome Society **20**(8): 462-475.
- Margadant, C. and A. Sonnenberg (2010). "Integrin-TGF- β crosstalk in fibrosis, cancer and wound healing." EMBO reports **11**(2): 97-105.
- Marsden, R. L., L. J. McGuffin, et al. (2002). "Rapid protein domain assignment from amino acid sequence using predicted secondary structure." Protein science : a publication of the Protein Society **11**(12): 2814-2824.
- Martin, F. N. and J. G. Clark (2009). Introduction to audiology. Boston, Mass. ; London, Pearson.
- Mason, P. S., E. Adam, et al. (2002). "Effect of bacterial endotoxin and middle ear effusion on ciliary activity: implications for otitis media." The Laryngoscope **112**(4): 676-680.

- Maynard, J. E., J. K. Fleshman, et al. (1972). "Otitis media in Alaskan Eskimo children. Prospective evaluation of chemoprophylaxis." JAMA : the journal of the American Medical Association **219**(5): 597-599.
- Mburu, P., M. Mustapha, et al. (2003). "Defects in whirlin, a PDZ domain molecule involved in stereocilia elongation, cause deafness in the whirler mouse and families with DFNB31." Nature genetics **34**(4): 421-428.
- Megerian, C. A., M. T. Semaan, et al. (2008). "A mouse model with postnatal endolymphatic hydrops and hearing loss." Hearing research **237**(1-2): 90-105.
- Meyerhoff, W. L., C. S. Kim, et al. (1978). "Pathology of chronic otitis media." The Annals of otology, rhinology, and laryngology **87**(6 Pt 1): 749-760.
- Michaud, E. J., C. T. Culiati, et al. (2005). "Efficient gene-driven germ-line point mutagenesis of C57BL/6J mice." BMC genomics **6**: 164.
- Miller, F., A. Burghard, et al. (2014). "Treatment of middle ear ventilation disorders: sheep as animal model for stenting the human Eustachian tube--a cadaver study." PloS one **9**(11): e113906.
- Monasta, L., L. Ronfani, et al. (2012). "Burden of disease caused by otitis media: systematic review and global estimates." PloS one **7**(4).
- Moraes, F., A. Nóvoa, et al. (2005). "Tbx1 is required for proper neural crest migration and to stabilize spatial patterns during middle and inner ear development." Mechanisms of development **122**(2): 199-212.
- Morizono, T. (1990). "Toxicity of ototopical drugs: animal modeling." The Annals of otology, rhinology & laryngology. Supplement **148**: 42-45.
- Morizono, T. and T. Tono (1991). "Middle ear inflammatory mediators and cochlear function." Otolaryngologic clinics of North America **24**(4): 835-843.
- Mouse Genome Sequencing, C., R. H. Waterston, et al. (2002). "Initial sequencing and comparative analysis of the mouse genome." Nature **420**(6915): 520-562.
- Mucha, D. R., C. L. Myers, et al. (2003). "Endothelial contraction and monolayer hyperpermeability are regulated by Src kinase." American journal of physiology. Heart and circulatory physiology **284**(3).
- Mundy, G. R., B. Boyce, et al. (1995). "The effects of cytokines and growth factors on osteoblastic cells." Bone **17**(2 Suppl).
- Munger, J. S., J. G. Harpel, et al. (1998). "Interactions between growth factors and integrins: latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1." Molecular biology of the cell **9**(9): 2627-2638.
- Murphy, J. P. (1981). "Two years of otolaryngology in Ghana, West Africa." Archives of otolaryngology (Chicago, Ill. : 1960) **107**(7): 422-424.
- Murphy, T. F. and L. C. Bartos (1988). "Human bactericidal antibody response to outer membrane protein P2 of nontypeable Haemophilus influenzae." Infection and immunity **56**(10): 2673-2679.
- Murphy, T. F., L. C. Bartos, et al. (1986). "Antigenic characterization of the P6 protein of nontypable Haemophilus influenzae." Infection and immunity **54**(3): 774-779.
- Nagy, A. and L. Mar (2001). "Creation and use of a Cre recombinase transgenic database." Methods in molecular biology (Clifton, N.J.) **158**: 95-106.
- Nagy, J. A., L. Benjamin, et al. (2008). "Vascular permeability, vascular hyperpermeability and angiogenesis." Angiogenesis **11**(2): 109-119.

- Nelson, J. D. (1988). "Chronic suppurative otitis media." The Pediatric infectious disease journal **7**(6): 446-448.
- Ng, P. C. and S. Henikoff (2001). "Predicting deleterious amino acid substitutions." Genome research **11**(5): 863-874.
- Ng, P. C. and S. Henikoff (2002). "Accounting for human polymorphisms predicted to affect protein function." Genome research **12**(3): 436-446.
- Ng, P. C. and S. Henikoff (2003). "SIFT: Predicting amino acid changes that affect protein function." Nucleic acids research **31**(13): 3812-3814.
- Ng, P. C. and S. Henikoff (2006). "Predicting the effects of amino acid substitutions on protein function." Annual review of genomics and human genetics **7**: 61-80.
- Noben-Trauth, K. and J. R. Latoche (2011). "Ectopic mineralization in the middle ear and chronic otitis media with effusion caused by RPL38 deficiency in the Tail-short (Ts) mouse." The Journal of biological chemistry **286**(4): 3079-3093.
- Nolan, P. M., J. Peters, et al. (2000). "A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse." Nature genetics **25**(4): 440-443.
- Nuytinck, L., E. De Meester, et al. (2006). "Role of mannose-binding lectin (MBL2) genotyping in predicting the risk of recurrent otitis media (rOM)." Advances in experimental medicine and biology **586**: 281-290.
- Ohashi, Y., Y. Nakai, et al. (1995). "Effects of human middle ear effusions on the mucociliary system of the tubotympanum in the guinea pig." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery **252**(1): 35-41.
- Okabe, Y., T. Sano, et al. (2009). "Regulation of the innate immune response by threonine-phosphatase of Eyes absent." Nature **460**(7254): 520-524.
- Okutani, D., M. Lodyga, et al. (2006). "Src protein tyrosine kinase family and acute inflammatory responses." American journal of physiology. Lung cellular and molecular physiology **291**(2): 41.
- Ortega-Velázquez, R., M. L. Díez-Marqués, et al. (2003). "Arg-Gly-Asp-Ser (RGDS) peptide stimulates transforming growth factor beta1 transcription and secretion through integrin activation." FASEB journal : official publication of the Federation of American Societies for Experimental Biology **17**(11): 1529-1531.
- Palva, T., P. Häyry, et al. (1980). "Immunologic aspects of otitis media." Acta oto-laryngologica **89**(3-4): 177-181.
- Palva, T., P. Pääkkö, et al. (2003). "Apoptosis and regression of embryonic mesenchyme in the development of the middle ear spaces." Acta oto-laryngologica **123**(2): 209-214.
- Palva, T. and H. Ramsay (2002). "Fate of the mesenchyme in the process of pneumatization." Otology & neurotology : official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology **23**(2): 192-199.
- Paparella, M. M., T. Morizono, et al. (1984). "Sensorineural hearing loss in otitis media." The Annals of otology, rhinology, and laryngology **93**(6 Pt 1): 623-629.
- Paparella, M. M., P. A. Schachern, et al. (1990). "Otopathologic correlates of the continuum of otitis media." Ann Otol Rhinol Laryngol Suppl **148**: 17-22.

- Parkinson, N., R. E. Hardisty-Hughes, et al. (2006). "Mutation at the Evi1 locus in Junbo mice causes susceptibility to otitis media." PLoS genetics **2**(10).
- Parks, W. T., D. B. Frank, et al. (2001). "Sorting nexin 6, a novel SNX, interacts with the transforming growth factor-beta family of receptor serine-threonine kinases." J Biol Chem **276**(22): 19332-19339.
- Parrini, M. C., M. Lei, et al. (2002). "Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1." Molecular cell **9**(1): 73-83.
- Patel, J. A., S. Nair, et al. (2006). "Association of proinflammatory cytokine gene polymorphisms with susceptibility to otitis media." Pediatrics **118**(6): 2273-2279.
- Paten, B., J. Herrero, et al. (2009). "Sequence progressive alignment, a framework for practical large-scale probabilistic consistency alignment." Bioinformatics (Oxford, England) **25**(3): 295-301.
- Paten, B., J. Herrero, et al. (2008). "Enredo and Pecan: genome-wide mammalian consistency-based multiple alignment with paralogs." Genome research **18**(11): 1814-1828.
- Pau, H., H. Fuchs, et al. (2005). "Hush puppy: a new mouse mutant with pinna, ossicle, and inner ear defects." The Laryngoscope **115**(1): 116-124.
- Pennock, B. E., C. P. Cox, et al. (1979). "A noninvasive technique for measurement of changes in specific airway resistance." J Appl Physiol Respir Environ Exerc Physiol **46**(2): 399-406.
- Perl, A. K. and J. A. Whitsett (1999). "Molecular mechanisms controlling lung morphogenesis." Clinical genetics **56**(1): 14-27.
- Perona, R., S. Montaner, et al. (1997). "Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins." Genes & development **11**(4): 463-475.
- Petak, F., W. Habre, et al. (2001). "Hyperoxia-induced changes in mouse lung mechanics: forced oscillations vs. barometric plethysmography." J Appl Physiol (1985) **90**(6): 2221-2230.
- Pettigrew, M. M., J. F. Gent, et al. (2006). "Association of surfactant protein A polymorphisms with otitis media in infants at risk for asthma." BMC medical genetics **7**: 68.
- Pettigrew, M. M., M. Khodaei, et al. (2003). "Duration of breastfeeding, daycare, and physician visits among infants 6 months and younger." Annals of epidemiology **13**(6): 431-435.
- Pfister, M., T. Tóth, et al. (2002). "A 4-bp insertion in the eya-homologous region (eyaHR) of EYA4 causes hearing impairment in a Hungarian family linked to DFNA10." Molecular medicine (Cambridge, Mass.) **8**(10): 607-611.
- Piletz, J. E., T. R. Ivanov, et al. (2000). "Imidazoline receptor antisera-selected (IRAS) cDNA: cloning and characterization." DNA and cell biology **19**(6): 319-329.
- Politzer, A., M. J. Ballin, et al. (1902). A Text-Book of the Diseases of the Ear ... Translated ... and edited by M. J. Ballin ... and C. L. Heller, M.D. Fourth edition, revised and enlarged, etc. London, Baillie & Co.
- Preciado, D., S. Goyal, et al. (2010). "MUC5B Is the predominant mucin glycoprotein in chronic otitis media fluid." Pediatric research **68**(3): 231-236.
- Prellner, K., T. Hallberg, et al. (1985). "Recurrent otitis media: genetic immunoglobulin markers in children and their parents." International journal of ...

