

A novel role for *Atmin* as a transcription factor controlling ciliogenesis

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Abstract

Cilia are cellular organelles involved in processing components of the hedgehog (hh) signalling pathway and determining left-right (L-R) axis formation in the embryo. An embryonic lethal mouse mutant, called *gasping 6* (*gpg6*), was identified that demonstrated morphological and molecular defects associated with L-R development and hh signalling. *gpg6* mutant embryos also demonstrate abnormally short cilia, which was hypothesised to be the primary morphological defect in *gpg6* mutants.

The underlying genetic lesion in *gpg6* is a mutation in the DNA repair gene *Atmin*. The base pair change results in an amino acid substitution in a critical residue in the third zinc finger of *Atmin*. The consequence of this change is the failure to activate transcriptional targets of *Atmin*. This raised the possibility that previously unidentified *Atmin* target genes are important for ciliogenesis. Consistent with this hypothesis, *Dynein light chain-LC8* (*Dynll1*) is downregulated in *gpg6* mutants. *LC8* (a homolog of mouse *Dynll1*) is required, in the single cell eukaryotic organism *Chlamydomonas*, for retrograde intraflagellar transport (IFT), a process crucial for ciliogenesis.

These data led to the following hypothesis: *Atmin* activates expression of *Dynll1*, which functions in retrograde IFT to enable normal ciliogenesis. Knockdown of *Atmin* in a ciliated kidney cell line resulted in abnormally short cilia. Thus, *Atmin* functions in ciliogenesis. Investigation of *gpg6* has therefore identified a novel role for *Atmin* in ciliogenesis and has added to the growing knowledge of genes that control cilia formation and embryonic development.

Abbreviations

53bp1	p53 interacting protein 1
A	Adenosine
AMP	Adenosine monophosphate
Amp	Ampicillin
A-P	Antero-posterior
Atmin	ATM interacting protein
ATP	Adenosine Triphosphate
Balb/c	Balb/cAnn
BBR	Boehinger Mannheim Blocking Reagent
BBS	Bardet-Biedl syndrome
Bmp	Bone morphogenetic protein
bp	Base pair
C	Cytosine
C3H	C3H/HeH
Ca ²⁺	Calcium ions
Cetn4	Centrin 4
CICR	Calcium induced calcium release
cAMP	Cyclic AMP
cDNA	Copy DNA
ci	Cubitus interruptus
CO ₂	Carbon Dioxide
cos2	Costal 2
CSF	Cerebrospinal fluid

dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytosine Triphosphate
dGTP	Deoxyguanine Triphosphate
dTTP	Deoxythymine Triphosphate
dNTP	Mixture of dATP, dCTP, dGTP, dTTP
Dhh	Desert Hedgehog
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic Acid
DORV	Double outlet right ventricle
dpc	Days post coitum
dsRNA	Double stranded RNA
D-V	Dorso-ventral
Dynll1	Dynein light chain LC8-type 1
Dynlt1	Dynein light chain Tctex-type 1
EMMA	European Mouse Mutagenesis Consortium
ENU	N-ethyl-N-nitrosourea
ES cells	Embryonic stem cells
EUCOMM	European Conditional Mouse Mutagenesis Program
FCS	Fetal Calf Serum
Fgf	Fibroblast growth factor
fu	Fused
G	Guanine
G1/2/3	Generation 1/2/3

GFP	Green fluorescent protein
GKAP	Guanylate kinase domain associated protein
Gli3A	Activated Gli3
Gli3FL	Full length Gli3
Gli3R	Gli3 Repressor
GO	Gene Ontology
gpg	Gasping
hh	Hedgehog
Hyb	Hybridisation
IAA	Interrupted aortic arch
Ic	Intermediate chain
IFT	Intraflagellar Transport
Ihh	Indian hedgehog
IMCD3	Inner medullary collecting duct
IVF	In vitro fertilisation
kb	Kilobase
kDa	KiloDalton
KOMP	Knockout mouse project
KV	Kupffers Vesicle
LB	Luria Bertani
L-R	Left Right
MAB	Maleic acid buffer
MAP	Microtubule-associated protein

Mb	Megabase
MEFs	Mouse embryonic fibroblasts
Mg ²⁺	Magnesium
MRC	Medical Research Council
mRNA	Messenger RNA
NCBI	National Centre of Bioinformatics
NEB	New England Biolabs
NIH	National Institute of Health
NORCOMM	North America Conditional Mouse Mutagenesis Project
NP40	Nonidet P40
NTC	Non template control
NVPs	Nodal vesicular parcels
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
pBS	Bluescript plasmid
PBT	Phosphate Buffered Triton
PCR	Polymerase chain reaction
Pen/strep	Penicillin/streptomycin
PK	Proteinase K
Pka	Protein kinase A
PKD	Polycystic kidney disease
Ptch	Patched
RNA	Ribonucleic acid
RNAi	RNA interference

RISC	RNA induced silencing complex
RTPCR	Reverse transcriptase PCR
Shh	Sonic Hedgehog
siRNA	Short interfering RNA
Smo	Smoothed
SNPs	Single nucleotide polymorphisms
snRNA	Small nuclear RNA
Sufu	Suppressor of fused
T	Thymine
TBE	Tris borate EDTA
TGA	Transposition of the great arteries
TIGM	Texas A&M institute for genomic medicine
UV	Ultraviolet
WISH	Whole mount in situ hybridisation
YFP	Yellow fluorescent protein
ZPA	Zone of polarising activity

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Chapter 1

Introduction

1.1 Cilia

Cilia, once seen as relatively obscure organelles, have emerged during the past 12 years as key cellular structures that sense the extracellular environment and process intracellular signalling molecules to ensure correct embryonic development and adult homeostasis.

1.1.1 Structure of Cilia

Cilia were first described in the kidney and thyroid gland in 1898 (Badano et al., 2006; Zimmerman, 1898) and have since been found on nearly every vertebrate cell (Gherman et al., 2006). Cilia project from the cell surface, can vary in length (typically 3-5 μ m), and contain a central structure called the axoneme, which is made up of nine doublet microtubules. The axoneme is a polarised structure, containing arrays of minus to plus end microtubule subunits orientated from the base to the tip of cilia (Allen and Borisy, 1974). Two types of cilia are observed, motile and non-motile (Porter, 1955). Typically motile cilia, in addition to the nine doublet microtubules, contain a central microtubule pair (9+2) and each of these microtubule doublets contains inner and outer dynein arms (Barnes, 1961; Porter, 1955). Radial spokes are also present in motile cilia and attach to the outer doublet microtubule and central pair. They functionally link the central microtubules to the dynein arms to control their function in cilia motility (Goodenough and Heuser, 1985; Warner and Satir, 1974; Witman et al., 1978). Non-motile, primary cilia (9+0) lack the central pair, dynein arms and radial spokes (Barnes, 1961; Porter, 1955). In all cilia the axoneme is surrounded by a ciliary membrane, which is continuous with the plasma membrane of the

cell (Barnes, 1961; Porter, 1955) (Ringo, 1967). The base of the cilium is made up of the basal body, a structure that develops from a mature centriole (Barnes, 1961; Porter, 1955; Rieder et al., 1979).

1.1.2 Cilia and the Cell Cycle

In 1979, Tucker and colleagues made the important observation that NIH 3T3 cells grown in low serum, or at high cell density, formed more cilia. In these growth conditions they observed cells entering G0 phase of the cell cycle and forming primary cilia from an unduplicated centriole. When serum was added back to growth media cells responded by re-entering the cell cycle, disassembling their cilia and duplicating the deciliated centriole ready for mitosis. Thus, they demonstrated that assembly and disassembly of cilia is intimately linked to the cell cycle (Tucker et al., 1979).

1.1.3 Intraflagellar Transport

The data generated by Tucker and colleagues (1979) raised the question of how the cell mechanically builds and dismantles the cilium during the cell cycle. Work on the equivalent structure, the flagella, of the single cell eukaryotic organism *Chlamydomonas* suggested an answer. Flagella are normally motile and propel *Chlamydomonas* through the water.

Kozminski and colleagues (1993) analysed paralysed mutant flagella and observed by DIC optics movement of particles (initially called “rafts”) up and down the length of flagella. They called this movement “Intraflagellar Transport” (IFT). It was discovered that the rate of base to tip movement (“anterograde IFT”) is slower than tip to base movement

(“retrograde IFT”), suggesting that separate molecular motors drive anterograde IFT and retrograde IFT (Kozminski et al., 1993).

IFT rafts were successfully extracted from flagella and initially found to contain 13 proteins (Piperno and Mead, 1997) although this was later revised to 15 proteins (Cole et al., 1998). Further analysis of these 15 proteins identified two complexes, complex A (comprising four proteins) and complex B (comprising 11 proteins). These complexes were collectively called IFT particles (Cole et al., 1998).

Subsequent studies in *Chlamydomonas* attempted to identify the IFT molecular motors; analysis of temperature sensitive mutants affecting a component of Kinesin II (see below), showed a failure of anterograde IFT. This revealed Kinesin II to be the molecular motor responsible for anterograde IFT (Cole et al., 1998; Kozminski et al., 1995; Piperno and Mead, 1997; Walther et al., 1994). An insertional mutant affecting the Cytoplasmic Dynein (see below) component *LC8* (the homolog of mouse *DynIII*) demonstrated short flagella and an accumulation of IFT particles at the flagella tip caused by a lack of retrograde IFT. This demonstrated that Cytoplasmic Dynein is the molecular motor responsible for retrograde IFT (Pazour et al., 1998).

Collectively, this led to the following model of IFT: flagella components (e.g. Kinesin II, Cytoplasmic Dynein, IFT particle complexes A and B, structural components of flagella and ATP) accumulate at the basal body. Kinesin II then complexes with IFT particles, which bind other flagella components (including Cytoplasmic Dynein). The anterograde IFT motor Kinesin II is activated and transports its cargo along microtubules to the flagella tip. Once at

the flagella tip, Kinesin II is inactivated and flagella components dissociate from IFT particles. Flagella proteins present at the tip that are targeted for turnover become bound to IFT particles and Cytoplasmic Dynein. The retrograde IFT motor Cytoplasmic Dynein is then activated and transports its cargo back towards the basal body (Figure 1) (Cole et al., 1998; Iomini et al., 2001; Qin et al., 2004). IFT was also observed in the sensory neuron cilia of *C.elegans* utilising GFP-tagged Kinesin II and IFT particles, thereby demonstrating that IFT is a common mechanism for building and dismantling both flagella and cilia (Orozco et al., 1999).

This model was refined with three observations. Firstly, IFT particles are transported from the golgi apparatus to cilia, suggesting they are specifically targeted to the basal body from the golgi (Follit et al., 2006). Secondly, two distinct phases of anterograde IFT powered by different anterograde motors were seen in *C.elegans* neuron sensory cilia. Kinesin II was shown to drive anterograde IFT along the middle segment of cilia while OSM3-Kinesin drives IFT particles throughout the entire cilia. Mutant analysis showed these motors have redundant roles in the middle segment of cilia (Snow et al., 2004). Thirdly, it was shown that cilia disassembly is induced by HEF1 dependent activation of Aurora A, which in turn activates the tubulin deacetylase HDAC6 (Pugacheva et al., 2007). Aurora A is also activated by *Pitchfork*, a gene required for cilia disassembly, centrosome release, mitosis and L-R development (Kinzel et al., 2010).

Recently, it has been shown in mouse cells that increasing levels of intracellular cyclic AMP (cAMP) inhibits release of intracellular pools of calcium (Ca^{2+}) from cells, activates Protein kinase A (PKA) and induces longer cilia. An increase in the rate of anterograde IFT was

shown to occur in the absence of Ca^{2+} , which was proposed to be responsible for the increase in cilia assembly and cilia length. Interestingly, Ca^{2+} levels were increased by cilia-based flow sensing via Pkd2, leading to reductions in levels of cAMP. Therefore, Pkd1/Pkd2-dependent release of Ca^{2+} in response to flow regulates the levels of cAMP, the activation of PKA, the rate of anterograde IFT and cilia length (Besschetnova et al., 2010).

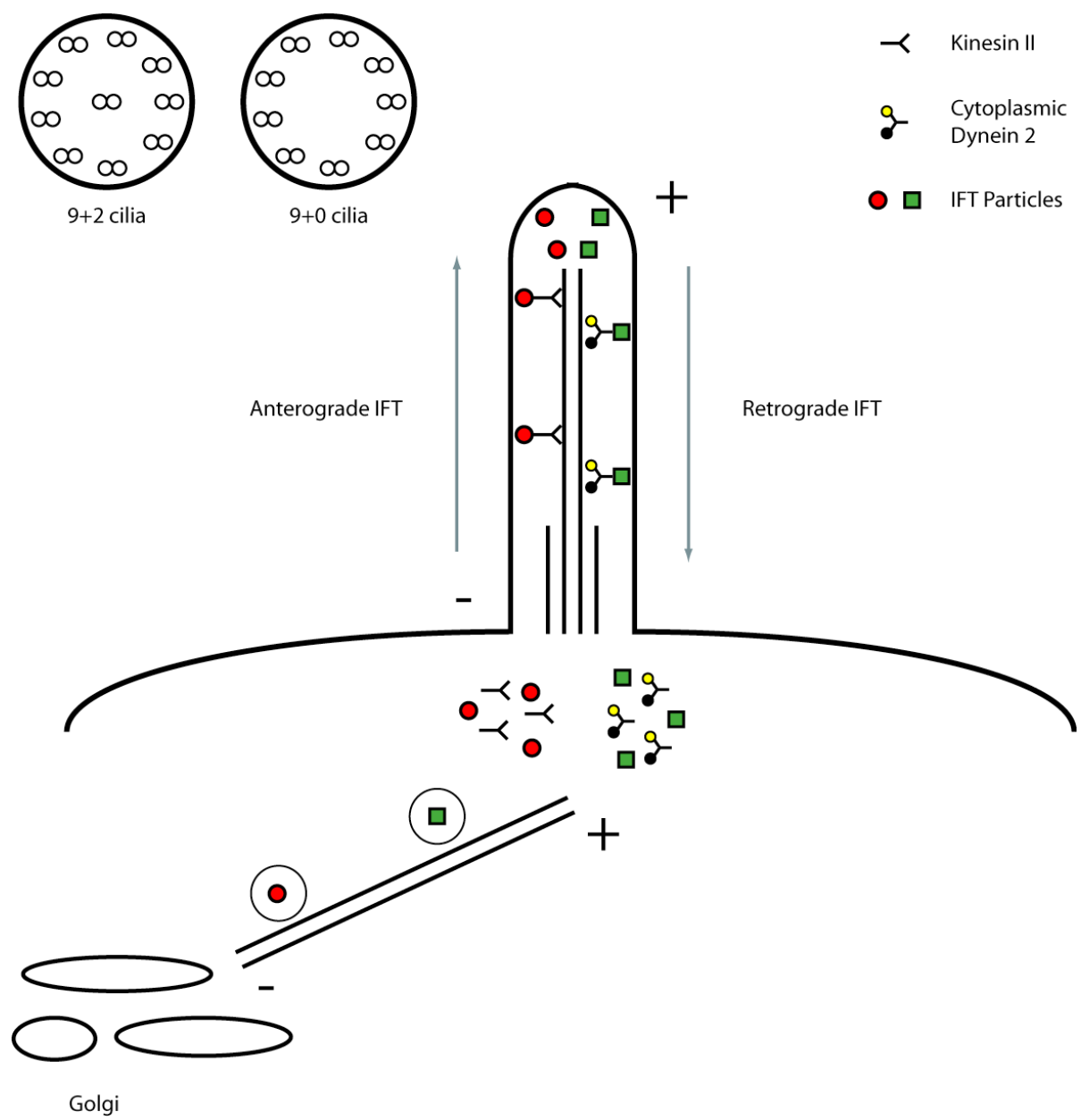


Figure 1. Model of Intraflagellar Transport (IFT)

Cilia are organelles that project from the cell surface and are typically made up of 9 microtubule doublets with (“9+2”) or without (“9+0”) a central microtubule doublet.

Intraflagellar transport (IFT) is a specialised transport mechanism within cilia. Components of the IFT machinery (including Kinesin II, Cytoplasmic Dynein 2 and IFT particles) and their cargo accumulate at the base of cilia. The molecular motor Kinesin II transports these components from the base to the tip of cilia (“anterograde IFT”). Conversely, the molecular motor Cytoplasmic Dynein 2 transports these components from the tip to the base of cilia (“retrograde IFT”).

1.1.4 The Anterograde Motor Kinesin II

Kinesin II was first identified as a component of squid axoplasm and optic lobes that caused ATP-dependent movement of purified microtubules along a glass slide (Vale et al., 1985). Extraction of this component showed it was composed of 3 proteins of different sizes (70kDa, 80kDa and 110kDa) (Vale et al., 1985). The 70kDa component of kinesin II shows homology to Kinesin-like proteins (KIF) in mouse (Aizawa et al., 1992; Cole et al., 1993; Walther et al., 1994). Antibodies against KIF family members Kif3a and Kif3b identified the 70kDa and 80kDa protein, respectively. The 110kDa protein was called Kinesin associated protein 3 (Kap3) (Kondo et al., 1994; Yamazaki et al., 1995). Kif3a alone or in complex with Kif3b could move extracted *Chlamydomonas* axonemes towards their minus end, demonstrating that Kinesin II is a plus end motor and moves in an anterograde (minus to plus) direction along microtubules (Kondo et al., 1994; Yamazaki et al., 1995). Absence of Kap3 did not affect the motor activity of Kif3a or Kif3a/Kif3b, arguing it is auxillary to the motor function of Kinesin II (Yamazaki et al., 1996).

1.1.5 The Retrograde Motor Cytoplasmic Dynein

In parallel to the discovery of Kinesin II, two classes of high molecular weight microtubule-associated proteins (MAPs), MAP1 (MAP1A, MAP1B and MAP1C) and MAP2 (MAP2A and MAP2B) were identified (Bloom et al., 1984b). MAP1A colocalised with extracted microtubules (Bloom et al., 1984a), while MAP1C was shown to cause microtubule movement (Paschal et al., 1987). Purification of MAP1C yielded proteins of varying sizes, suggesting that MAP1C is a complex made up of two catalytic heavy chains (532kDa),

intermediate chains (74kDa) and light intermediate chains (59-53kDa) (Paschal et al., 1987). MAP1C was shown to be the cytoplasmic equivalent of axonemal Dynein (Vallee et al., 1988). Subsequent work identified light chains (11-8kDa) as additional components of Cytoplasmic Dynein (King and Patel-King, 1995).

Purified MAP1C caused *Chlamydomonas* axonemes to move towards their plus end, the opposite direction to that seen for Kinesin II (Kondo et al., 1994; Paschal and Vallee, 1987; Yamazaki et al., 1995). Therefore, Cytoplasmic Dynein is a minus end directed motor and moves in a retrograde (plus to minus) direction along microtubules (Paschal and Vallee, 1987). This was confirmed by the observation that cleavage (Schroer et al., 1989) or mutation (Gee et al., 1997) of the ATP binding domain of Cytoplasmic Dynein abolished retrograde transport.

Subsequent work has identified two Cytoplasmic Dynein complexes with distinct functions (Gibbons et al., 1994), called Cytoplasmic Dynein 1 and 2 (Pfister et al., 2006). Analysis of Cytoplasmic Dynein 1 mutants revealed defects in golgi positioning leading to early embryonic death (Harada et al., 1998). In contrast, Cytoplasmic Dynein 2 mutants survive until later in development and demonstrate cilia-related phenotypes (Huangfu and Anderson, 2005). Thus, Cytoplasmic Dynein 1 is involved in intracellular transport and Cytoplasmic Dynein 2 is involved in IFT (Pfister et al., 2006). Table 1 details known components of the IFT machinery (reviewed by Cole and Snell, 2009; Pfister et al., 2006)).

<i>Anterograde Motor Kinesin-II</i>	<i>IFT Particle Complex A</i>	<i>IFT Particle Complex B</i>	<i>Retrograde Motor Cytoplasmic Dynein 1</i>	<i>Retrograde Motor Axonemal Cytoplasmic Dynein 2</i>
Kif3a	IFT44	IFT172	DYNC1H1 (Heavy Chain)	DYNC2H1 (Heavy Chain)
Kif3b	IFT140	IFT88	DYNC1I (Intermediate Chain)	DYNC1I (Intermediate Chain)
KAP	IFT139	IFT81	DYNC1L1 (Light Intermediate Chain)	DYNC2L1 (Light Intermediate Chain)
	IFT122	IFT80	DYNLRB (Light Chain – Roadblock)	?
	IFT121	IFT74	DYNLT (Light Chain – Tctex1)	?
	IFT43	IFT70	DYNLL (Light Chain - Dynll1/2)	DYNLL (Light Chain - Dynll1/2)
		IFT57		
		IFT52		
		IFT46		
		IFT27		
		IFT25		
		IFT22		
		IFT20		

Table 1. Components of the IFT machinery.

The mechanism of moving proteins into and out of cilia (called Intraflagellar Transport (IFT)) involves three major components; the anterograde motor Kinesin II, IFT particles and the retrograde motor Cytoplasmic Dynein 2. All are multimeric complexes. There are two Cytoplasmic Dynein complexes. Cytoplasmic Dynein 1 is involved in intracellular retrograde transport while Cytoplasmic Dynein 2 powers retrograde IFT in cilia (Bloom et al., 1984b; Cole et al., 1998; Gibbons et al., 1994; King and Patel-King., 1995; Kondo et al., 1994; Paschal et al., 1987; Vale et al., 1985; Yamazaki et al., 1995). Adapted from Pfister et al, 2006 and Cole and Snell, 2009.

1.2 Left-Right Development

During development, the embryo acquires three axes, head to toe (anterior-posterior (A-P)), front to back (dorsoventral (D-V)) and left to right (L-R). The L-R axis is the last to form and is set up in relation to the A-P and D-V axes (Brown and Wolpert, 1990). The L-R axis is manifested when the left and right sides of the embryo, which are separated by a midline, become differentiated from each other allowing organ asymmetry to form. One of the earliest, and most influential, models of how this occurs was proposed by Brown and Wolpert (1990). They hypothesized that during development a handed molecule becomes orientated in respect to the A-P and D-V axes. This positional information is then “converted” into cellular asymmetry, which biases a randomly generated asymmetry. Subsequent “interpretation” of L-R cellular asymmetry leads to morphological asymmetry (Brown and Wolpert, 1990).

1.2.1 The node and cilia beating

It remained a mystery within the theoretical framework of Brown and Wolpert (1990) how bilateral symmetry is broken in the early embryo. The first indication of how this happens came from a study by Afzelius (1976) linking Kartengers syndrome and the incidence of *situs inversus* (reversal of the L-R axis) with lack of cilia motility. Afzelius made the key prediction that cilia beating in the embryo determined L-R patterning (Afzelius, 1976). A ciliated structure that forms during early embryonic development is the node. The node arises at the caudal end of the primitive streak and is the source of cells that form the notochord. It is made up of two cell layers, a ventral layer consisting of ciliated cells (with 9+0 cilia) and an unciliated dorsal layer. The node is a transient structure and is seen from

about 7.0-8.5dpc (Sulik et al., 1994). Analysis of wild type nodes by video microscopy revealed that node cilia are motile with a vortical beating pattern (Nonaka et al., 1998).