- Prellner, K., O. Kalm, et al. (1989). "Pneumococcal serum antibody concentrations during the first three years of life: a study of otitis-prone and non-otitis-prone children." International journal of
- Pukander, J., J. Luotonen, et al. (1985). "Risk factors affecting the occurrence of acute otitis media among 2-3-year-old urban children." Acta oto-
- Quwailid, M. M., A. Hugill, et al. (2004). "A gene-driven ENU-based approach to generating an allelic series in any gene." Mammalian genome : official journal of the International Mammalian Genome Society **15**(8): 585-591.
- Rajewsky, K., H. Gu, et al. (1996). "Conditional gene targeting." The Journal of clinical investigation **98**(3): 600-603.
- Ram, G. and J. Chinen (2011). "Infections and immunodeficiency in Down syndrome." Clinical and experimental immunology **164**(1): 9-16.
- Rämet, M., J. Löfgren, et al. (2001). "Surfactant protein-A gene locus associated with recurrent otitis media." The Journal of pediatrics.
- Rasmussen, F. (1993). "Protracted secretory otitis media. The impact of familial factors and day-care center attendance." International journal of pediatric otorhinolaryngology **26**(1): 29-37.
- Reddig, P. J., D. Xu, et al. (2005). "Regulation of p21-activated kinase-independent Rac1 signal transduction by nischarin." The Journal of biological chemistry **280**(35): 30994-31002.
- Revai, K., J. A. Patel, et al. (2009). "Association between cytokine gene polymorphisms and risk for upper respiratory tract infection and acute otitis media." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **49**(2): 257-261.
- Richter, C. A., S. Amin, et al. (2010). "Defects in middle ear cavitation cause conductive hearing loss in the Tcof1 mutant mouse." Hum Mol Genet **19**(8): 1551-1560.
- Richtsmeier, J. T., L. L. Baxter, et al. (2000). "Parallels of craniofacial maldevelopment in Down syndrome and Ts65Dn mice." Developmental dynamics : an official publication of the American Association of Anatomists **217**(2): 137-145.
- Ricketts, T., P. McGoldrick, et al. (2014). "A nonsense mutation in mouse Tardbp affects TDP43 alternative splicing activity and causes limb-clasping and body tone defects." PloS one **9**(1).
- Ringwald, M., V. Iyer, et al. (2011). "The IKMC web portal: a central point of entry to data and resources from the International Knockout Mouse Consortium." Nucleic acids research **39**(Database issue): 55.
- Rivkin, A. Z., S. D. Palacios, et al. (2005). "The role of Fas-mediated apoptosis in otitis media: observations in the lpr/lpr mouse." Hearing research **207**(1-2): 110-116.
- Roberts, D. S. and S. A. Miller (1998). "Apoptosis in cavitation of middle ear space." The Anatomical record **251**(3): 286-289.
- Roier, S., D. R. Leitner, et al. (2012). "Intranasal immunization with nontypeable Haemophilus influenzae outer membrane vesicles induces cross-protective immunity in mice." PloS one **7**(8).
- Rosenfeld, R. M., L. Culpepper, et al. (2004). "Clinical practice guideline: Otitis media with effusion." Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery **130**(5 Suppl): 118.

- Rosenfeld, R. M. and D. Kay (2003). Natural history of untreated otitis media. Evidence-based otitis media. R. M. Rosenfeld and C. D. Bluestone. Hamilton ; London, BC Decker: 180-198.
- Rosenthal, N. and S. Brown (2007). "The mouse ascending: perspectives for human-disease models." Nat Cell Biol **9**(9): 993-999.
- Rosito, L. P., S. S. da Costa, et al. (2007). "Contralateral ear in chronic otitis media: a histologic study." The Laryngoscope **117**(10): 1809-1814.
- Rovers, M., M. Haggard, et al. (2002). "Heritability of symptom domains in otitis media: a longitudinal study of 1,373 twin pairs." American journal of ...
- Rovers, M. M., A. G. M. Schilder, et al. (2004). "Otitis media." The Lancet.
- Rowe, D. S. (1975). "Acute suppurative otitis media." Pediatrics **56**(2): 285-294.
- Roy, M. G., A. Livraghi-Butrico, et al. (2014). "Muc5b is required for airway defence." Nature **505**(7483): 412-416.
- Ruoslahti, E. (1996). "RGD and other recognition sequences for integrins." Annual review of cell and developmental biology **12**: 697-715.
- Russell, W. L., E. M. Kelly, et al. (1979). "Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse." Proceedings of the National Academy of Sciences of the United States of America **76**(11): 5818-5819.
- Ruuskanen, O. and T. Heikkinen (1994). "Viral-bacterial interaction in acute otitis media." The Pediatric infectious disease ...
- Ryan, A. F., J. Ebmeyer, et al. (2006). "Mouse models of induced otitis media." Brain research **1091**(1): 3-8.
- Rye, M. S., M. F. Bhutta, et al. (2011). "Unraveling the genetics of otitis media: from mouse to human and back again." Mammalian genome : official journal of the International Mammalian Genome Society **22**(1-2): 66-82.
- Rye, M. S., N. M. Warrington, et al. (2012). "Genome-wide association study to identify the genetic determinants of otitis media susceptibility in childhood." PloS one **7**(10).
- Rye, M. S., S. P. Wiertsema, et al. (2011). "FBXO11, a regulator of the TGFbeta pathway, is associated with severe otitis media in Western Australian children." Genes Immun **12**(5): 352-359.
- Rye, M. S., S. P. Wiertsema, et al. (2013). "Genetic and functional evidence for a role for SLC11A1 in susceptibility to otitis media in early childhood in a Western Australian population." Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases **16**: 411-418.
- Sabirov, A., R. G. Hamilton, et al. (2008). "Role of local immunoglobulin E specific for *Alternaria alternata* in the pathogenesis of nasal polyposis." Laryngoscope **118**(1): 4-9.
- Sabirov, A. and D. W. Metzger (2008). "Mouse models for the study of mucosal vaccination against otitis media." Vaccine **26**(12): 1501-1524.
- Sabrautski, S., I. Rubio-Aliaga, et al. (2012). "New mouse models for metabolic bone diseases generated by genome-wide ENU mutagenesis." Mammalian genome : official journal of the International Mammalian Genome Society **23**(7-8): 416-430.
- Sadé, J. and A. Ar (1997). "Middle ear and auditory tube: middle ear clearance, gas exchange, and pressure regulation." Otolaryngology--head and neck surgery :

- official journal of American Academy of Otolaryngology-Head and Neck Surgery **116**(4): 499-524.
- Sale, M. M., W.-M. M. Chen, et al. (2011). "Evaluation of 15 functional candidate genes for association with chronic otitis media with effusion and/or recurrent otitis media (COME/ROM)." PloS one **6**(8).
- Salvinelli, F., M. Trivelli, et al. (1999). "Chronic otitis media: histopathological changes: a post mortem study on temporal bones." European review for medical and pharmacological sciences **3**(4): 175-178.
- Schachern, P. A., S. Cureoglu, et al. (2007). "Age-related functional and histopathological changes of the ear in the MPS I mouse." International journal of pediatric otorhinolaryngology **71**(2): 197-203.
- Schmidt-Ullrich, R., T. Aebischer, et al. (2001). "Requirement of NF-kappaB/Rel for the development of hair follicles and other epidermal appendices." Development (Cambridge, England) **128**(19): 3843-3853.
- Segade, F., K. A. Daly, et al. (2006). "Association of the FBXO11 gene with chronic otitis media with effusion and recurrent otitis media: the Minnesota COME/ROM Family Study." Archives of otolaryngology--head & neck surgery **132**(7): 729-733.
- Sekiyama, K., J.-i. Ohori, et al. (2011). "The role of vascular endothelial growth factor in pediatric otitis media with effusion." Auris, nasus, larynx **38**(3): 319-324.
- Selaimen da Costa, S., L. P. Rosito, et al. (2008). "The contralateral ear in chronic otitis media: a series of 500 patients." Archives of otolaryngology--head & neck surgery **134**(3): 290-293.
- Selby, P. B. (1987). "A rapid method for preparing high quality alizarin stained skeletons of adult mice." Stain technology **62**(3): 143-146.
- Senger, D. R., S. J. Galli, et al. (1983). "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid." Science (New York, N.Y.) **219**(4587): 983-985.
- Seo, S. R., N. Ferrand, et al. (2006). "Nuclear retention of the tumor suppressor cPML by the homeodomain protein TGIF restricts TGF-beta signaling." Molecular cell **23**(4): 547-559.
- Sharma, S. K., J. R. Casey, et al. (2012). "Reduced serum IgG responses to pneumococcal antigens in otitis-prone children may be due to poor memory B-cell generation." The Journal of infectious diseases **205**(8): 1225-1229.
- Shi, W., S. Bellusci, et al. (2007). "Lung development and adult lung diseases." Chest **132**(2): 651-656.
- Shi, Y. and J. Massagué (2003). "Mechanisms of TGF- β signaling from cell membrane to the nucleus." Cell.
- Shimada, J., S. K. Moon, et al. (2008). "Lysozyme M deficiency leads to an increased susceptibility to Streptococcus pneumoniae-induced otitis media." BMC infectious diseases **8**: 134.
- SIGN (2003). Diagnosis and management of childhood otitis media in primary care: A national clinical guideline. Edinburgh, Scottish Intercollegiate Guidelines Network.
- Simon, M. M., S. Greenaway, et al. (2013). "A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains." Genome Biol **14**(7): R82.

- Sipilä, M., P. Karma, et al. (1988). "The Bayesian approach to the evaluation of risk factors in acute and recurrent acute otitis media." Acta oto-laryngologica **106**(1-2): 94-101.
- Sipilä, P. (1984). "Aspects of biological potentials of mononuclear cells in middle ear effusions." Acta oto-laryngologica. Supplementum **414**: 138-142.
- Skarnes, W. C., B. Rosen, et al. (2011). "A conditional knockout resource for the genome-wide study of mouse gene function." Nature **474**(7351): 337-342.
- Skotnicka, B., A. Stasiak-Barmuta, et al. (2005). "Lymphocyte subpopulations in middle ear effusions: flow cytometry analysis." Otology & neurotology : official publication of the American Otological Society, American Neurotology Society [and] European Academy of Otology and Neurotology **26**(4): 567-571.
- Sloyer, J. L., J. H. Ploussard, et al. (1981). "Efficacy of pneumococcal polysaccharide vaccine in preventing acute otitis media in infants in Huntsville, Alabama." Reviews of infectious diseases **3 Suppl**: 23.
- Smallman, L. A., S. L. Hill, et al. (1984). "Reduction of ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect." Thorax **39**(9): 663-667.
- Smith, D. F. and E. F. Boss (2010). "Racial/ethnic and socioeconomic disparities in the prevalence and treatment of otitis media in children in the United States." The Laryngoscope.
- Sood, S. and A. Waddell (2007). "Accurate consent for insertion and later removal of grommets." The Journal of laryngology and otology **121**(4): 338-340.
- Spauwen, P. H., B. Hillen, et al. (1991). "Three-dimensional computer reconstruction of the eustachian tube and paratubal muscles." Cleft Palate Craniofac J **28**(2): 217-219; discussion 220.
- Stevens, G., S. Flaxman, et al. (2013). "Global and regional hearing impairment prevalence: an analysis of 42 studies in 29 countries." European journal of public health **23**(1): 146-152.
- Stevenson, R. E. and J. G. Hall (2005). Human malformations and related anomalies. New York ; Oxford, Oxford University Press.
- Stol, K., S. van Selm, et al. (2009). "Development of a non-invasive murine infection model for acute otitis media." Microbiology (Reading, England) **155**(Pt 12): 4135-4144.
- Sullivan, K. E., A. F. Jawad, et al. (1998). "Lack of correlation between impaired T cell production, immunodeficiency, and other phenotypic features in chromosome 22q11.2 deletion syndromes." Clinical immunology and immunopathology **86**(2): 141-146.
- Sun, M., K. Mondal, et al. (2012). "Multiplex Chromosomal Exome Sequencing Accelerates Identification of ENU-Induced Mutations in the Mouse." G3 (Bethesda, Md.) **2**(1): 143-150.
- Sunderman, J. and H. Dyer (1984). "Chronic ear disease in Australian Aborigines." The Medical journal of Australia **140**(12): 708-711.
- Tabor, H. K., N. J. Risch, et al. (2002). "Candidate-gene approaches for studying complex genetic traits: practical considerations." Nature reviews. Genetics **3**(5): 391-397.
- Takeuchi, K., S. Saida, et al. (1990). "The effect of middle ear effusions from children on in vitro ciliary activity." European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-

- Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery **247**(5): 323-325.
- Tammela, T. and K. Alitalo (2010). "Lymphangiogenesis: Molecular mechanisms and future promise." Cell **140**(4): 460-476.
- Tateossian, H., R. E. Hardisty-Hughes, et al. (2009). "Regulation of TGF-beta signalling by Fbxo11, the gene mutated in the Jeff otitis media mouse mutant." Pathogenetics **2**(1): 5.
- Tateossian, H., S. Morse, et al. (2013). "Otitis media in the Tgif knockout mouse implicates TGF β signalling in chronic middle ear inflammatory disease." Human molecular genetics **22**(13): 2553-2565.
- Teasdale, R. D. and B. M. Collins (2012). "Insights into the PX (phox-homology) domain and SNX (sorting nexin) protein families: structures, functions and roles in disease." The Biochemical journal **441**(1): 39-59.
- Teele, D. W., J. O. Klein, et al. (1981). "Use of pneumococcal vaccine for prevention of recurrent acute otitis media in infants in Boston. The Greater Boston Collaborative Otitis Media Study Group." Reviews of infectious diseases **3** **Suppl**: 8.
- Teele, D. W., J. O. Klein, et al. (1989). "Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study." The Journal of infectious diseases **160**(1): 83-94.
- Teele, D. W., J. O. Klein, et al. (1984). "Otitis media with effusion during the first three years of life and development of speech and language." Pediatrics **74**(2): 282-287.
- Thaung, C., K. West, et al. (2002). "Novel ENU-induced eye mutations in the mouse: models for human eye disease." Human molecular genetics **11**(7): 755-767.
- Thomas, K. R., K. R. Folger, et al. (1986). "High frequency targeting of genes to specific sites in the mammalian genome." Cell **44**(3): 419-428.
- Thornton, C. E., S. D. Brown, et al. (1999). "Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses." Mammalian genome : official journal of the International Mammalian Genome Society **10**(10): 987-992.
- Tian, C., H. Yu, et al. (2012). "Otitis media in a new mouse model for CHARGE syndrome with a deletion in the Chd7 gene." PloS one **7**(4).
- Tomonaga, K., Y. Kurono, et al. (1988). "The role of nasal allergy in otitis media with effusion: a clinical study." Acta oto-laryngologica.
- Tong, H. H., Y. X. Li, et al. (2010). "Enhanced susceptibility to acute pneumococcal otitis media in mice deficient in complement C1qa, factor B, and factor B/C2." Infection and immunity **78**(3): 976-983.
- Torres, M. and F. Giráldez (1998). "The development of the vertebrate inner ear." Mechanisms of development **71**(1-2): 5-21.
- Trapp, S., S. J. Tucker, et al. (2011). "Respiratory responses to hypercapnia and hypoxia in mice with genetic ablation of Kir5.1 (Kcnj16)." Exp Physiol **96**(4): 451-459.
- Trune, D. R. and Q. Y. Zheng (2009). "Mouse models for human otitis media." Brain research **1277**: 90-103.
- Tsuchiya, K., M. Komori, et al. (2008). "Interleukin-10 is an essential modulator of mucoid metaplasia in a mouse otitis media model." The Annals of otology, rhinology, and laryngology **117**(8): 630-636.

- Tyrer, H. E., M. Crompton, et al. (2013). "What have we learned from murine models of otitis media?" Curr Allergy Asthma Rep **13**(5): 501-511.
- Ubell, M. L., P. Khampang, et al. (2010). "Mucin gene polymorphisms in otitis media patients." The Laryngoscope **120**(1): 132-138.
- Uhari, M. and K. Mäntysaari (1996). "Meta-analytic review of the risk factors for acute otitis media." Clinical Infectious
- Venter, J. C., M. D. Adams, et al. (2001). "The sequence of the human genome." Science (New York, N.Y.) **291**(5507): 1304-1351.
- Vergison, A., R. Dagan, et al. (2010). "Otitis media and its consequences: beyond the earache." The Lancet infectious
- Vogel, P., G. Hansen, et al. (2010). "Tubulin tyrosine ligase-like 1 deficiency results in chronic rhinosinusitis and abnormal development of spermatid flagella in mice." Veterinary pathology **47**(4): 703-712.
- Vogel, P., R. W. Read, et al. (2012). "Congenital hydrocephalus in genetically engineered mice." Veterinary pathology **49**(1): 166-181.
- Vogler, C., B. Levy, et al. (2001). "A novel model of murine mucopolysaccharidosis type VII due to an intracisternal a particle element transposition into the beta-glucuronidase gene: clinical and pathologic findings." Pediatric research **49**(3): 342-348.
- Voronina, V. A., K.-I. Takemaru, et al. (2009). "Inactivation of Chibby affects function of motile airway cilia." The Journal of cell biology **185**(2): 225-233.
- Wake, M. and L. A. Smallman (1992). "Ciliary beat frequency of nasal and middle ear mucosa in children with otitis media with effusion." Clinical otolaryngology and allied sciences **17**(2): 155-157.
- Wald, E. R., B. Dashefsky, et al. (1988). "Frequency and severity of infections in day care." The Journal of
- Wang, D., L. Sun, et al. (1999). "Control of type II transforming growth factor-beta receptor expression by integrin ligation." The Journal of biological chemistry **274**(18): 12840-12847.
- Wang, H., H. Yang, et al. (2013). "One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering." Cell **153**(4): 910-918.
- Wang, W., A. Zhou, et al. (2014). "Interleukin 17A promotes pneumococcal clearance by recruiting neutrophils and inducing apoptosis through a p38 mitogen-activated protein kinase-dependent mechanism in acute otitis media." Infection and immunity **82**(6): 2368-2377.
- Wang, Y., G. Jin, et al. (2006). "Integrins regulate VE-cadherin and catenins: dependence of this regulation on Src, but not on Ras." Proceedings of the National Academy of Sciences of the United States of America **103**(6): 1774-1779.
- Warren, M., W. Wang, et al. (2007). "A Sall4 mutant mouse model useful for studying the role of Sall4 in early embryonic development and organogenesis." Genesis (New York, N.Y. : 2000) **45**(1): 51-58.
- Wayne, S., N. G. Robertson, et al. (2001). "Mutations in the transcriptional activator EYA4 cause late-onset deafness at the DFNA10 locus." Human molecular genetics **10**(3): 195-200.
- WHO (1993). World development report 1993: Investing in Health, World Bank: 213-225.

- WHO (2004). Chronic suppurative otitis media: Burden of Illness and Management Options. Geneva, World Health Organisation.
- WHO (2008). The global burden of disease: 2004 update. Geneva, World Health Organisation.
- Wiertsema, S. P., B. L. Herpers, et al. (2006). "Functional polymorphisms in the mannan-binding lectin 2 gene: effect on MBL levels and otitis media." The Journal of allergy and clinical immunology **117**(6): 1344-1350.
- Wiertsema, S. P., S. K. K. Khoo, et al. (2006). "Association of CD14 promoter polymorphism with otitis media and pneumococcal vaccine responses." Clinical and vaccine immunology : CVI **13**(8): 892-897.
- Wiet, R. J. (1979). "Patterns of ear disease in the southwestern American Indian." Archives of otolaryngology (Chicago, Ill. : 1960) **105**(7): 381-385.
- Williamson, I., S. Benge, et al. (2006). "Consultations for middle ear disease, antibiotic prescribing and risk factors for reattendance: a case-linked cohort study." British journal of general ...
- Williamson, M. P. (1994). "The structure and function of proline-rich regions in proteins." The Biochemical journal **297 (Pt 2)**: 249-260.
- Wilson, R., D. Roberts, et al. (1985). "Effect of bacterial products on human ciliary function in vitro." Thorax **40**(2): 125-131.
- Wipff, P.-J. J. and B. Hinz (2008). "Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship." European journal of cell biology **87**(8-9): 601-615.
- Witschi, H. and J. A. Last (2001). Toxic responses of the respiratory system. Casarett and Doull's toxicology : the basic science of poisons. L. J. Casarett, J. Doull and C. D. Klaassen. New York ; London, McGraw-Hill Medical Pub. Division: 515-534.
- Wong, J. and M. Hawke (1983). "The cytopathology of middle ear effusions (a new technique)." The Journal of otolaryngology **12**(6): 356-360.
- Wotton, D., R. S. Lo, et al. (1999). "A Smad transcriptional corepressor." Cell **97**(1): 29-39.
- Wozniak, M. A., K. Modzelewska, et al. (2004). "Focal adhesion regulation of cell behavior." Biochimica et biophysica acta **1692**(2-3): 103-119.
- Wu, L., J. A. Bernard-Trifilo, et al. (2008). "Distinct FAK-Src activation events promote alpha5beta1 and alpha4beta1 integrin-stimulated neuroblastoma cell motility." Oncogene **27**(10): 1439-1448.
- Xu, P. X., J. Adams, et al. (1999). "Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia." Nature genetics **23**(1): 113-117.
- Xu, X., C.-H. H. Woo, et al. (2012). "EVI1 acts as an inducible negative-feedback regulator of NF- κ B by inhibiting p65 acetylation." Journal of immunology (Baltimore, Md. : 1950) **188**(12): 6371-6380.
- Xu, Y., L. F. Seet, et al. (2001). "The Phox homology (PX) domain, a new player in phosphoinositide signalling." The Biochemical journal **360**(Pt 3): 513-530.
- Yamanaka, N. and H. Faden (1993). "Antibody response to outer membrane protein of nontypeable Haemophilus influenzae in otitis-prone children." The Journal of pediatrics **122**(2): 212-218.

- Yang, A., N. Walker, et al. (2000). "p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours." Nature **404**(6773): 99-103.
- Yang, B., C. Tian, et al. (2011). "Sh3pxd2b mice are a model for craniofacial dysmorphology and otitis media." PloS one **6**(7).
- Yang, J. T., H. Rayburn, et al. (1993). "Embryonic mesodermal defects in alpha 5 integrin-deficient mice." Development (Cambridge, England) **119**(4): 1093-1105.
- Yang, Z., Z. Mu, et al. (2007). "Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice." The Journal of cell biology **176**(6): 787-793.
- Yates, L., F. McMurray, et al. (2009). "ENU mutagenesis as a tool for understanding lung development and disease." Biochemical Society transactions **37**(Pt 4): 838-842.
- Yoon, T. H., M. M. Paparella, et al. (1990). "Morphometric studies of the continuum of otitis media." Ann Otol Rhinol Laryngol Suppl **148**: 23-27.
- Yoshimura, A., Y. Wakabayashi, et al. (2010). "Cellular and molecular basis for the regulation of inflammation by TGF-beta." Journal of biochemistry **147**(6): 781-792.
- Yücetürk, A. V., H. H. Unlü, et al. (1997). "The evaluation of eustachian tube function in patients with chronic otitis media." Clinical otolaryngology and allied sciences **22**(5): 449-452.
- Yueh, B., N. Shapiro, et al. (2003). "Screening and management of adult hearing loss in primary care: scientific review." Jama.
- Zhang, L., T. Y. Zhao, et al. (2013). "Generation and primary phenotypes of imidazoline receptor antisera-selected (IRAS) knockout mice." CNS Neurosci Ther **19**(12): 978-981.
- Zhang, X., J. E. Groopman, et al. (2005). "Extracellular matrix regulates endothelial functions through interaction of VEGFR-3 and integrin alpha5beta1." Journal of cellular physiology **202**(1): 205-214.
- Zhang, Y., H. Yu, et al. (2012). "Pathological features in the LmnaDhe/+ mutant mouse provide a novel model of human otitis media and laminopathies." The American journal of pathology **181**(3): 761-774.
- Zhang, Z., D. Alpert, et al. (2009). "Massively parallel sequencing identifies the gene Megf8 with ENU-induced mutation causing heterotaxy." Proceedings of the National Academy of Sciences of the United States of America **106**(9): 3219-3224.
- Zheng, Q. Y., R. Hardisty-Hughes, et al. (2006). "Mouse models as a tool to unravel the genetic basis for human otitis media." Brain Res **1091**(1): 9-15.