1.2.2 Nodal Flow

Nonaka and colleagues (1998), after discovering cilia beating in the node, made the important observation of a leftward flow of liquid across the node. They called this “nodal flow” (Nonaka et al., 1998). Nodal flow is absent in *Kif3a* and *Kif3b* mutants (which lack node cilia) and in *iv* mutants (which have immotile cilia); therefore cilia and their motility are responsible for nodal flow. Importantly, *Kif3a*, *Kif3b* and *iv* mutants demonstrate L-R morphological defects (randomised heart looping and embryonic turning), thereby linking nodal flow to morphological L-R development (Marszalek et al., 1999; Nonaka et al., 1998; Okada et al., 1999; Supp et al., 1999; Supp et al., 1997; Takeda et al., 1999; Hummel, 1959).

To directly test if nodal flow is sufficient to initiate L-R patterning in the mouse an innovative experiment was carried out by Nonaka and colleagues (2002) that applied an artificial flow to cultured mouse embryos. Leftward artificial flow applied to wild type embryos resulted in normal L-R development. In contrast, rightward artificial flow mostly reversed the *situs* of cultured embryos. This reversal is more complete in *iv* mutant embryos. In combination with the analysis of nodal flow in *Kif3a*, *Kif3b* and *iv* mutants (Nonaka et al., 1998; Okada et al., 1999), these data are direct evidence for a necessary and sufficient role for nodal flow in L-R development (Nonaka et al., 2002).

1.2.3 Cilia tilt and the origin of leftward nodal flow

The origin of *directional* nodal flow in the embryo was addressed by various groups; they asked how could leftward flow result from cilia rotating around a vertical axis. Detailed analysis of rotating cilia demonstrated they have a 40° posterior tilt. This means that cilia rotate in two distinct phases: the “effective stroke”, which rotates the cilia leftward away from the cell surface and the “recovery stroke”, which moves the cilia rightward close to the cell surface. The effective stroke generates the most efficient force to give a net leftward directionality to nodal flow (Nonaka et al., 2005; Okada et al., 2005).

Recently, the origin of the posterior tilt of node cilia and the leftward force of nodal flow has been shown to result from the posterior shift in the position of the basal body (Hashimoto et al., 2010). A candidate that was proposed for the control of this posterior shift was Wnt associated planar cell polarity (PCP). When basal body shift was measured in mutants of the PCP gene Dishevelled (*Dvl*), the shift was reduced and nodal flow was disorganised. This suggests that non-canonical Wnt signalling determines the posterior tilt of node cilia (Hashimoto et al., 2010). Consistent with the role of PCP in node cilia positioning, double mutants of the PCP genes *Vangl1* and *Vangl2* demonstrate situs defects, disrupted nodal flow and abnormally positioned node cilia (Song et al., 2010).

Nodal flow is now firmly established as the mechanism responsible for the breaking of bilateral symmetry and the initiation of L-R patterning in the mouse. Motile cilia and nodal flow have also been observed in rabbit, medaka fish (Okada et al., 2005), zebrafish (Kramer-Zucker et al., 2005) and *Xenopus* (Schweickert et al., 2007). The exception to this is the

chick embryo where flow is not observed. Instead, asymmetric distribution of cells occurs at Henson's node leading to asymmetric gene expression and the breaking of bilateral symmetry (Gros et al., 2009). The underlying mechanism for how this occurs remains unknown.

1.2.4 Morphogen Model

Three models have emerged to explain the downstream consequences of nodal flow. The first to be proposed was the “morphogen model”: cilia driven nodal flow carries a morphogen to the left side of the node. The asymmetric distribution of this morphogen results in activation of genes on the left side of the embryo, which direct subsequent L-R morphological development (Nonaka et al., 1998). This explains the observation of randomised *situs* and the absence of nodal flow in *Kif3a*, *Kif3b* and *iv* mutants; in the absence of nodal flow, Brownian motion will randomly distribute a morphogen around the node, causing random activation of L-R genes in both left and right lateral plates.

1.2.5 Two Cilia Model

An alternative to the morphogen model, based on the mechanical force of nodal flow in the absence of a morphogen, has been proposed. McGrath and colleagues (2003) observed two distinct populations of cilia in the node. One population, located in the centre of the node, contain *Dnahc11* (the gene mutated in *iv* mutants (Supp et al., 1999; Supp et al., 1997)); these central cilia are motile. The other population of cilia, located on the periphery of the node, contain only *Pkd2* and are immotile.

Previously, *Pkd2* null mutants had been shown to demonstrate L-R defects (right pulmonary isomerism and randomised heart looping) (Pennekamp et al., 2002), arguing that the presence of Pkd2 in node cilia is functionally required for L-R development. In other studies, Pkd2 had been shown to interact with Pkd1 in kidney cilia forming a calcium channel upstream of calcium induced calcium release (CICR): bending of cilia brings Pkd1 and Pkd2 together to induce influx of extracellular Ca^{2+} , which releases intracellular stores of Ca^{2+} (Hanaoka et al., 2000; Nauli et al., 2003; Praetorius and Spring, 2001, 2003; Yoder et al., 2002). McGrath and colleagues (2003) demonstrated the presence of a calcium signal on the left side of the node. This signal is randomised in *iv* mutants, suggesting nodal flow is upstream of calcium signalling. Collectively, these data led to the “two cilia hypothesis”: cilia in the centre of the node rotate to generate leftward flow which causes mechanical bending of *Pkd2*-containing cilia on the left periphery of the node. This generates a left sided calcium signal that subsequently directs L-R development (McGrath et al., 2003).

1.2.6 NVP Model

The “NVP model”, a variation of the “morphogen model”, was proposed by Tanaka and colleagues (2005). The basis of this model came from the observation that large molecular weight proteins form a stable asymmetric distribution at the node, while smaller proteins do not (Okada et al., 2005). Consistent with this, high molecular weight membraneous particles called “nodal vesicular parcels” (NVPs) are observed emerging from the surface of the node and are carried to the left side by nodal flow. Once at the left side of the node, NVPs fragment in a cilia dependent manner. The NVP model states that once NVPs fragment on

the left side of the node, they release Shh and retinoic acid to activate left sided calcium signalling (Tanaka et al., 2005).

It remains controversial whether the morphogen model, the two cilia hypothesis or the NVP model is correct. The models are not necessarily mutually exclusive. For example, it could be argued that the force of the NVPs fragmenting on the left side of the node (as the NVP model proposes) could produce the mechanical force needed to bend Pkd2-containing cilia at the periphery of the node to initiate calcium signalling (as the two cilia hypothesis proposes).

1.2.7 Investigating the link between the node and lateral plates

A major gap in current understanding of L-R development is the link between nodal flow at the node and the activation of genes in the left lateral plate mesoderm. Nakamura and colleagues (2006) observed that culturing the right lateral plate together with the node causes ectopic right lateral plate *Nodal* expression. This shows that both left and right lateral plates can receive activating signals from the node, arguing that relative levels of signal received due to leftward nodal flow results in robust asymmetric gene expression on the left (Nakamura et al., 2006). McGrath and colleagues (2003) propose calcium to fulfil this signalling role; however it remains unknown whether calcium signalling directly activates L-R gene expression. Alternatively, Oki and colleagues (2007) argue that Nodal itself moves from the node to the lateral plate. Cryptic is a co-receptor of Nodal and *Cryptic* mutants fail to activate *Nodal* expression in the left lateral plate (Yan et al., 1999). However, in these mutants *Nodal* expression at the node is unaffected (Yan et al., 1999). Therefore, *Cryptic* mutants cannot respond to *Nodal* signalling in the node and lateral plate mesoderm, despite

the presence of Nodal at the node. Oki and colleagues (2007) demonstrated that transgenic rescue of lateral plate *Cryptic* expression in *Cryptic* mutants restored left lateral plate expression of *Lefty1*, *Lefty2* and *Pitx2*, targets of Nodal signalling (see below). This was argued to show that Nodal from the node acts directly to initiate *Nodal* expression in the lateral plates (Oki et al., 2007).

Squint (one of the zebrafish homologs of mouse *Nodal*) was shown to act as a morphogen (Chen and Schier, 2001). Mouse Nodal interacts with sulfated glycosaminoglycans (which are known to be involved in movement of other morphogens such as Wnt and hh (Lin, 2004)) and this interaction is required to activate left lateral plate expression of *Nodal* (Oki et al., 2007). If Nodal was moving from the node to the lateral plate it should be possible to detect Nodal moving between these structures. However, tagged Nodal expressed from the node is only detected close to node cells (Oki et al., 2007). It is possible that sulfated glycosaminoglycans may be transporting other factors to the left lateral plate to initially activate *Nodal* expression in a *Cryptic*-independent manner; *Cryptic* is then needed to maintain *Nodal* expression.

1.2.8 Events Downstream of Nodal Flow in Lateral Plate Mesoderm – the Nodal Pathway

The TGF β family loci *Nodal*, *Lefty1*, *Lefty2* and the transcription factor *Pitx2c* are components of the Nodal pathway. *Nodal* activates its own expression and that of *Lefty1*, *Lefty2* and *Pitx2c* through the transcription factor FoxH1 (Saijoh et al., 1999; Shiratori et al., 2001). *Nodal*, *Lefty2* and *Pitx2c* are expressed specifically in the left lateral plate mesoderm downstream of nodal flow; *Nodal* and *Lefty2* are expressed for only 6-8 hours (Campione et

al., 1999; Collignon et al., 1996; Logan et al., 1998; Lowe et al., 1996; Meno et al., 1997; Meno et al., 1996; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). *Lefty1* is expressed on the left side of the midline at the same stage of development (Meno et al., 1997; Meno et al., 1996). In contrast, *Pitx2c* expression is maintained, via the transcription factor *Nkx2.5* (Shiratori et al., 2001), later in development on the left side of organ primordia (Campioni et al., 1999; Kitamura et al., 1999; Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). *Pitx2c* is a major determinant of left identity in later development but does not have roles in determining the initial direction of embryonic turning or heart looping (Kitamura et al., 1999; Lin et al., 1999).

Various *Nodal* mutants have been generated to investigate its role during development. *Nodal* null mutants fail to gastrulate, demonstrating an early embryonic role for *Nodal* (Collignon et al., 1996) (Conlon et al., 1994; Zhou et al., 1993). Deletion of the enhancers controlling different aspects of *Nodal* expression results in the absence of *Lefty 1*, *Lefty 2* and *Pitx2* expression and subsequent morphological L-R defects, demonstrating a later role for *Nodal* in L-R development (Brennan et al., 2002; Norris et al., 2002; Norris and Robertson, 1999).

Lefty2 null mutants gastrulate but mesoderm fails to move from the primitive streak, demonstrating an early embryonic role for *Lefty2* (Meno et al., 1999). *Lefty2* was first shown to inhibit *Nodal* expression during gastrulation (Meno et al., 1999); these genes were subsequently shown to also have an antagonistic relationship during L-R development (Meno et al., 2001). *Lefty1* has a similar inhibitory effect on *Nodal* expression since *Lefty1* null mutants demonstrate bilateral expression of *Nodal*, *Lefty2* and *Pitx2* (Meno et al., 1998).

This led Meno and colleagues (1998) to propose the “midline barrier model”: *Lefty1* midline expression sets up a midline barrier to prevent diffusion of Nodal into the right lateral plate mesoderm. *Lefty1* therefore acts to confine left identity to the left side of the embryo (Meno et al., 1998).

1.2.9 The SELI model

Nakamura and colleagues (2006) observed that ectopically expressing *Nodal* in the right lateral plate inhibited left lateral plate expression of *Nodal* (Nakamura et al., 2006). This suggested a self-enhancement and lateral inhibition (SELI) model for setting up robust asymmetric *Nodal* expression: When left lateral plate levels of *Nodal* are high, the regulatory feedback loop of *Nodal* amplifies this asymmetry. High levels of Nodal will also activate high levels of its inhibitor *Lefty2*. Thus, *Nodal* is expressed at high levels for a limited time only (2-6 somites (Lowe et al., 1996)). In the right lateral plate, initial levels of Nodal are small, resulting in the activation of *Lefty2* expression at similar levels to that of *Nodal* and the rapid inhibition of *Nodal* on the right. High levels of *Lefty1* and *Lefty2* expression in the midline and left lateral plate (respectively) also inhibit *Nodal* expression on the right side. This model explains the random lateral plate expression of *Nodal* in mutants lacking nodal flow: random signalling from the node due to a lack of nodal flow can activate initially high levels of *Nodal* either on the left, the right or on both sides. SELI will subsequently cause, respectively, robust left, right or bilateral expression of *Nodal*. Alternatively, small initial levels of *Nodal* expression on both sides will be rapidly inhibited, resulting in absence of lateral plate *Nodal* expression (Nakamura et al., 2006).

1.3 L-R Development and the hedgehog signalling pathway

Another signalling pathway that has been implicated in L-R development is the hedgehog (hh) pathway. In the chick, *Sonic hedgehog* (*Shh*) is asymmetrically expressed on the left side of the node and activates left lateral plate expression of *Nodal* to direct L-R development (Levin et al., 1995). In the mouse node, *Shh* is symmetrically expressed at the node (Echelard et al., 1993). The difference in *Shh* expression in chick and mouse may reflect the different mechanisms (cell movement in chick (Gros et al., 2009) and cilia-based nodal flow in mouse (Nonaka et al., 2002; Nonaka et al., 1998)) that break bilateral symmetry in these species. *Shh* has been associated with NVPs and FGF-dependent calcium signalling (Tanaka et al., 2005), although its precise role at the mouse node remains obscure.

Analysis of mutants disrupting hh components *Shh*, *Ihh*, *Smo* and *Gli3* in mouse extended understanding of the influence hh signalling has on L-R development. *Shh* null mutants demonstrate bilateral *Lefty2* and *Pitx2* expression and left pulmonary isomerism (Chiang et al., 1996; Litingtung et al., 1998; Meyers and Martin, 1999; Pepicelli et al., 1998). This is caused by degeneration of the notochord in *Shh* mutants, absence of a midline and therefore lack of a *Lefty1* midline barrier (Chiang et al., 1996; Meno et al., 1998; Meyers and Martin, 1999; Tsukui et al., 1999). In contrast, *Smo* mutants and *Shh/Ihh* double mutants fail to activate lateral plate expression of *Nodal*, *Lefty2* and *Pitx2* (Zhang et al., 2001). This was associated with reduced levels of *Cryptic* and *Gdf1* expression in the node and lateral plate mesoderm in *Smo* mutants, suggesting the hh pathway may activate *Cryptic* and *Gdf1*; both needed for *Nodal* expression (Zhang et al., 2001).

Recently, rescue of lateral plate *Smo* expression in *Smo* mutants was shown to restore *Nodal*, *Lefty2* and *Pitx2* expression on a *Smo* mutant background, further suggesting that hh is needed for the propagation of *Nodal* in the lateral plate mesoderm. This is mediated by both Shh and Ihh dependent activation of *Cryptic* expression (Tsiairis and McMahon, 2009). Furthermore, *Pitx2* expression was restored in *Smo/Gli3* double mutants, suggesting that the repressor form of Gli3 acts to inhibit *Pitx2* expression. Additional analysis demonstrated *Foxf1* and *Bmp4* to be downregulated in *Smo* mutants, suggesting they are activated by hh. Previously, *Bmp4* was shown to activate *Cryptic* and *Nodal* expression (Fujiwara et al., 2002). Together these data argue that hh signalling activates *Gli3* to induce *Foxf1* expression, which activates *Bmp4*, *Cryptic* and the *Nodal* pathway (Figure 2) (Tsiairis and McMahon, 2009).

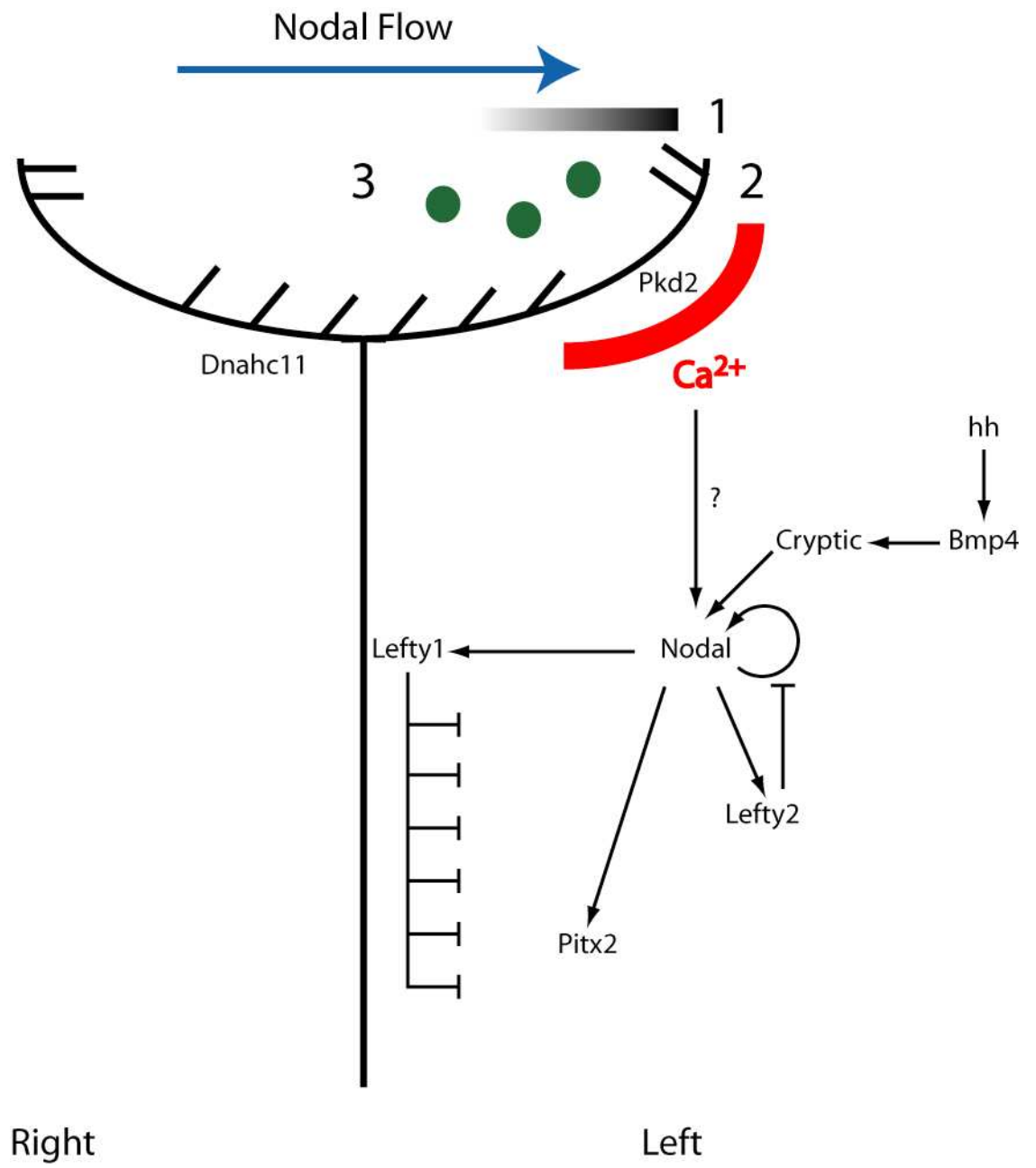


Figure 2. Model of L-R Development

Cilia in the embryonic node rotate (in a *Dnahc11*-dependent manner) to generate a leftward “nodal flow”. Various models have been proposed to describe the downstream consequences of nodal flow: (1) “Morphogen model”; a morphogen is asymmetrically distributed to the left side of the node, where it activates gene expression on the left side. (2) “Two cilia hypothesis”; the mechanical force of nodal flow bends Pkd2-containing cilia on the left periphery of the node to induce a left sided calcium (Ca^{2+}) signal. (3) “NVP model”; nodal vesicular parcels (NVPs; green circles) are released from the surface of the node and carried, by nodal flow, to the left side of the node. Once there, NVPs fragment releasing Shh and Retinoic acid to induce a left sided Ca^{2+} signal.

It is currently unknown how the symmetry-breaking event at the node is transmitted to the left lateral plate mesoderm. Nevertheless, *Nodal* is expressed in the left lateral plate downstream of nodal flow. Once *Nodal* expression is initiated (which is dependent on hedgehog (hh) activating, via *Bmp4*, expression of the co-receptor *Cryptic*), Nodal activates expression of itself and that of *Lefty1*, *Lefty2* and *Pitx2*. *Lefty1* is expressed in the midline to provide a “midline barrier”, inhibiting *Nodal* expression and preventing Nodal moving to the right lateral plate; thereby confining left identity to the left side. *Lefty2* also inhibits *Nodal* expression, while *Pitx2* is a major downstream determinant of left identity in the embryo.

1.4 hh Signalling Pathway

1.4.1 hh pathway in *Drosophila*

Since the initial discovery of Hh mutants in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), a generally accepted model of the hh pathway has emerged, primarily from work on *Drosophila* (Figure 3).

In the absence of Hh ligand, the receptor Patched (Ptch) inhibits the hh pathway by promoting the dephosphorylation and downregulation of the transmembrane G coupled receptor Smoothened (Smo) (Denef et al., 2000; Ingham et al., 1991). Inhibition of Smo allows Costal2 (Cos2), Fused (Fu), Suppressor of fused (Sufu) and the transcription factor Cubitus interruptus (Ci) to form a complex that is tethered to microtubules. This prevents Ci from accumulating in the nucleus. It also promotes Protein kinase A (Pka) associated proteolytic cleavage of ci; this liberates Ci-75, the repressor form of Ci. Subsequently, Ci-75 enters the nucleus and inhibits transcription of hh target genes (Methot and Basler, 2000; Wang et al., 2000).

In Hh-expressing cells, Hh is cleaved and the N-terminal region (Hh-N) is modified by the addition of cholesterol (Lee et al., 1994; Lee et al., 1992; Porter et al., 1996a; Porter et al., 1995; Porter et al., 1996b). Hh-N is secreted from the cell and acts as a morphogen (Heemskerk and DiNardo, 1994).

In the presence of Hh-N, binding of Hh-N to Ptch inhibits the repressor function of Ptch, resulting in internalisation of Ptch, phosphorylation of Smo and accumulation of Smo at the cell surface (Ingham et al., 1991) (Denef et al., 2000). Activation of Smo inhibits Pka-mediated cleavage of Ci, resulting in the liberation of full length Ci (Ci-155) from microtubules. Ci-155 is then activated by Fu. Smo also inhibits sufu to allow accumulation of Ci-155 in the nucleus (Methot and Basler, 2000; Ohlmeyer and Kalderon, 1998; Wang et al., 2000). Nuclear Ci-155 activates expression of hh target genes (Methot and Basler, 2000; Wang et al., 2000).