CHAPTER 10: Appendix

Appendix

Chromosome 1					
SNP location (Mb)	36	73	93	135	170
C3H	T	A	T	G	T
B6J	C	G	C	A	C
PEDV/7.1b	C3H	C3H	C3H	C3H	C3H
PEDV/7.9e	Het	Het	Het	Het	Het
EDSN/3.1c	C3H	C3H	C3H	C3H	C3H
EDSN/3.3a	C3H	C3H	C3H	C3H	C3H
EDSN/3.3c	C3H	C3H	C3H	C3H	C3H
EDSN/4.3a	C3H	Het	C3H	C3H	C3H
EDSN/6.1f	C3H	C3H	C3H	C3H	C3H
EDSN/5.3d	C3H	C3H	C3H	C3H	C3H
EDSN/6.7f	C3H	C3H	C3H	C3H	C3H
EDSN/5.5c	C3H	C3H	C3H	C3H	C3H
EDSN/6.4a	Het	C3H	C3H	C3H	C3H
EDSN/6.4d	C3H	C3H	C3H	C3H	C3H
EDSN/6.4e	C3H	C3H	C3H	C3H	C3H

Chromosome 2				
SNP location (Mb)	38	99	133	181
C3H	G	T	T	T
B6J	T	C	C	C
PEDV/7.1b	Het	B6J	Het	Het
PEDV/7.9e	C3H	Het	Het	Het
EDSN/3.1c	Het	C3H	C3H	C3H
EDSN/3.3a	C3H	Het	Het	C3H
EDSN/3.3c	Het	C3H	C3H	C3H
EDSN/4.3a	Het	F	C3H	C3H
EDSN/6.1f	C3H	C3H	C3H	C3H
EDSN/5.3d	Het	Het	Het	C3H
EDSN/6.7f	Het	Het	Het	C3H
EDSN/5.5c	Het	C3H	C3H	C3H
EDSN/6.4a	C3H	C3H	C3H	C3H
EDSN/6.4d	C3H	C3H	C3H	C3H
EDSN/6.4e	C3H	C3H	C3H	C3H

Chromosome 3				
SNP location (Mb)	26	88	105	132
C3H	G	C	C	A
B6J	A	A	T	G
PEDV/7.1b	Het	C3H	C3H	C3H
PEDV/7.9e	Het	Het	C3H	C3H
EDSN/3.1c	B6J	Het	Het	C3H
EDSN/3.3a	Het	C3H	C3H	C3H
EDSN/3.3c	C3H	C3H	C3H	C3H
EDSN/4.3a	C3H	C3H	C3H	C3H
EDSN/6.1f	Het	C3H	C3H	C3H
EDSN/5.3d	C3H	C3H	C3H	C3H
EDSN/6.7f	C3H	B6J	B6J	C3H
EDSN/5.5c	Het	Het	Het	C3H
EDSN/6.4a	C3H	C3H	C3H	C3H
EDSN/6.4d	C3H	C3H	C3H	C3H
EDSN/6.4e	C3H	C3H	C3H	C3H

Chromosome 4				
SNP location (Mb)	20	42	103	145
C3H	A	G	T	C
B6J	T	T	C	G
PEDV/7.1b	Het	Het	C3H	C3H
PEDV/7.9e	C3H	C3H	F	C3H
EDSN/3.1c	C3H	C3H	Het	Het
EDSN/3.3a	Het	F	C3H	F
EDSN/3.3c	C3H	C3H	C3H	C3H
EDSN/4.3a	C3H	C3H	C3H	C3H
EDSN/6.1f	Het	C3H	Het	B6J
EDSN/5.3d	Het	C3H	Het	B6J
EDSN/6.7f	Het	C3H	Het	Het
EDSN/5.5c	C3H	C3H	C3H	Het
EDSN/6.4a	C3H	C3H	C3H	C3H
EDSN/6.4d	Het	C3H	C3H	Het
EDSN/6.4e	C3H	C3H	Het	B6J

Chromosome 5				
SNP location (Mb)	23	76	104	137
C3H	T	G	T	C
B6J	G	A	C	T
PEDV/7.1b	Het	B6J	B6J	Het
PEDV/7.9e	C3H	B6J	B6J	Het
EDSN/3.1c	Het	C3H	Het	Het
EDSN/3.3a	Het	Het	Het	Het
EDSN/3.3c	B6J	B6J	B6J	C3H
EDSN/4.3a	C3H	Het	Het	Het
EDSN/6.1f	C3H	Het	C3H	C3H
EDSN/5.3d	Het	Het	Het	C3H
EDSN/6.7f	Het	B6J	B6J	F
EDSN/5.5c	Het	Het	Het	Het
EDSN/6.4a	Het	B6J	B6J	Het
EDSN/6.4d	Het	B6J	B6J	C3H
EDSN/6.4e	Het	B6J	Het	C3H

Chromosome 6					
SNP location (Mb)	22	52	87	115	146
C3H	A	G	T	T	T
B6J	C	T	C	C	G
PEDV/7.1b	B6J	B6J	B6J	Het	Het
PEDV/7.9e	Het	C3H	Het	Het	Het
EDSN/3.1c	Het	Het	C3H	C3H	C3H
EDSN/3.3a	C3H	Het	C3H	C3H	Het
EDSN/3.3c	C3H	C3H	C3H	C3H	C3H
EDSN/4.3a	Het	Het	C3H	C3H	C3H
EDSN/6.1f	C3H	Het	Het	Het	F
EDSN/5.3d	C3H	C3H	C3H	C3H	Het
EDSN/6.7f	C3H	C3H	C3H	F	F
EDSN/5.5c	Het	C3H	C3H	F	C3H
EDSN/6.4a	C3H	C3H	Het	Het	B6J
EDSN/6.4d	C3H	Het	C3H	C3H	C3H
EDSN/6.4e	Het	Het	Het	C3H	C3H

Chromosome 7			
SNP location (Mb)	11	54	102
C3H	G	C	G
B6J	T	A	A
PEDV/7.1b	F	C3H	C3H
PEDV/7.9e	Het	Het	B6J
EDSN/3.1c	C3H	C3H	Het
EDSN/3.3a	F	C3H	Het
EDSN/3.3c	C3H	C3H	Het
EDSN/4.3a	C3H	C3H	C3H
EDSN/6.1f	C3H	C3H	Het
EDSN/5.3d	C3H	C3H	C3H
EDSN/6.7f	C3H	C3H	Het
EDSN/5.5c	C3H	C3H	C3H
EDSN/6.4a	C3H	C3H	Het
EDSN/6.4d	C3H	C3H	Het
EDSN/6.4e	C3H	C3H	Het

Chromosome 8				
SNP location (Mb)	14	65	95	123
C3H	T	G	T	A
B6J	G	A	C	G
PEDV/7.1b	Het	B6J	B6J	Het
PEDV/7.9e	Het	Het	Het	Het
EDSN/3.1c	Het	C3H	Het	C3H
EDSN/3.3a	Het	C3H	Het	C3H
EDSN/3.3c	C3H	C3H	Het	C3H
EDSN/4.3a	B6J	C3H	Het	Het
EDSN/6.1f	Het	C3H	B6J	B6J
EDSN/5.3d	F	C3H	C3H	C3H
EDSN/6.7f	F	C3H	B6J	Het
EDSN/5.5c	F	C3H	B6J	B6J
EDSN/6.4a	C3H	Het	F	C3H
EDSN/6.4d	C3H	C3H	Het	C3H
EDSN/6.4e	F	C3H	B6J	B6J

Chromosome 9			
SNP location (Mb)	33	62	100
C3H	A	T	C
B6J	G	C	T
PEDV/7.1b	Het	Het	Het
PEDV/7.9e	Het	Het	C3H
EDSN/3.1c	C3H	C3H	C3H
EDSN/3.3a	C3H	C3H	C3H
EDSN/3.3c	C3H	C3H	C3H
EDSN/4.3a	C3H	Het	C3H
EDSN/6.1f	C3H	C3H	C3H
EDSN/5.3d	C3H	C3H	C3H
EDSN/6.7f	C3H	C3H	C3H
EDSN/5.5c	C3H	C3H	C3H
EDSN/6.4a	C3H	C3H	C3H
EDSN/6.4d	C3H	C3H	C3H
EDSN/6.4e	C3H	C3H	C3H

Appendix

Chromosome 10				
SNP location (Mb)	28	56	98	
C3H	A	C	T	
B6J	G	T	A	
PEDV/7.1b	Het	Het	Het	
PEDV/7.9e	C3H	C3H	C3H	
EDSN/3.1c	Het	Het	Het	
EDSN/3.3a	Het	C3H	C3H	
EDSN/3.3c	Het	F	B6J	
EDSN/4.3a	C3H	C3H	C3H	
EDSN/6.1f	Het	C3H	C3H	
EDSN/5.3d	Het	Het	B6J	
EDSN/6.7f	C3H	C3H	Het	
EDSN/5.5c	Het	Het	C3H	
EDSN/6.4a	C3H	C3H	C3H	
EDSN/6.4d	Het	Het	Het	
EDSN/6.4e	B6J	Het	Het	

Chromosome 11				
SNP location (Mb)	22	54	94	
C3H	A	C	T	
B6J	C	A	C	
PEDV/7.1b	Het	Het	Het	
PEDV/7.9e	Het	C3H	C3H	
EDSN/3.1c	C3H	C3H	C3H	
EDSN/3.3a	C3H	C3H	C3H	
EDSN/3.3c	C3H	C3H	C3H	
EDSN/4.3a	C3H	C3H	C3H	
EDSN/6.1f	C3H	C3H	C3H	
EDSN/5.3d	C3H	C3H	C3H	
EDSN/6.7f	C3H	C3H	C3H	
EDSN/5.5c	C3H	C3H	C3H	
EDSN/6.4a	C3H	C3H	C3H	
EDSN/6.4d	C3H	C3H	C3H	
EDSN/6.4e	C3H	C3H	C3H	

Chromosome 12			
SNP location (Mb)	24	68	85
C3H	C	A	C
B6J	T	T	T
PEDV/7.1b	C3H	C3H	C3H
PEDV/7.9e	C3H	C3H	C3H
EDSN/3.1c	C3H	Het	C3H
EDSN/3.3a	C3H	C3H	C3H
EDSN/3.3c	C3H	C3H	C3H
EDSN/4.3a	C3H	C3H	C3H
EDSN/6.1f	C3H	C3H	C3H
EDSN/5.3d	C3H	Het	C3H
EDSN/6.7f	C3H	C3H	C3H
EDSN/5.5c	C3H	C3H	C3H
EDSN/6.4a	C3H	C3H	C3H
EDSN/6.4d	C3H	C3H	C3H
EDSN/6.4e	C3H	C3H	C3H

LEGEND	
C3H	C3H/HeH
Het	C3H/HeH; C57BL/6J
B6J	C57BL/6J
F	Failed 2x

Chromosome 13			
SNP location (Mb)	20	43	93
C3H	A	G	A
B6J	G	T	G
PEDV/7.1b	Het	Het	Het
PEDV/7.9e	C3H	C3H	Het
EDSN/3.1c	C3H	Het	Het
EDSN/3.3a	C3H	C3H	B6J
EDSN/3.3c	C3H	Het	C3H
EDSN/4.3a	B6J	B6J	B6J
EDSN/6.1f	C3H	C3H	C3H
EDSN/5.3d	C3H	C3H	C3H
EDSN/6.7f	C3H	C3H	C3H
EDSN/5.5c	C3H	C3H	C3H
EDSN/6.4a	Het	Het	Het
EDSN/6.4d	Het	C3H	C3H
EDSN/6.4e	B6J	B6J	C3H