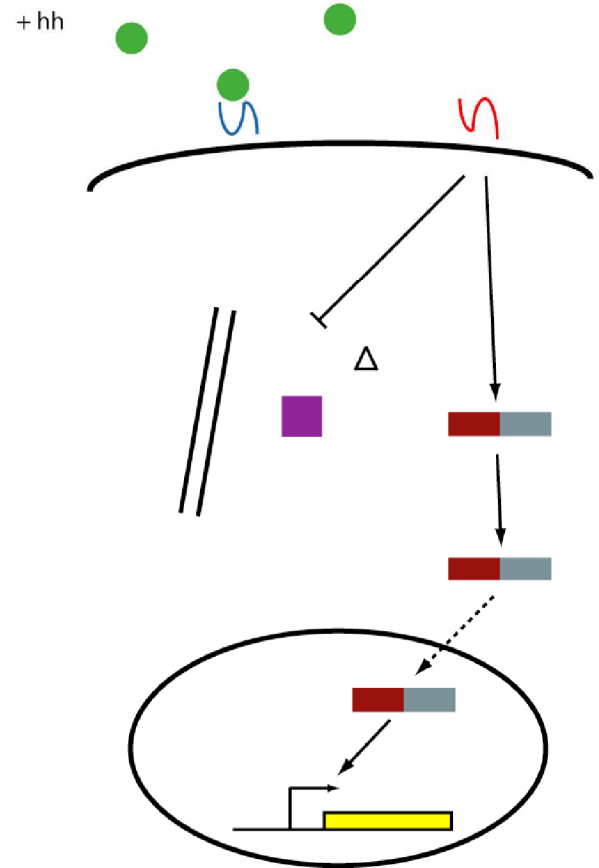
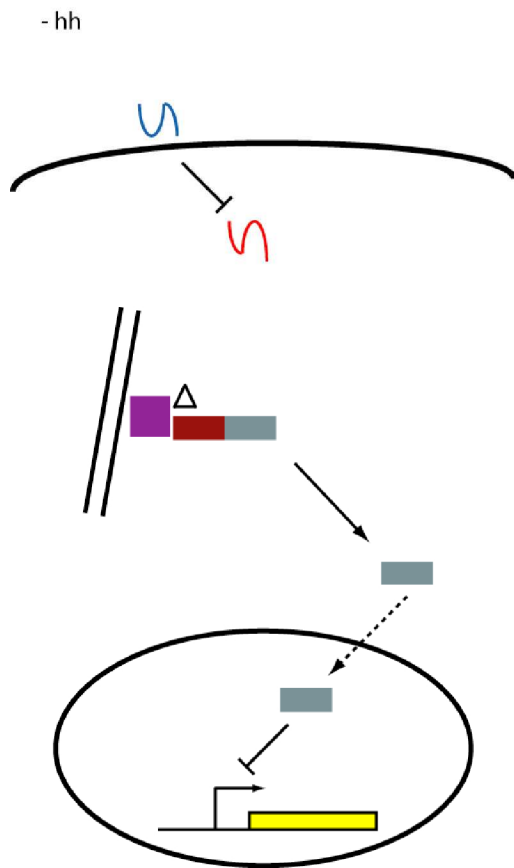


Figure 3. Hh signalling pathway

In the absence of Hh-N ligand, Patched (Ptch) inhibits Smoothened (Smo) to allow the transcription factor Cubitus interruptus (Ci), in complex with Costal2 (Cos2), Fused (Fu) and Suppressor of fused (Sufu), to become tethered to microtubules. This prevents nuclear accumulation of full length and promotes Protein kinase A (Pka) mediated proteolytic cleavage of Ci to liberate Ci-75. Ci-75 enters the nucleus to inhibit transcription of Hh target genes.

In the presence of Hh-N ligand, Hh-N inhibits Ptch to relieve inhibition of Smo. Smo then blocks proteolytic cleavage of full length Ci and releases Ci from microtubules. Ci enters the nucleus to activate transcription of Hh target genes.

1.4.2 Hh signalling during vertebrate development

Three hh homologs are found in mammals, *Sonic hedgehog* (*Shh*), *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*) (Echelard et al., 1993; Riddle et al., 1993). All three homologs are predicted to be secreted proteins and function like *Drosophila* hh (Echelard et al., 1993; Riddle et al., 1993).

1.4.2.1 Functional analysis of Shh

Early work on *Shh* suggested a key role during embryogenesis. *Shh* is expressed in the node, notochord, ventral floor plate of the neural tube and in the zone of polarising activity (ZPA) of the limb in the chick, zebrafish, rat and mouse (Chang et al., 1994; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). Ectopic expression of *Shh* in the neural tube of mouse induced expression of *Hnf3 β* (MGI: *FoxA2*); in the notochord and ventral neural tube *Hnf3 β* is normally expressed before *Shh* (Echelard et al., 1993). The following model was proposed: *Hnf3 β* induces expression of *Shh* in the notochord. Subsequently, *Shh* signals to the floor plate of the neural tube where it activates *Hnf3 β* ; *Hnf3 β* activates *Shh* expression in floor plate cells (Echelard et al., 1993). *Shh* in floor plate cells induces ventral cell identity in the neural tube (Roelink et al., 1994).

Extensive work from a number of groups has elucidated how the neural tube is patterned. A gradient of *Shh* from the ventral side (Chiang et al., 1996; Ericson et al., 1996; Ericson et al., 1997; Hynes et al., 2000; Marigo and Tabin, 1996; Marti et al., 1995; Roelink et al., 1995) and a gradient of *Bmp* activity from the dorsal side (Barth et al., 1999; Liem et al., 2000;

Nguyen et al., 2000) determines the position of various neuronal cell types across the D-V axis. From the ventral side, these are floor plate cells, V3 neurons, motor neurons, V2 neurons, V1 neurons, V0 neurons and pD cells. Class I and Class II genes, under the control of Shh and Bmp, are expressed in the neural tube and determine the specification of neuronal cell types (Table 2) (Briscoe et al., 2000; Briscoe et al., 1999; Mansouri and Gruss, 1998; Pfaff et al., 1996; Sander et al., 2000; Tanabe et al., 1998). Once neuron-specific Class I and Class II genes are activated, they mutually repress each other setting up and maintaining neuron cell type boundaries (Table 3) (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997; Novitsch et al., 2001; Vallstedt et al., 2001).

				Activated by Shh				Repressed by Shh							
					Class II				Class I						
				Shh	Hnf3β	Nkx6.1	Nkx2.2	Olig2	Nkx6.2	Pax6	Irx3	Dbx2	Dbx1	Pax7	
Dorsal	pD														
	P0	V0								Pax6	Irx3	Dbx2	Dbx1	Pax7	
	P1	V1							Nkx6.2	Pax6	Irx3	Dbx2			
	P2	V2				Nkx6.1				Pax6	Irx3				
	pMN	Motor Neuron				Nkx6.1		Olig2		Pax6					
	P3	V3				Nkx6.1	Nkx2.2								
		Floor Plate		Shh	Hnf3β	Nkx6.1									
Ventral		Notochord		Shh	Hnf3β										

Table 2. Dorso-ventral patterning of the vertebrate neural tube.

Two classes of genes (Class I and Class II) are activated in distinct regions along the dorso-ventral axis of the neural tube. Different combinations of these genes are required to specify neuronal cell types (V3, motor neurons, V2, V1, V0). *Shh*, which diffuses from the floor plate of the neural tube, activates expression of Class II genes and represses Class I genes. Class I genes are activated by Bmp signalling in the dorsal neural tube. Therefore, Class II genes are present in the ventral half of the neural tube (where *Shh* is present) and Class I genes are present in the dorsal half (where *Shh* is absent). Adapted from Dessaud *et al*, 2008.

<i>Gene...</i>	<i>Repressed by...</i>	<i>In cell type...</i>	<i>To allow expression...</i>	<i>And set up boundary...</i>	<i>Reference</i>
Dbx1	Nkx6.2	P1	Dbx1 in p0	p1 / p0	Vallstedt <i>et al</i> , 2001
Nkx6.1	Dbx2	P1	Nkx6.1 in p2	p2 / p1	Briscoe <i>et al</i> , 2000
Dbx2	Nkx6.1	P2	Dbx2 in p1	p1 / p2	Briscoe <i>et al</i> , 2000
Nkx6.2	Nkx6.1	P2	Nkx6.2 in p1	p2 / p1	Vallstedt <i>et al</i> , 2001
Irx3	Olig2	pMN	Irx3 in p2	pMN / p2	Novitch <i>et al</i> , 2001
Olig2	Irx3	P2	Olig2 in pMN	p2 / pMN	Novitch <i>et al</i> , 2001
Olig2	Nkx2.2	P3	Olig2 in pMN	pMN / p3	Novitch <i>et al</i> , 2001
Pax6	Nkx2.2	P3	Pax6 in pMN	pMN / p3	Briscoe <i>et al</i> , 2000
Nkx2.2	Pax6	pMN	Nkx2.2 in p3	p3 / pMN	Ericson <i>et al</i> , 1997

Table 3. Mutually repressive interactions between Class I and Class II genes set up boundaries between neuronal cell fates.

Once Class I and Class II genes are activated by gradients of Bmp and Shh activity (respectively) they mutually repress each other to set up boundaries between different neuronal cell types. For example, Nkx2.2 represses *Pax6* in pV3 cells. Reciprocally, Pax6 represses *Nkx2.2* in pMN cells. In this way the boundary between V3 and Motor neurons is set up (see Table 2). Adapted from Dessaud *et al*, 2008.

1.4.2.2 Mutational analysis of components of the hh pathway in mouse

1.4.2.2.1 Shh

The role of *Shh* in the notochord, neural tube and limb was confirmed when a *Shh* null allele was generated in mouse (Chiang et al., 1996). *Shh* mutant embryos have a large proboscis extending from the rostral midline and fused optic vesicles due to a failure to form anterior midline. The notochord initially forms in *Shh* mutants but has reduced *Hnf3 β* expression, which causes its subsequent degradation. This reflects the requirement for Shh to maintain *Hnf3 β* expression and notochord integrity. The lack of a notochord results in failure to activate *Hnf3 β* and *Shh* in the floor plate of the neural tube, leading to a dorsalised neural tube. *Shh* mutants also fail to pattern the fore- and hind limbs, resulting in absence of digits and the formation of a single bony extension from the humerus. Together, this confirms the role of *Shh* in the neural tube and limb ZPA (Chiang et al., 1996).

Shh mutants lack the entire vertebral column and show defects in heart, lung and kidney patterning (Chiang et al., 1996). *Shh* is essential for endoderm morphogenesis to form a proper foregut, a tracheo-oesophageal septum and is required for proper lung lobation and branching (Litingtung et al., 1998; Pepicelli et al., 1998). Subsequent analysis demonstrated that *Shh* has extensive roles in the development of the frontonasal and maxillary processes in the face (Hu and Helms, 1999). Moreover, human mutations in *SHH* are known to give rise to coloboma (failure to fully form the iris) (Schimmenti et al., 2003).

Shh, like *Drosophila* hh, is processed into an active N terminal protein containing a cholesterol modification (Porter et al., 1996). To understand the function of Shh-N, transgenic embryos expressing Shh-N were generated and found to ectopically express *Ptch1* and *Gli1* in the anterior of the limb. This demonstrates that processing of Shh into Shh-N is required for long range signalling (Li et al., 2006).

1.4.2.2.2 Ihh

Ihh mutants demonstrate hypoplastic bone formation (without patterning defects) in limbs, ribs, facial bones and vertebrae. This is associated with downregulation of *Ptch1* and *Gli1* and failure to maintain chondrocyte proliferation (St-Jacques et al., 1999). However, *Shh/Ihh* double mutants have a severe, *Smo* mutant-like phenotype (see below), which suggests *Shh* and *Ihh* have redundant roles during development; both signal through the receptor *Smo* (Zhang et al., 2001).

1.4.2.2.3 Dhh

The only defects found in *Dhh* mutants is reduced testicular growth, lack of germ cells and reduced *Ptch1* expression in the testis. Thus, *Dhh* has a restricted role during male sexual development (Bitgood et al., 1996).

1.4.2.2.4 Ptch

There are two homologs of the *Drosophila* receptor *ptch* in mammals; *Ptch1* (Concordet et al., 1996; Goodrich et al., 1996; Marigo et al., 1996) and *Ptch2* (Motoyama et al., 1998b). *Ptch2* mutants are viable and fertile but develop epidermal hypoplasia (Nieuwenhuis et al., 2006). In contrast, *Ptch1* mutants have a severe, early embryonic lethal phenotype. At 9.5dpc they fail to turn, have an open and ventralised neural tube and show increased levels of *Ptch1* and *Gli1* expression (Goodrich et al., 1997). Partial rescue of the *Ptch1* null phenotype (using an inducible *Ptch1* transgene) demonstrated defects in later embryos such as exencephaly, ventralised neural tube and ectopic *Shh* expression in the anterior limb bud and subsequent polydactyly (Milenkovic et al., 1999). Overexpression of *Ptch1* throughout the entire neural tube resulted in an open, dorsalised neural tube and reduction of endogenous *Ptch1* and *Gli1* expression (Goodrich et al., 1999). These studies confirmed that *Ptch1* is a negative regulator of the mammalian hh pathway.

1.4.2.2.5 Smo

Smo mutants have a severe embryonic lethal phenotype. They die around 9.5dpc, fail to undergo embryonic turning and heart looping and have ventral cyclopia and holoprosencephaly (Zhang et al., 2001). *Ptch1/Smo* double mutants have a *Smo* mutant phenotype, demonstrating that *Smo* acts downstream of *Ptch1* (Zhang et al., 2001). To analyse the requirement of *Smo* in the neural tube, chimeric mice were generated with *Smo* mutant ES cells (Wijgerde et al., 2002). When *Smo* mutant cells populated the ventral neural tube they failed to activate either *Shh* or *Hnf3 β* in the floor plate, leading to dorsalised cell

identities and reduced *Ptch1* expression. This demonstrates that hh signalling, acting through *Smo*, is required for ventral identity in the neural tube. This requirement is partially dependent on *Gli3* since *Smo/Gli3* double mutants fail to form floor plate cells and have intermingled ventral cell types. Therefore, the specification of the ventral most cell types (floor plate or V3) is dependent on the inhibition of Gli3 repressor by Smo (Wijgerde et al., 2002).

1.4.2.2.6 Gli family members

There are 3 homologs of *Drosophila ci* in vertebrates, *Gli1*, *Gli2* and *Gli3* (Kinzler et al., 1988; Ruppert et al., 1988). Like *ci*, *Gli2* (Pan et al., 2006) and *Gli3* (Dai et al., 1999; Wang et al., 2000a) are proteolytically cleaved into a repressor form (*Gli2R* and *Gli3R*). There is no evidence that *Gli1* is similarly processed (Dai et al., 1999). *Gli3* processing to liberate the repressor form is inhibited by Shh and induced by Pka (Dai et al., 1999; Wang et al., 2000a). Full length *Gli3* (*Gli3FL*) is needed to mediate the transcriptional activation of *Gli1* (Dai et al., 1999; Wang et al., 2000a). In the chick and mouse limb there are increased levels of *Gli3FL* in posterior regions of the limb (where *Shh* is expressed) and more *Gli3R* form in anterior regions of the limb (where Shh is absent), demonstrating that levels of *Gli3R* are a read out of the activity of the hh pathway (Wang et al., 2000a).

Recently, it has been shown that in the absence of Shh, *Gli3FL* locates to the cytoplasm and complexes with Sufu while *Gli3R* is located in the nucleus. In the presence of Shh, *Gli3FL* dissociates from Sufu, translocates to the nucleus and becomes phosphorylated to form active *Gli3* (*Gli3A*); formation of *Gli3A* is inhibited by Pka (Humke et al., 2010).

The function of the Gli genes was investigated by mutagenesis in the mouse. *Gli1* null mice are phenotypically normal and viable (Park et al., 2000). In contrast, *Gli2* mutants fail to induce *Hnf3 β* and *Shh* expression in the ventral midline, resulting in the absence of floor plate cells. However, *Gli2* is not required for forebrain development (Ding et al., 1998; Matise et al., 1998; Mo et al., 1997). The role of *Gli2* in the neural tube was further investigated when a *Pka* insensitive mutant form of *Gli2* was generated. This constitutively active form of *Gli2* cannot be processed into Gli2R causing increased expression of *Gli1* and *Ptch1* and ventralisation of the neural tube (Pan et al., 2009). *Gli2* null mutants also demonstrate skeletal abnormalities (Mo et al., 1997) and hypoplastic lungs with left pulmonary isomerism (Motoyama et al., 1998a).

A mutant of *Gli3*, called *extratoes*, demonstrates polydactyly, exencephaly, eye defects and oedema (Hui and Joyner, 1993). *Gli3* mutants demonstrate a dorsal expansion of intermediate neuronal cell types in the neural tube (Persson et al., 2002), arguing that *Gli3* specifies intermediate regions of the neural tube, whereas *Gli2* specifies more ventral regions.

To address the question of functional redundancy between the three *Gli* genes during development, genetic interaction and epistasis studies were carried out. *Gli1*^{-/-}; *Gli3*^{-/-} double mutants resemble *Gli3*^{-/-} mutants, suggesting that *Gli1* and *Gli3* are non-redundant (Park et al., 2000). Although *Gli1*^{-/-} mutants are viable, *Gli1*^{-/-}; *Gli2*^{-/-} double mutants show smaller lungs than *Gli2*^{-/-} mutants, showing *Gli1* has minor roles in development. However, *Gli1*^{-/-}; *Gli2*^{-/-} double mutants show similar D-V patterning defects as *Gli2*^{-/-} mutants, suggesting that

Gli1 has no role in the neural tube (Park et al., 2000). *Ptch1*^{-/-}; *Gli2*^{-/-} double mutants demonstrate normal *Ptch1* expression in the rostral neural tube but reduced *Ptch1* expression in the caudal neural tube. *Ptch1*^{-/-}; *Gli2*^{-/-}; *Gli3*^{-/-} triple mutants show a severe reduction in *Ptch1* expression. These data argue that *Gli2* is the major mediator of hh signalling in the caudal neural tube and that *Gli3* has overlapping ventralising functions throughout the neural tube (Motoyama et al., 2003).

Gli2^{-/-}; *Gli3*^{-/-} double mutants abolish expression of *Gli1*, fail to activate *Ptch1* expression above basal levels, lack floor plate cells and have intermingled ventral neurons (Bai et al., 2004; Lei et al., 2004). Furthermore, they fail to form lungs, trachea and oesophagus (Motoyama et al., 1998a), a more severe lung phenotype than either *Gli2*^{-/-} or *Shh*^{-/-} mutants (Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998; Mo et al., 1997). Therefore, *Gli2* and *Gli3* have redundant activator functions. This was further emphasized when Bai and colleagues (2004) replaced *Gli2* with *Gli3* by generating a knockin allele (*Gli2*^{3ki/3ki}) in mouse. *Gli2*^{3ki/3ki} embryos demonstrate partial rescue of the *Gli2* mutant phenotype (some floor plate cells and V3 neurons form). Therefore, *Gli3* can replace some functions of *Gli2* (Bai et al., 2004). In addition, *Gli2*^{3ki/3ki}; *Gli3*^{-/-} double mutants failed to form floor plate cells, showing that *Gli2* has important roles in specifying ventral cell types (Bai et al., 2004). Bai and colleagues (2004) proposed the following model to assign different roles to *Gli1*, *Gli2* and *Gli3* in the neural tube: At high levels of Shh, Gli3 repressor formation is inhibited and full length Gli2 and Gli3 are activated; *Gli1* expression is subsequently activated by Gli2 and Gli3. At moderate levels of Shh, a combination of Gli2 and Gli3 activators and repressors are formed, specifying intermediate motoneuron identity. At low levels of Shh, the majority of Gli3 is processed into the repressor form, inhibiting hh signalling and thereby allowing dorsal V0, V1 and V2 neurons to form (Bai et al., 2004).

1.4.2.2.7 Rab23

Rab23, a member of the Rab family of GTPases, was identified as the gene responsible for the *open brain (opb)* mutant phenotype (Eggenchwiler et al., 2001). Mutants demonstrate exencephaly, ventralisation of the neural tube, polydactyly and increased *Shh*, *Hnf3 β* and *Ptch1* expression (Eggenchwiler et al., 2001; Gunther et al., 1994; Kasarskis et al., 1998), arguing that *Rab23* is a negative regulator of the hh pathway (Eggenchwiler et al., 2001). Epistasis studies demonstrate that *Rab23* acts downstream of *Shh* and *Smo*, upstream of *Gli2* and independently of *Gli3* (Eggenchwiler et al., 2006; Eggenchwiler et al., 2001).

1.4.2.2.8 Pka

Embryos mutant for the C α and C β 1 subunits of *Pka* demonstrated spina bifida, overproliferation of dorsal neuroepithelium in the posterior neural tube, increased *Shh* and *Hnf3 β* expression and ventralisation of the posterior neural tube; arguing that *Pka* is a negative regulator of *Shh* signalling. Defects were seen only in the posterior of the embryo because *Pka* kinase activity is lower in the posterior half of wild type embryos, therefore making this part of the embryo more susceptible to the reduction of activity in *Pka* mutants (Huang et al., 2002). *Pka* inhibits the hh pathway by blocking the phosphorylation of Gli3FL into the active form of Gli3 (Gli3A) (Humke et al., 2010).

1.4.2.2.9 Sufu

Mouse Sufu interacts with all three Gli proteins and coexpression of *Sufu* with *Gli1*, *Gli2* or *Shh* inhibits Gli-mediated transcriptional activation (Ding et al., 1999). Similar to *Drosophila* *sufu* (Methot and Basler, 2000; Wang et al., 2000b), mouse Sufu inhibits the nuclear accumulation of Gli1 (Ding et al., 1999; Svard et al., 2006). This suggests that *Sufu* is a negative regulator of the hh pathway in mouse (Ding et al., 1999). *Sufu* mutant embryos demonstrate ectopic *Shh*, *Gli1* and *Ptch1* expression and a ventralised neural tube, confirming its negative regulator role in mouse (Cooper et al., 2005; Svard et al., 2006).

Recently, Sufu has been shown to form a complex with full length Gli3 and Gli2 to stabilise these proteins. Also, Sufu inhibits the nuclear translocation of Gli3FL to prevent activation of Gli3 by phosphorylation (Humke et al., 2010).

1.5 Cilia and the Hh signalling pathway

Mouse mutagenesis screens for neural tube phenotypes unexpectedly found that cilia are crucial for vertebrate hh signalling (Huangfu et al., 2003). This discovery has influenced all subsequent work on vertebrate hh signalling and has greatly raised the profile of cilia as important centres of signaling.

The first evidence of a link between cilia and hh signalling came from the analysis of ENU-derived mouse mutants of *Ift88* and *Ift172* (Huangfu et al., 2003). These mutants had characteristic defects associated with the absence of hh signalling, including a dorsalised neural tube and reduced *Ptch1* expression in the neural tube. Importantly, *Ift88* and *Ift172* mutants lacked cilia in the node. Analysis of *Kif3a* mutants found a similar neural tube patterning defect, confirming a role for cilia in D-V patterning of the neural tube. Epistatic analysis using *Ptch1*, *Rab23* and *Gli3* mutants demonstrated that cilia are downstream of *Ptch1*, *Smo* and *Rab23* and upstream of *Gli3*. Together these data strongly argued that cilia are required for hh signalling (Huangfu et al., 2003).

An important set of studies demonstrated that Smo (Corbit et al., 2005), Gli1, Gli2, Gli3, Sufu (Haycraft et al., 2005) and Ptch1 (Rohatgi et al., 2007) proteins localise to cilia. In the presence of Shh-N, Ptch1 is removed from cilia (Rohatgi et al., 2007) and Smo accumulates in cilia (Corbit et al., 2005). Smo is targeted to cilia independently of cytoplasmic microtubules (Kim et al., 2009), either intracellularly (Wang et al., 2009) or from the cell surface (Milenkovic et al., 2009). Consistent with the data of Huangfu and colleagues

(2003), in the absence of cilia Gli2 does not activate transcription of hh target genes and the repressor form of Gli3 (Gli3R) is not generated, arguing that processing of Gli2 and Gli3 is cilia dependent (Haycraft et al., 2005).

Together, these data strongly suggest the following model (Figure 4): in the absence of Shh-N, Ptch1 translocates to cilia where it blocks Smo from accumulating in cilia and allows Gli3R to form, thereby inhibiting the hh pathway. In the presence of Shh-N, Shh-N binds to Ptch1 and Ptch1 is removed from cilia. This allows Smo to accumulate in cilia where it activates the hh pathway by inhibiting cleavage of full length Gli3 (Gli3FL) into Gli3R; Gli3FL then leaves cilia and enters the nucleus to activate hh target genes (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007).