Chromosome 14				
SNP location (Mb)	8	22	59	80
C3H	C	A	T	T
B6J	T	G	G	C
PEDV/7.1b	Het	Het	C3H	C3H
PEDV/7.9e	B6J	B6J	Het	Het
EDSN/3.1c	B6J	B6J	C3H	C3H
EDSN/3.3a	B6J	B6J	C3H	C3H
EDSN/3.3c	Het	Het	C3H	C3H
EDSN/4.3a	B6J	B6J	C3H	C3H
EDSN/6.1f	B6J	B6J	C3H	C3H
EDSN/5.3d	B6J	B6J	C3H	C3H
EDSN/6.7f	Het	Het	C3H	C3H
EDSN/5.5c	C3H	C3H	C3H	C3H
EDSN/6.4a	B6J	B6J	C3H	Het
EDSN/6.4d	B6J	B6J	C3H	Het
EDSN/6.4e	B6J	B6J	C3H	C3H

Chromosome 15			
SNP location (Mb)	32	52	80
C3H	A	T	C
B6J	G	C	T
PEDV/7.1b	Het	Het	C3H
PEDV/7.9e	C3H	C3H	C3H
EDSN/3.1c	C3H	C3H	F
EDSN/3.3a	C3H	C3H	C3H
EDSN/3.3c	C3H	C3H	C3H
EDSN/4.3a	B6J	B6J	B6J
EDSN/6.1f	Het	Het	Het
EDSN/5.3d	C3H	C3H	C3H
EDSN/6.7f	C3H	C3H	Het
EDSN/5.5c	Het	Het	B6J
EDSN/6.4a	C3H	C3H	B6J
EDSN/6.4d	C3H	C3H	C3H
EDSN/6.4e	Het	B6J	B6J

Chromosome 16		
SNP location (Mb)	37	59
C3H	C	A
B6J	T	G
PEDV/7.1b	Het	C3H
PEDV/7.9e	Het	Het
EDSN/3.1c	Het	Het
EDSN/3.3a	B6J	B6J
EDSN/3.3c	B6J	B6J
EDSN/4.3a	B6J	B6J
EDSN/6.1f	C3H	C3H
EDSN/5.3d	B6J	B6J
EDSN/6.7f	Het	Het
EDSN/5.5c	Het	Het
EDSN/6.4a	C3H	C3H
EDSN/6.4d	Het	Het
EDSN/6.4e	Het	Het

Chromosome 17		
SNP location (Mb)	31	71
C3H	A	C
B6J	G	T
PEDV/7.1b	C3H	Het
PEDV/7.9e	C3H	C3H
EDSN/3.1c	C3H	Het
EDSN/3.3a	C3H	C3H
EDSN/3.3c	C3H	C3H
EDSN/4.3a	B6J	Het
EDSN/6.1f	C3H	Het
EDSN/5.3d	C3H	Het
EDSN/6.7f	C3H	Het
EDSN/5.5c	C3H	Het
EDSN/6.4a	Het	C3H
EDSN/6.4d	C3H	Het
EDSN/6.4e	C3H	B6J

Chromosome 18		
SNP location (Mb)	25.4	53.7
C3H	C	G
B6J	G	C
PEDV/7.1b	Het	Het
PEDV/7.9e	C3H	Het
EDSN/3.1c	C3H	C3H
EDSN/3.3a	C3H	C3H
EDSN/3.3c	C3H	C3H
EDSN/4.3a	C3H	C3H
EDSN/6.1f	Het	Het
EDSN/5.3d	C3H	C3H
EDSN/6.7f	Het	Het
EDSN/5.5c	C3H	C3H
EDSN/6.4a	Het	C3H
EDSN/6.4d	Het	Het
EDSN/6.4e	C3H	C3H

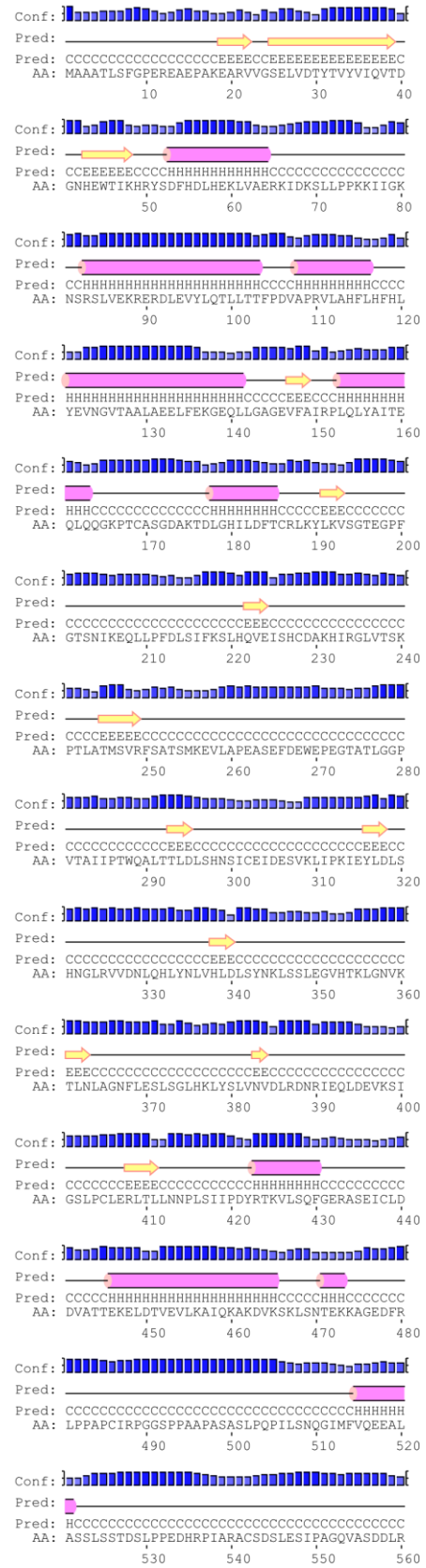
Chromosome 19		
SNP location (Mb)	19	56
C3H	A	T
B6J	C	C
PEDV/7.1b	Het	Het
PEDV/7.9e	C3H	C3H
EDSN/3.1c	C3H	C3H
EDSN/3.3a	C3H	C3H
EDSN/3.3c	C3H	C3H
EDSN/4.3a	C3H	C3H
EDSN/6.1f	C3H	C3H
EDSN/5.3d	C3H	C3H
EDSN/6.7f	C3H	C3H
EDSN/5.5c	C3H	C3H
EDSN/6.4a	B6J	C3H
EDSN/6.4d	C3H	C3H
EDSN/6.4e	C3H	C3H

Figure 10.1| Whole genome scan using 63 SNP markers and 13 affected *edison* mice with OM shows a strong linkage to Chromosome 14.

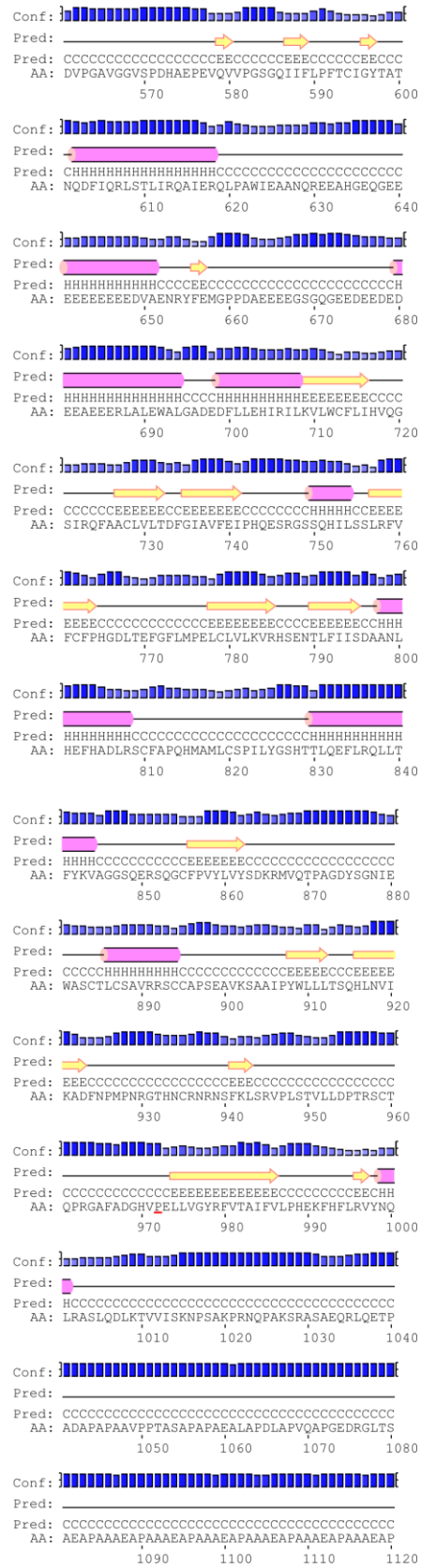
Table 10.1| Annotations used to categorise the single nucleotide variants identified through NGS.

NGS annotations	Definition
Essential Splice Site	located in the first two or the last two bases of an intron
Stop Gained	adds a stop codon to a coding sequence
Stop Lost	removes a stop codon from a coding sequence
Non-Synonymous Coding	changes a coding sequence such that an amino acid change occurs in the encoded protein sequence
Splice Site	located 1-3 bases into an exon or 3-8 bases into an intron
Partial Codon	located within the final, incomplete codon of a transcript with a shortened coding sequence where the end is unknown
Synonymous Coding	changes a coding sequence such that no amino acid change occurs in the encoded protein sequence
Regulatory Region	located in regulatory regions annotated in Ensembl
Within Mature miRNA	located within a microRNA
5' UTR	located in the five prime untranslated region (5' UTR) of a coding transcript
3' UTR	located in the three prime untranslated region (3' UTR) of a coding transcript
Intronic	located in the intron of a gene
Within Non-Coding Gene	located within a gene that does not code for a protein
Upstream	within 5 kb upstream of the 5' end of a transcript
Downstream	within 5 kb downstream of the 3' end of a transcript
Intergenic	more than 5 kb either upstream or downstream of a transcript

Nisch^{edsn}



Nisch^{edsn}



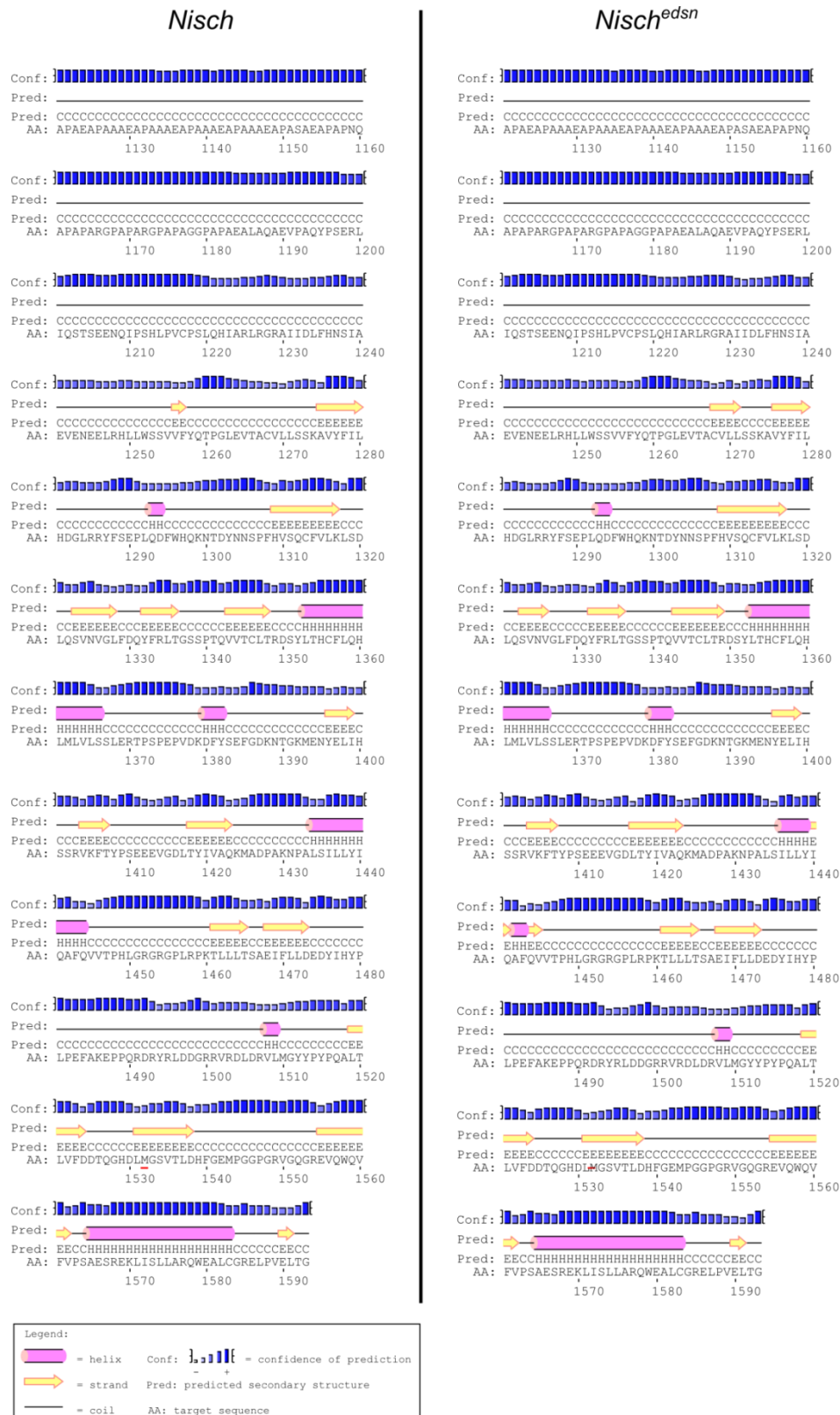


Figure 10.2| DomPred protein domain prediction of NISCH.

Computational analysis using the DomPred protein domain prediction server showed that the L972P substitution in *edison* NISCH would result in a significant change in the predicted domain structure.

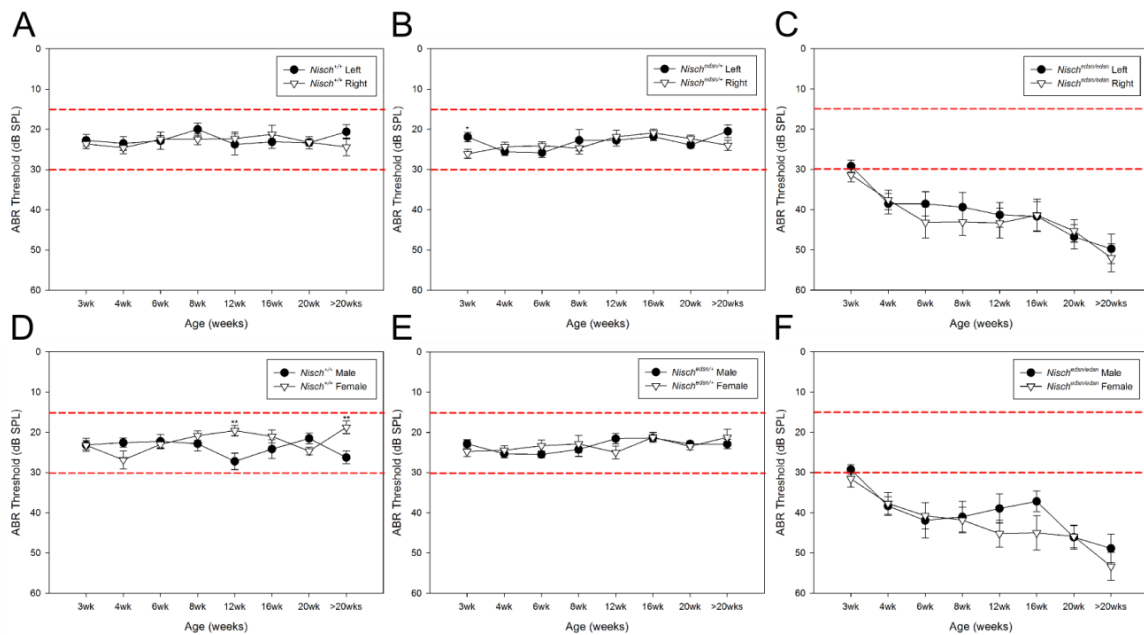


Figure 10.3| Comparisons of click-evoked ABR thresholds in *edison* mice for left-right orientation and gender.

(A-C) Left-right orientation comparisons of mean ABR thresholds for (A) $Nisch^{+/+}$, (B) $Nisch^{edsn/+}$ and (C) $Nisch^{edsn/edsn}$ mice. **(D-F)** Gender comparisons of mean ABR thresholds for (D) $Nisch^{+/+}$, (E) $Nisch^{edsn/+}$ and (F) $Nisch^{edsn/edsn}$ mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). * $P < 0.05$; ** $P < 0.01$.

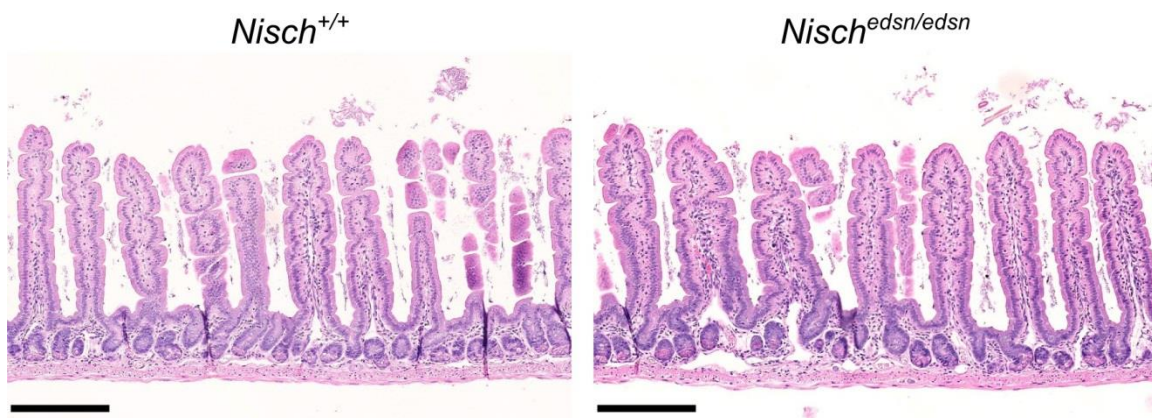


Figure 10.4| Assessment of colon from $Nisch^{edsn/edsn}$ and wild-type animals.

Representative H&E stained section from a $Nisch^{+/+}$ and $Nisch^{edsn/edsn}$ animal showing a normal colon with no inflammation. There are no differences observed between genotypes in colon morphology. Scale bar = 200 μ m.

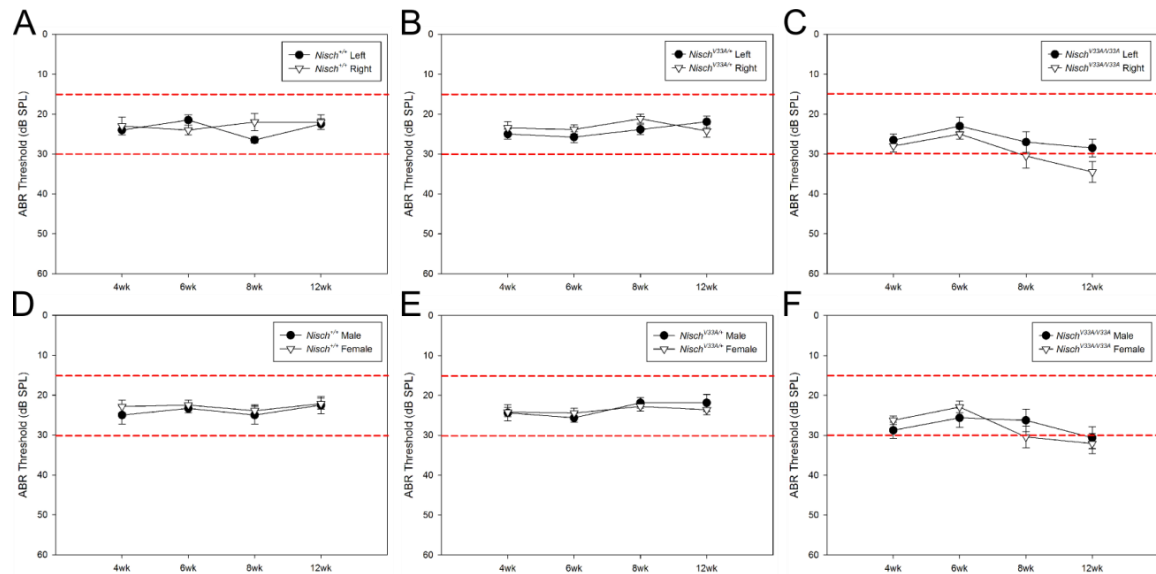


Figure 10.5| Comparisons of click-evoked ABR thresholds in *Nisch*^{V33A}|*Nisch*^{V33A} littermates for left-right orientation and gender.

(A-C) Left-right orientation comparisons of mean ABR thresholds for (A) *Nisch*^{+/+}, (B) *Nisch*^{V33A/+} and (C) *Nisch*^{V33A/V33A} mice. **(D-F)** Gender comparisons of mean ABR thresholds for (D) *Nisch*^{+/+}, (E) *Nisch*^{V33A/+} and (F) *Nisch*^{V33A/V33A} mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines).

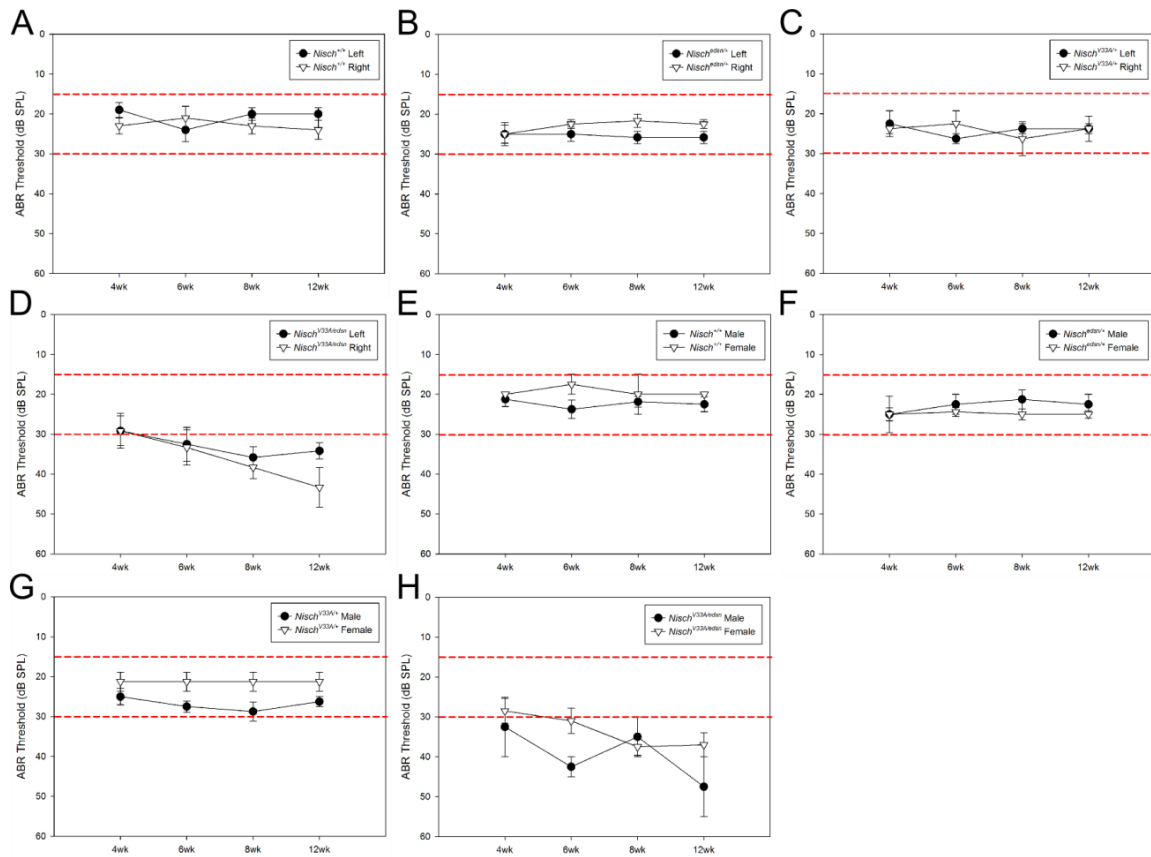


Figure 10.6] Comparisons of click-evoked ABR thresholds in $Nisch^{V33A}|Nisch^{edn}$ littermates for left-right orientation and gender.