1.5.1 IFT particle mutants and hh signalling

Analysis of a growing number of mouse mutants has revealed that IFT particle complexes A and B are required for different aspects of IFT and hh signalling (Table 4). Mutations disrupting *Kif3a*, an essential component of the anterograde IFT motor Kinesin II, result in loss of cilia and a reduction in hh signalling (Huangfu et al., 2003; Marszalek et al., 1999; Takeda et al., 1999). Similarly, mutants of IFT particle complex B components *Ift172*, *Ift88*, *Ift52* and *Ift57* also demonstrate loss of cilia and reduced hh signalling (Houde et al., 2006; Huangfu et al., 2003; Liu et al., 2005). In contrast, mutants of *Ift122* and *Ift139*, components of IFT particle complex A, demonstrate short cilia, which is characteristic of retrograde IFT mutants. *Ift122* and *Ift139* mutants demonstrate increased hh signalling, whereas decreased hh signalling is found in *Dync2h1* mutants (Cortellino et al., 2009; Huangfu and Anderson,

2005; May et al., 2005; Tran et al., 2008). Thus, IFT particle complex B functions in anterograde IFT while IFT particle complex A functions in retrograde IFT. Furthermore, mutations disrupting IFT particle complex B reduces hh signalling while disruption of IFT particle complex A increases hh signalling, arguing that IFT particles can differentially influence hh signalling.

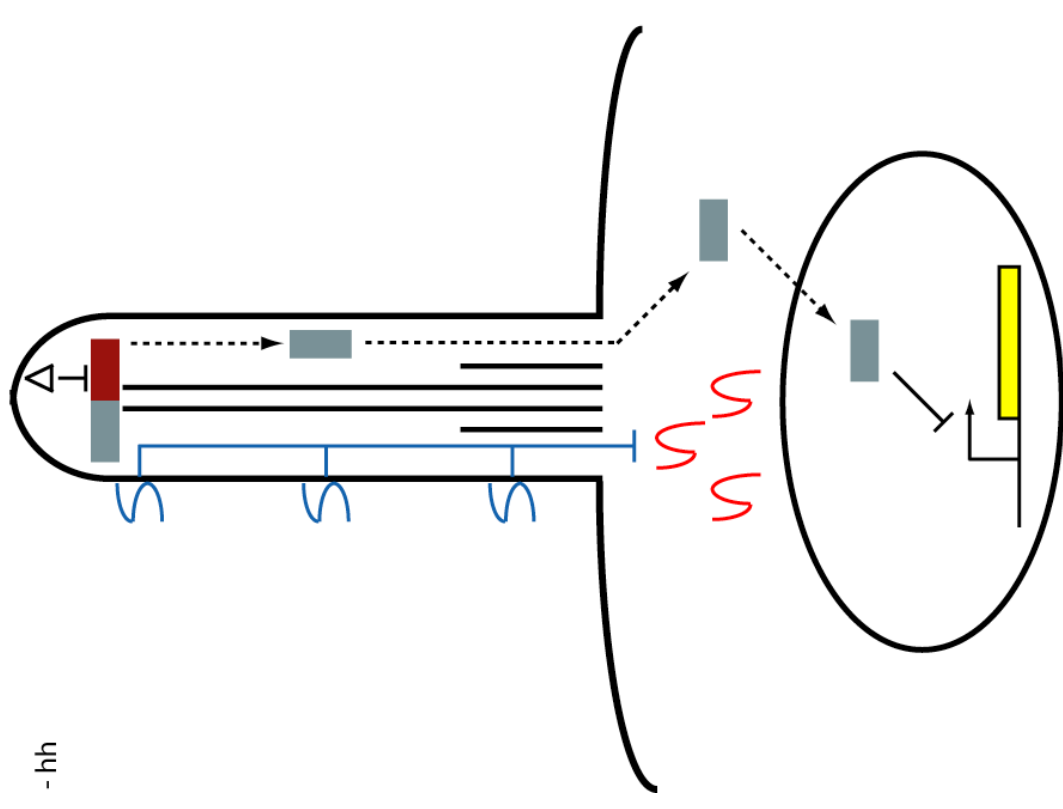
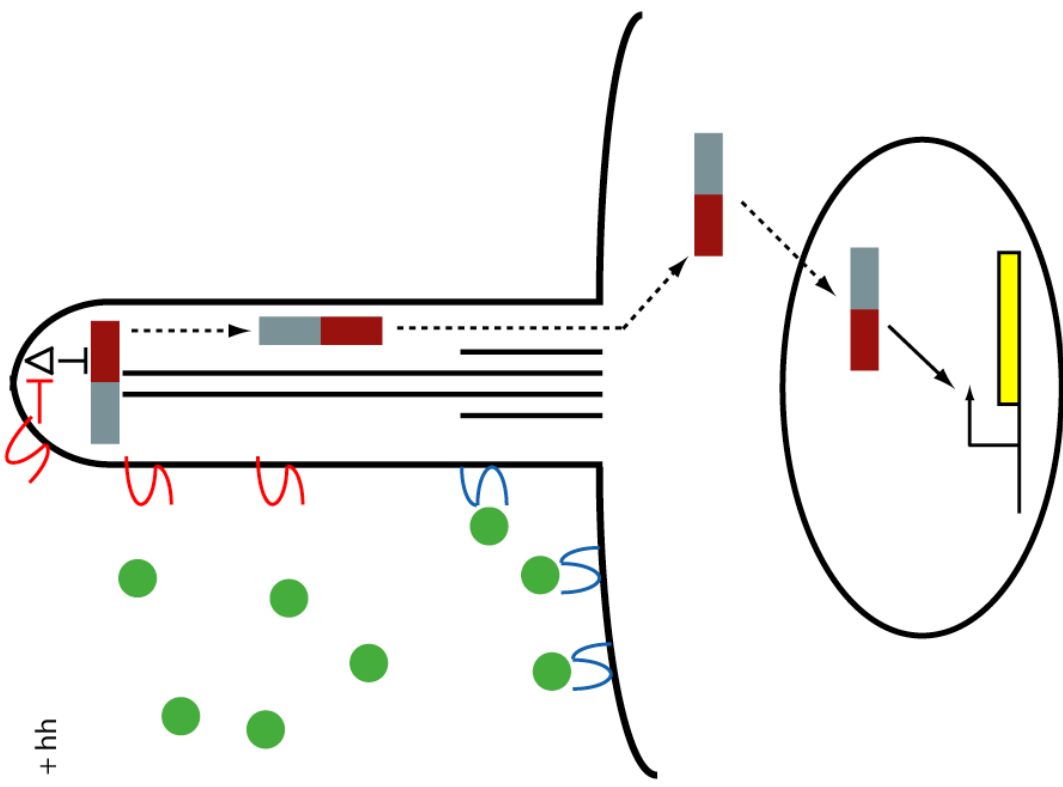
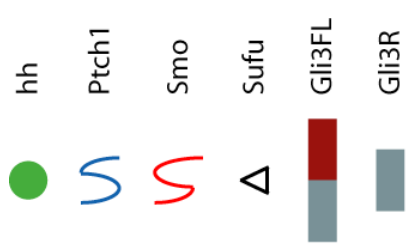


Figure 4. hh signalling and cilia

In the absence of hedgehog (hh) ligand, Patched1 (Ptch1) accumulates in cilia and prevents translocation of Smoothened (Smo) to cilia. This allows Suppressor of fused (Sufu) to inhibit full length Gli3 (Gli3FL) at the cilia tip by promoting the proteolytic cleavage of Gli3FL and thereby liberating Gli3R, the repressor form of Gli3. Gli3R leaves cilia and enters the nucleus to repress hh target genes.

In the presence of hh, hh binds to Ptch1 causing the removal of Ptch1 from cilia. This allows Smo to accumulate in cilia, where it inhibits Sufu and proteolytic cleavage of Gli3FL. Gli3FL subsequently leaves cilia and enters the nucleus to activate hh target genes.

1.5.2 Sufu and cilia

The role of cilia in the function of Sufu has been controversial. Hemke and colleagues (2010) demonstrated that in the absence of Shh, a complex forms between Sufu and Gli3FL in the cytoplasm, preventing nuclear translocation of Gli3FL and formation of active Gli3 (Gli3A); this is independent of cilia. In the presence of Shh, Sufu and Gli3FL dissociate in a cilia dependent manner. Gli3FL is then free to enter the nucleus, form Gli3A and initiate transcription of hh target genes (Humke et al., 2010). This model is consistent with the observation that overexpressing *Sufu* in the absence of cilia blocks activation of hh target genes (Chen et al., 2009).

The model proposed by Hemke and colleagues (2010) predicts that in the absence of Sufu cilia dependent disassociation of Sufu from Gli3FL would not be needed, resulting in activation of hh target genes. Knockdown of *Sufu* in the absence of cilia, however, blocked activation of hh target genes (Ocbina and Anderson, 2008). It could be speculated that following disassociation from Sufu within cilia, Gli3FL is “primed” in a cilia-dependent manner for subsequent phosphorylation in the nucleus to form Gli3A. This “priming” is missing in cells that lack cilia, resulting in the failure to initiate hh signalling in the absence of Sufu and cilia. Further work is needed to fully explain the activation of Gli3FL and the role of cilia in this process.

	Cilia function	Exencephaly	Polydactyly	Node Cilia	Heart looping	<i>Ptch1</i> expression	Patterning of Neural tube	Reference
<i>Kif3a</i>	Anterograde IFT			Absent	Randomised	Reduced	Severely Dorsalised	Nonaka et al 1998; Huangfu 2003
<i>Ift88</i>	IFT particle	Yes	Yes	Absent	Randomised	Reduced	Severely Dorsalised	Huangfu 2003
<i>Ift172</i>	IFT particle	Yes		Absent	Randomised	Reduced	Severely Dorsalised	Huangfu 2003
<i>Ift52</i>	IFT particle	Yes	Yes	?	Randomised	Reduced	Dorsalised	Liu 2005
<i>Ift57</i>	IFT particle	Yes	Yes	Absent	Randomised	Reduced	Dorsalised	Houde 2006
<i>Dnchc2</i>	Retrograde IFT		Yes	Short	Randomised	Reduced	Dorsalised	May 2005
<i>Ift122</i>	IFT particle			Short			Ventralised	Cortellino 2009
<i>Thm1/Ift139</i>	Retrograde IFT	Yes	Yes	Short	?	Increased	Ventralised	Tran 2008

Table 4. Analysis of various cilia mutants and their effect on Left-right development and hh signalling.

1.6 Cilia and Human Disease

1.6.1 Ciliopathies - an overlapping spectrum of disease

Defects in cilia are found in a range of human diseases collectively called ciliopathies (Table 5). Since primary, nonmotile cilia are found on nearly every cell in the human body (Gherman et al., 2006), it is perhaps unsurprisingly that ciliopathies can affect multiple organ systems; they include the complex, embryonic lethal Meckel-Gruber syndrome and complex adult diseases such as Bardet Biedl syndrome (BBS) and Alstrom syndrome. Strikingly however, some ciliopathies affect only single organs, for example the kidney in Junvenile Nephrophthisis and the eye in Retinotis Pigmontosa (Badano et al., 2006). Thus, ciliopathies cover a broad spectrum of possible phenotypes. Nevertheless, many ciliopathies share common phenotypes such as cystic kidneys or retinal degeneration (Table 5).

The overlapping spectrum of defects described in the ciliopathies is matched by an underlying expressivity; the same disease gene is often affected in different ciliopathies. For example, mutations in *TMEM216* are found in patients with Meckel-Gruber syndrome, Joubert syndrome and Orofaciodigital syndrome (Valente et al., 2010). The underlying cause of the expressivity could be the strength of the different mutant alleles; for example, *NPHP3* null mutations give rise to Meckel-Gruber syndrome (Bergmann et al., 2008) whereas hypomorphic *NPHP3* mutations can cause Junvenile Nephrophthisis (Olbrich et al., 2003). Alternatively, triallelic inheritance could account for variation within ciliopathies (e.g. inheritance of three mutant BBS genes causes Bardet Biedl syndrome (Katsanis et al., 2001)).