(A-D) Left-right orientation comparisons of mean ABR thresholds for (A) $Nisch^{+/+}$, (B) $Nisch^{edn/+}$, (C) $Nisch^{V33A/+}$ and (D) $Nisch^{V33A/edn}$ mice. **(E-H)** Gender comparisons of mean ABR thresholds for (E) $Nisch^{+/+}$, (F) $Nisch^{edn/+}$, (G) $Nisch^{V33A/+}$ and (H) $Nisch^{V33A/edn}$ mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines).

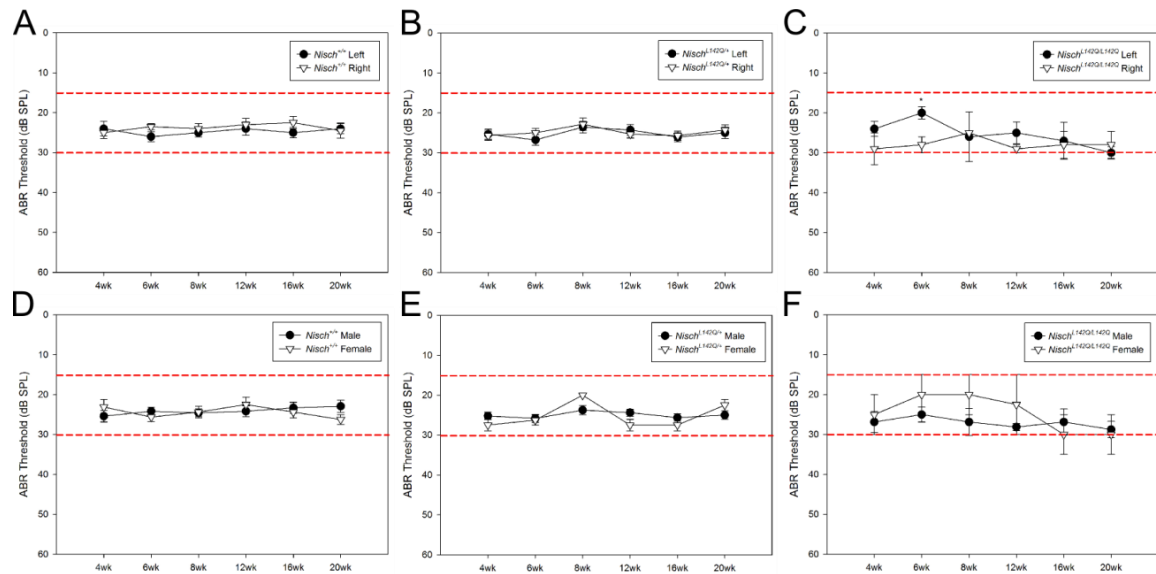


Figure 10.7| Comparisons of click-evoked ABR thresholds in *Nisch*^{L142Q}/*Nisch*^{L142Q} littermates for left-right orientation and gender.

(A-C) Left-right orientation comparisons of mean ABR thresholds for (A) *Nisch*^{+/+}, (B) *Nisch*^{L142Q/+} and (C) *Nisch*^{L142Q/L142Q} mice. **(D-F)** Gender comparisons of mean ABR thresholds for (D) *Nisch*^{+/+}, (E) *Nisch*^{L142Q/+} and (F) *Nisch*^{L142Q/L142Q} mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines).

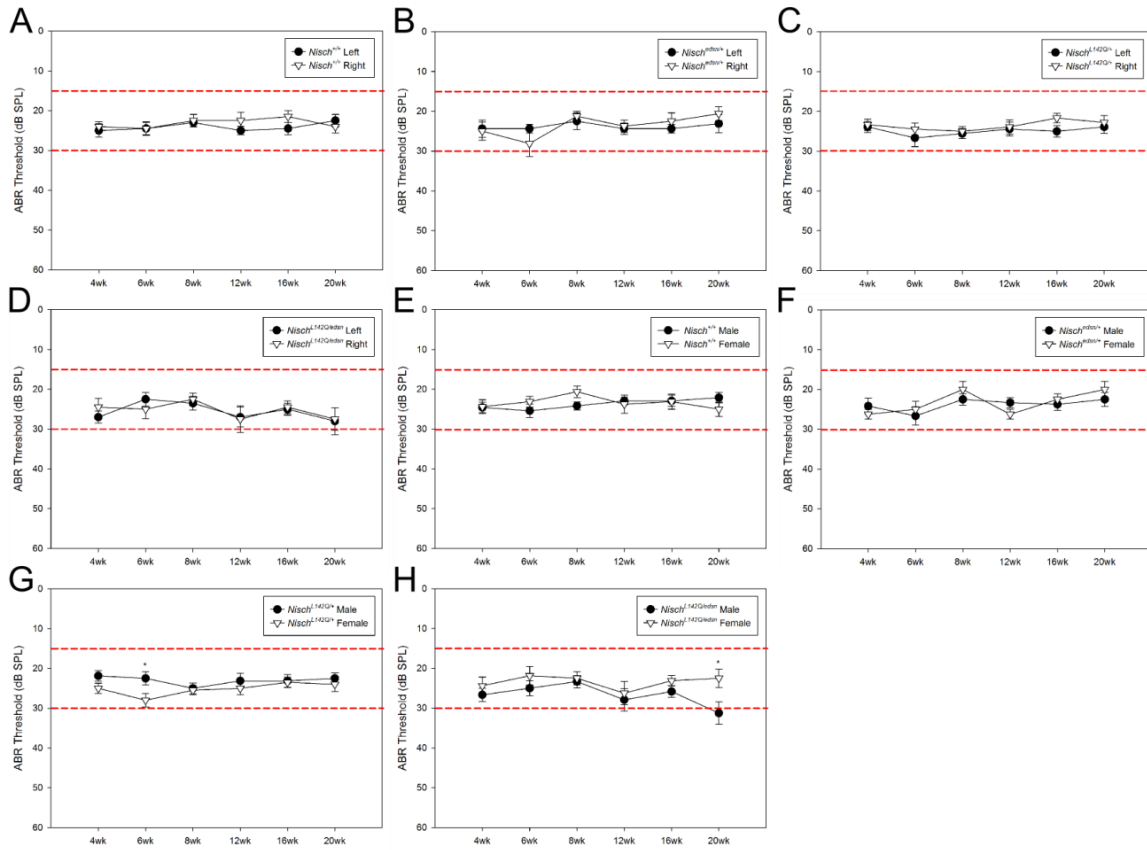


Figure 10.8| Comparisons of click-evoked ABR thresholds in $Nisch^{L142Q}/Nisch^{edn}$ littermates for left-right orientation and gender.

(A-D) Left-right orientation comparisons of mean ABR thresholds for (A) $Nisch^{+/+}$, (B) $Nisch^{edn/+}$, (C) $Nisch^{L142Q/+}$ and (D) $Nisch^{L142Q/edn}$ mice. **(E-H)** Gender comparisons of mean ABR thresholds for (E) $Nisch^{+/+}$, (F) $Nisch^{edn/+}$, (G) $Nisch^{L142Q/+}$ and (H) $Nisch^{L142Q/edn}$ mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). * $P < 0.05$.

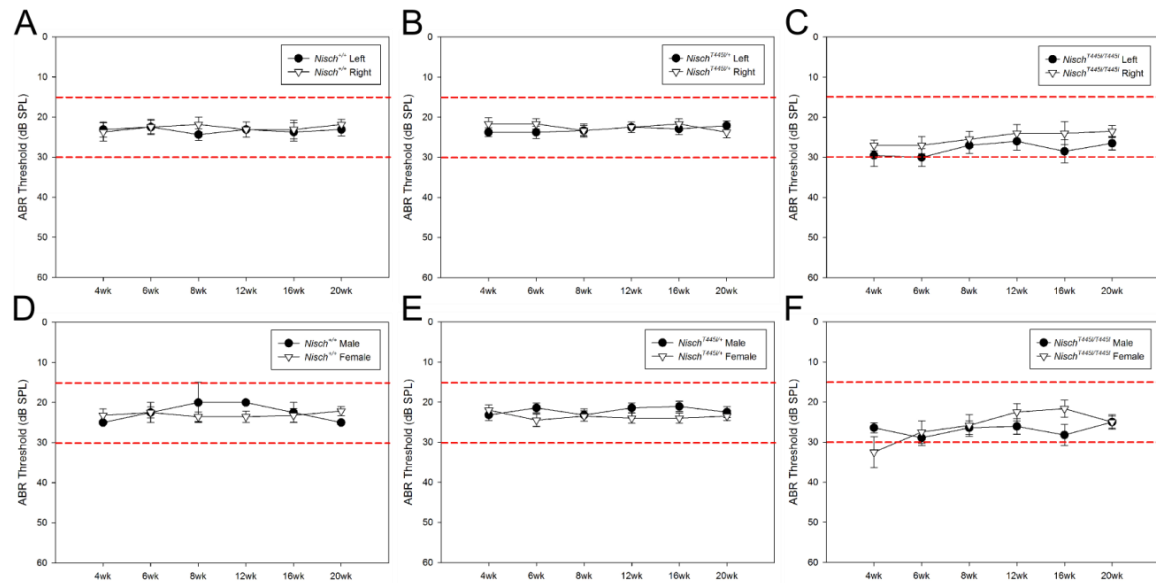


Figure 10.9| Comparisons of click-evoked ABR thresholds in $Nisch^{T445I}/Nisch^{T445I}$ littermates for left-right orientation and gender.

(A-C) Left-right orientation comparisons of mean ABR thresholds for (A) $Nisch^{+/+}$, (B) $Nisch^{T445I/+}$ and (C) $Nisch^{T445I/T445I}$ mice. **(D-F)** Gender comparisons of mean ABR thresholds for (D) $Nisch^{+/+}$, (E) $Nisch^{T445I/+}$ and (F) $Nisch^{T445I/T445I}$ mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines).

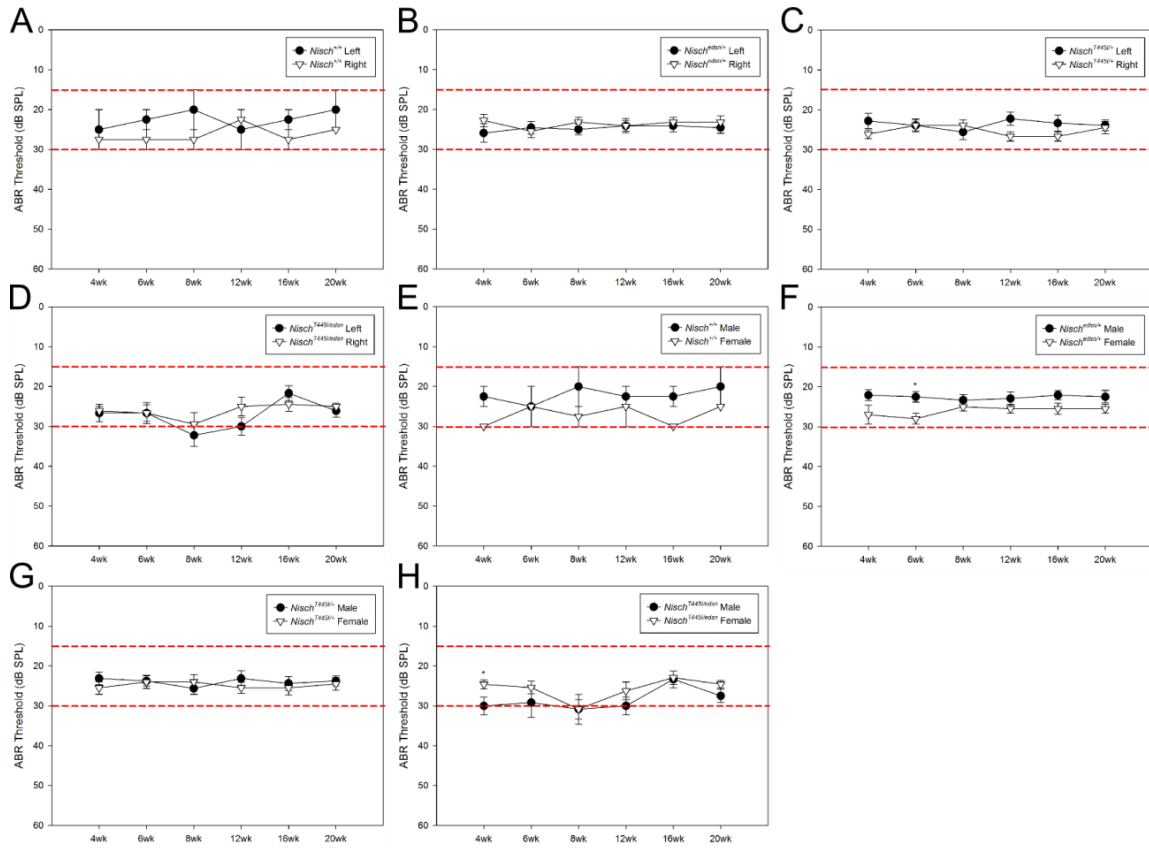


Figure 10.10| Comparisons of click-evoked ABR thresholds in *Nisch*^{T445I}/*Nisch*^{edn} littermates for left-right orientation and gender.

(A-D) Left-right orientation comparisons of mean ABR thresholds for (A) *Nisch*^{+/+}, (B) *Nisch*^{edn/+}, (C) *Nisch*^{T445I/+} and (D) *Nisch*^{T445I/edn} mice. **(E-H)** Gender comparisons of mean ABR thresholds for (E) *Nisch*^{+/+}, (F) *Nisch*^{edn/+}, (G) *Nisch*^{T445I/+} and (H) *Nisch*^{T445I/edn} mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). * $P < 0.05$.

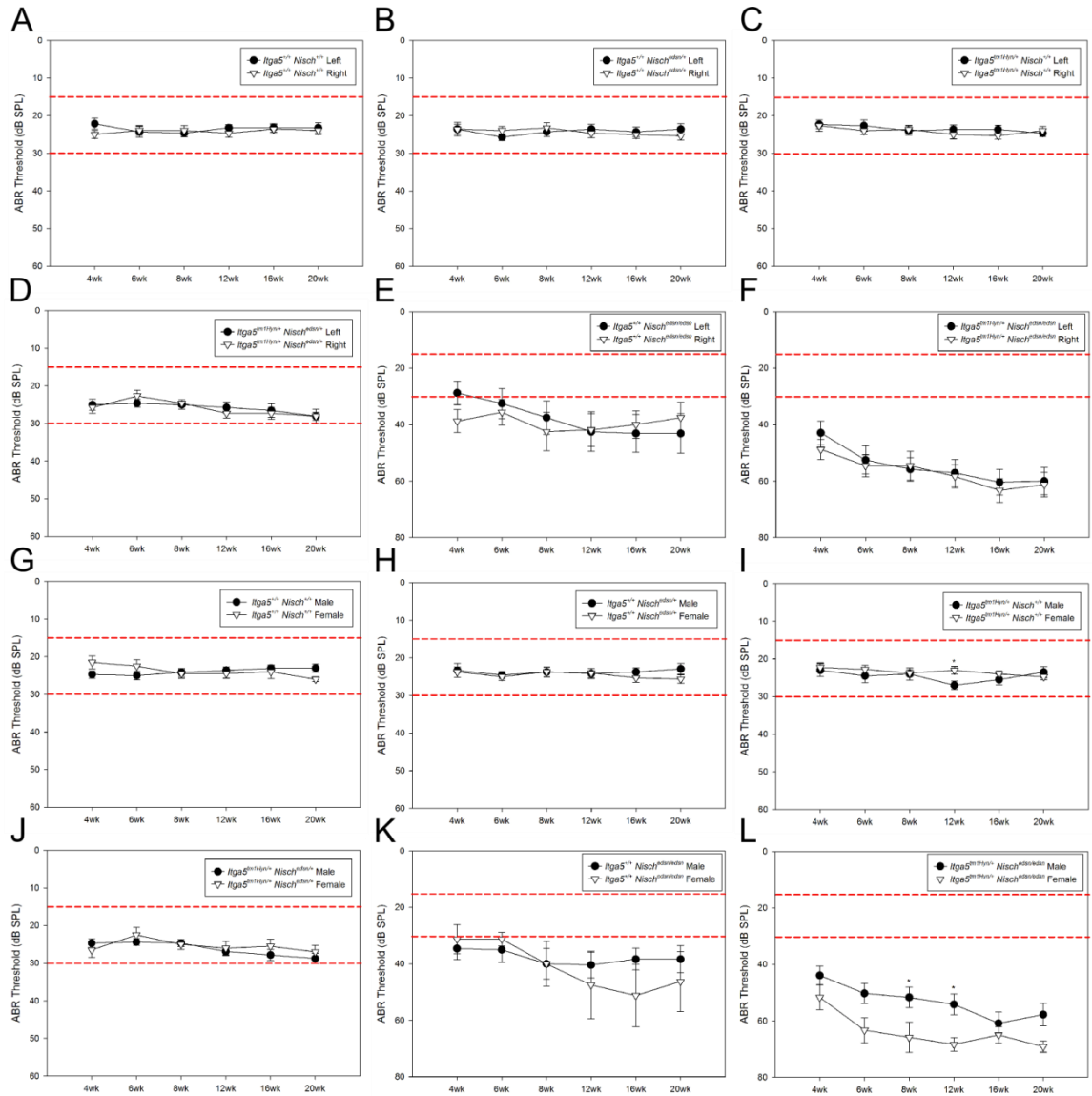


Figure 10.11| Comparisons of click-evoked ABR thresholds in *Itga5^{tm1Hyn}|Nisch^{edsn}* littermates for left-right orientation and gender.

(A-F) Left-right orientation comparisons of mean ABR thresholds for (A) *Itga5^{+/+} Nisch^{+/+}*, (B) *Itga5^{+/+} Nisch^{edsn/+}*, (C) *Itga5^{tm1Hyn/+} Nisch^{+/+}*, (D) *Itga5^{tm1Hyn/+} Nisch^{edsn/+}*, (E) *Itga5^{+/+} Nisch^{edsn/edsn}* and (F) *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. **(G-L)** Gender comparisons of mean ABR thresholds for (G) *Itga5^{+/+} Nisch^{+/+}*, (H) *Itga5^{+/+} Nisch^{edsn/+}*, (I) *Itga5^{tm1Hyn/+} Nisch^{+/+}*, (J) *Itga5^{tm1Hyn/+} Nisch^{edsn/+}*, (K) *Itga5^{+/+} Nisch^{edsn/edsn}* and (L) *Itga5^{tm1Hyn/+} Nisch^{edsn/edsn}* mice. Expected ABR threshold range for normal hearing was between 15-30 dB SPL (dashed red lines). * P < 0.05.

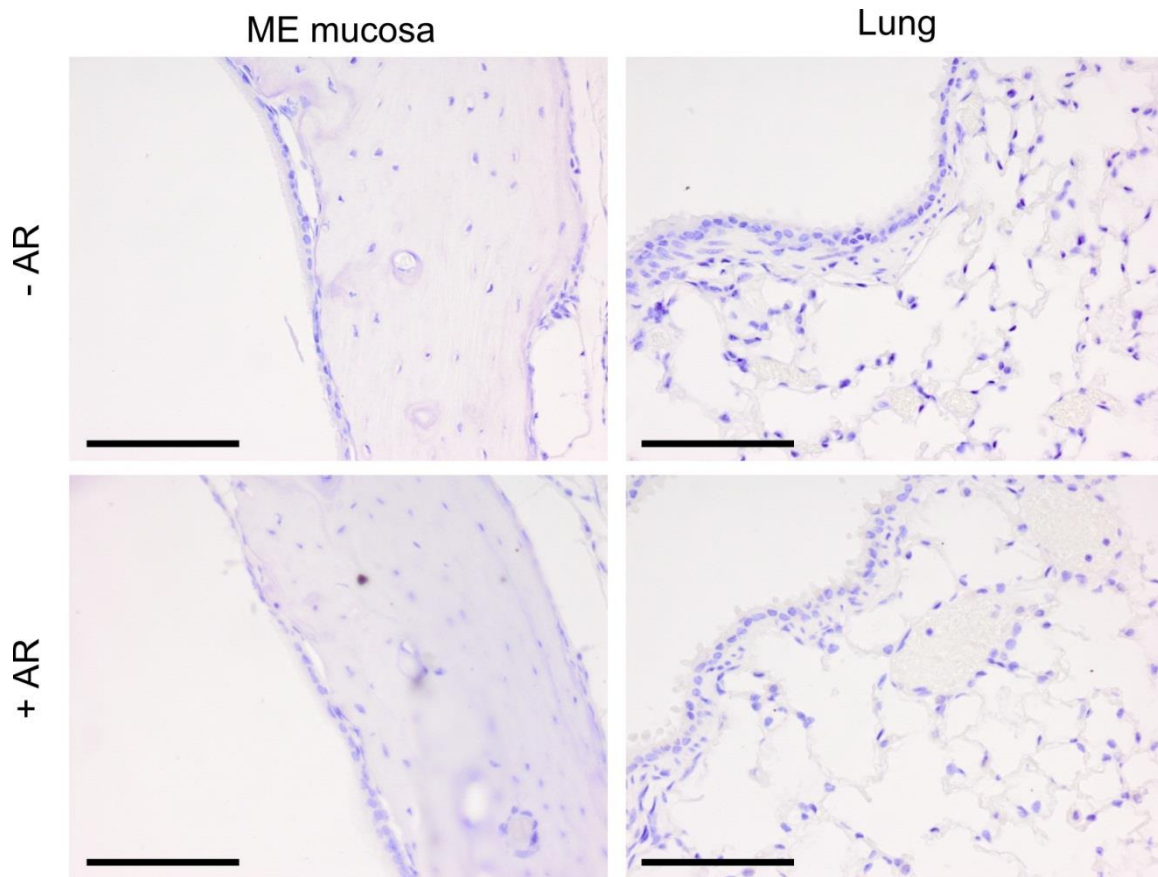


Figure 10.12| Negative control for IHC of ME and lung.

Representative ME and lung sections, with and without antigen retrieval (+AR/-AR), with no primary antibody showing no background staining. Scale bar = 100 μ m.