1.6.2 Functional analysis of Polycystic Kidney Disease

Mutations in *PKD1* and *PKD2* cause dysregulation of calcium signalling and formation of kidney cysts in PKD individuals. PKD1 and PKD2 interact to form a Ca^{2+} channel in the membrane of kidney cilia. Mechanical bending of cilia due to urine flow in the kidney brings PKD1 and PKD2 into contact to induce calcium induced calcium release (CICR), thereby regulating water homeostasis (Hanaoka et al., 2000; Nauli et al., 2003; Praetorius and Spring, 2001, 2003).

1.6.3 Functional analysis of Bardet Biedel syndrome

Nachury and colleagues (2007) found that *BBS 1, 2, 4, 5, 7, 8* and *9* form a complex they called the BBSome. The BBSome is located at the basal body and knockdown of components of the BBSome result in lack of cilia. The BBSome interacts with *Rabin8* at the basal body. Consistent with a role in ciliogenesis, knockdown of *Rabin8* also results in lack of cilia. *Rabin8* mediates loading of GTP onto *Rab8*, therefore suggesting that *Rab8*, which is located in cilia, is also needed for ciliogenesis. A mutant form of *Rab8* that fails to bind GTP is excluded from cilia and prevents ciliary membrane extension. Conversely, expression of activated *Rab8* (*Rab8*-GTP) promotes membrane extension. Therefore, the BBSome mediates activation of *Rabin8* to promote cilia accumulation of active *Rab8* and subsequent cilia axoneme and membrane extension (Nachury et al., 2007).

Recent analysis of *BBS3* (*Arl6*), an additional component of the BBSome, suggests a role for the BBSome in sorting membrane proteins that are transported to cilia. The BBSome interacts, via *Arl6*, with liposomes to form a membrane BBSome coat. One of the proteins transported to cilia by the BBSome coat is somatostatin receptor 3 (SSTR3). This suggests that the BBSome coat transports cargos into cilia (Jin et al., 2010).

1.6.4 Functional analysis of Meckel Gruber Syndrome

MKS1 and *MKS3* (encoding Meckelin) are mutated in Meckel-Gruber syndrome (Kyttala et al., 2006; Morgan et al., 2002; Paavola et al., 1995; Smith et al., 2006). Meckelin (which localises to cilia and the plasma membrane) and MKS1 (a basal body protein) are required for apical positioning of the centriole before it matures into a basal body and forms a cilium (Dawe et al., 2007). This requirement is mediated by actin remodelling controlled by RhoA signalling and Nesprin-2 (an actin binding protein), thereby implicating the actin cytoskeleton with centriole migration and the early stages of ciliogenesis (Dawe et al., 2009).

1.6.5 Primary Ciliary Dyskinesia – a Ciliopathy affecting cilia motility

Defects in non-motile primary cilia, which typically cause kidney cysts or retinal degeneration, form the basis of most Ciliopathies (Table 5). However, mutations disrupting motile cilia, which are present in Primary Ciliary Dyskinesia (PCD) and Kartegener syndrome, cause a different phenotype (Table 5). PCD and Kartegener syndrome are heterogeneous disorders; most patients present with chronic bronchitis, chronic otitis media,

male and female infertility and *situs inversus* (Leigh et al., 2009). Mutations have been found in PCD that disrupt the outer dynein arms and radial spokes of motile cilia, resulting in uncoordinated, ineffective or lack of cilia beating (Fliegauf et al., 2007; Leigh et al., 2009).

	OMIM	Chronic Bronchitis	Cleft Palate	Cystic Kidneys	Deafness	Diabetes	Immotile Sperm	Liver Fibrosis	Obesity	Occipital encephalocele	Polydactyly	Retinal degeneration	Situs inversus
<i>Alstrom</i>	203800				Yes	Yes			Yes			Yes	
<i>Bardet Biedl</i>	209900			Yes		Yes			Yes		Yes	Yes	Yes
<i>Joubert</i>	213300			Yes								Yes	
<i>Juvenile Nephronophthisis</i>	256100			Yes									
<i>Kartenger</i>	244400	Yes					Yes						Yes
<i>Meckel Gruber</i>	249000		Yes	Yes				Yes		Yes	Yes		
<i>Orofaciodigital</i>	311200		Yes	Yes							Yes		
<i>Primary Ciliary Dyskinesia</i>	242650	Yes					Yes						
<i>Polycystic Kidney Disease</i>	173900			Yes				Yes					
<i>Retinitis Pigmentosa</i>	268000											Yes	
<i>Senior Loken</i>	266900			Yes								Yes	

Table 5. Analysis of cilia-based Human diseases (collectively called Ciliopathies).

1.7 Understanding gene function by mutagenesis

During the past 15 years there has been an intensive effort to sequence the genomes of a wide variety of organisms; this included sequencing the human (Lander et al., 2001; Venter et al., 2001) and mouse genomes (Waterston et al., 2002). Sequencing data has been deposited into open databases, such as NCBI and Ensembl, which are freely accessible. The development of tools, such as BLAST and Clustal X, has greatly facilitated the mining of this data by enabling comparison of sequences within and between species (Flicek et al., 2010; Sayers et al., 2010). However, despite the growing accumulation of DNA sequencing data there is still a major challenge to fully understand the long list of nucleotides that make up a genome sequence. The availability of a genome sequence greatly facilitates the exploration of gene function but does not, in itself, provide knowledge of function. Moving from sequence to gene function is arguably the major challenge in the post genomics era.

1.7.1 Sequence to function – mutagenesis in the Mouse

Historically, gene function was studied by analysing the phenotypes resulting from mutation of genes. This so called forward genetic approach, a term coined in response to the development of gene targeting technology in the 1980s, allows study of gene function by first understanding the phenotype a gene contributes to. Spontaneous mutations or those resulting from intentional mutagenesis (chemical or radiation) causing a mutant phenotype are then mapped and the mutated gene identified. Reverse genetic approaches, in contrast, allow gene function to be tested by mutating a known gene in a specific way and studying the resulting phenotype.

Both forward and reverse genetic approaches allow analysis of a range of genetic defects, from absence of gene function (null) to reduced (hypomorph) and enhanced (hypermorph) gene function. This is of particular importance in understanding the genetic basis of human disease since a population normally has a range of genetic defects for a particular disease gene. To overcome the ethical and practical difficulties of forward and reverse genetic approaches in humans, model organisms are used to study aspects of gene function shared with humans (Nolan et al., 2002). An organism that has been extensively used to develop models of human disease is the mouse. Mice share significant aspects of their anatomy, physiology and gene function with humans, have well defined genetics, they can be bred in large numbers and both forward and reverse genetic tools exist to readily manipulate the mouse genome (Acevedo-Arozena et al., 2008).

While studies of simple genetic diseases have identified single genes as being responsible, it is possible for a gene to have multiple functions in various parts of the organism and at various times during the organism's life cycle. Comprehensive understanding of gene function depends on analysis in all of these different contexts. However, most phenotyping strategies focus on a particular developmental stage or function of a particular organ. The success of both forward and reverse genetic approaches is therefore dependent on the ability to accurately and comprehensively phenotype the mutant. Continuing efforts to develop standardised phenotyping strategies will further enable forward and reverse genetic approaches to be successfully used in the mouse to understand human disease (Acevedo-Arozena et al., 2008).

1.7.2 Reverse Genetic Approaches in the Mouse

Targeted mutagenesis by homologous recombination involves the manipulation of specific genes to modify their function. The Nobel Prize awarded to Capecchi, Evans and Smithies in 2007 honoured the development of the technologies needed for this approach. The first direct breakthrough came in 1981 with the publication of two studies that described the successful culturing of embryo derived stem cells (Evans and Kaufman, 1981; Martin, 1981). Cultured embryonic stem (ES) cells from one mouse strain were then successfully injected into blastocysts from another strain to generate chimeric mice. These mice were shown to transmit, through the germline, coat colour from the ES cell-derived strain (Bradley et al., 1984). The next breakthrough came when foreign DNA was successfully introduced and integrated into ES cells. These modified ES cells were incorporated into the germ line of chimeric mice, therefore allowing transmission of the foreign DNA from generation to generation (Gossler et al., 1986; Robertson et al., 1986). The following methodology was used: ES cells were grown in culture and exposed to retroviral vectors (Robertson et al., 1986) or plasmid vectors (Gossler et al., 1986) containing a neomycin selectable marker. Cells were selected for successful vector integration and then injected into blastocysts to generate chimeric mice, which were bred for germline transmission (Gossler et al., 1986; Robertson et al., 1986). Parallel developments in somatic cell homologous recombination (Koller and Smithies, 1992) in combination with this culturing technology raised the possibility of introducing specific changes into individual genes to generate heritable mutations. Manipulation of the *Hprt* locus proved this was possible (Doetschman et al., 1987; Doetschman et al., 1988; Hooper et al., 1987; Kuehn et al., 1987; Thomas and Capecchi, 1987; Thompson et al., 1989). Initially, ES cells were selected for variant *Hprt* function and *Hprt* null mice were generated from these ES cells (Hooper et al., 1987). The *Hprt* locus was also randomly mutated by retroviral insertion and ES cells that lacked *Hprt*

function were selected (Kuehn et al., 1987). Following these proof of principle experiments, the *Hprt* locus was then specifically disrupted by homologous recombination (Doetschman et al., 1988; Thomas and Capecchi, 1987). *Hprt* mutations were also rescued by reintroducing the promoter region and the first two exons of *Hprt* into *Hprt* mutant ES cells (Doetschman et al., 1987; Thompson et al., 1989). Targeted mutagenesis by homologous recombination was shown to be applicable to other loci by mutating *En-2* (Joyner et al., 1989). Since the development of this technology, approximately 9000 genes have been mutated in the mouse by gene trapping and homologous recombination (Collins et al., 2007).

Reverse genetic approaches are arguably limited in that they assume a certain amount of current knowledge of a specific gene and its predicted effect when mutated. When used to mutate a single gene it is biased to test what is already hypothesized and while it may reveal unexpected roles for that gene it is unlikely to identify novel genes acting in a specific process or pathway. However, this is no longer the case when large numbers of genes are mutated and the resulting mice are systematically phenotyped. Currently, major international efforts are attempting to do this. Collectively, the Knock Out Mouse Project (KOMP), European Conditional Mouse Mutagenesis Program (EUCOMM), North American Conditional Mouse Mutagenesis Project (NORCOMM) and Texas A&M Institute for Genomic Medicine (TIGM) aim to generate another 8500 knock out mice (Collins et al., 2007). These mutant mice will be systematically analysed in large scale phenotyping pipelines designed to comprehensively understand the mutant phenotype (Acevedo-Arozena et al., 2008).

1.7.3 Forward Genetic Approaches in the Mouse

Forward genetic approaches, in contrast to reverse genetics, move from phenotype to genotype by first identifying a phenotype arising from random mutagenesis and then uncovering the mutated gene. Importantly, forward genetic approaches do not assume prior knowledge of gene function or the nature of the genetic defect causing the phenotype and are therefore more likely to uncover novel genes acting in a pathway or process.

The process of identifying gene function through phenotype is exemplified in phenotype driven screens. In such screens, the genome of an individual is randomly mutated by irradiation, random integration of viral vectors or a chemical mutagen and the resulting progeny are screened for phenotypes of interest. Once a phenotype has been identified further work is needed to identify the causative gene (Chapter 4).

A highly effective mutagen in mice is N-ethyl-N-nitrosourea (ENU) (Russell et al., 1979) and is commonly used in phenotype driven screens. ENU is injected into male mice to introduce point mutations in the germ line. Following a period of sterility these G0 mutagenised males are bred and their progeny screened for dominant and recessive mutant phenotypes. Dominant mutations are evident in G1 progeny while recessive mutations are identified in the G3 generation (Figure 5). Using this approach, if the G1 founder male contains a recessive mutation, it is expected that one out of eight G3 progeny will be homozygous mutant, four will be heterozygous and three will be wild type.

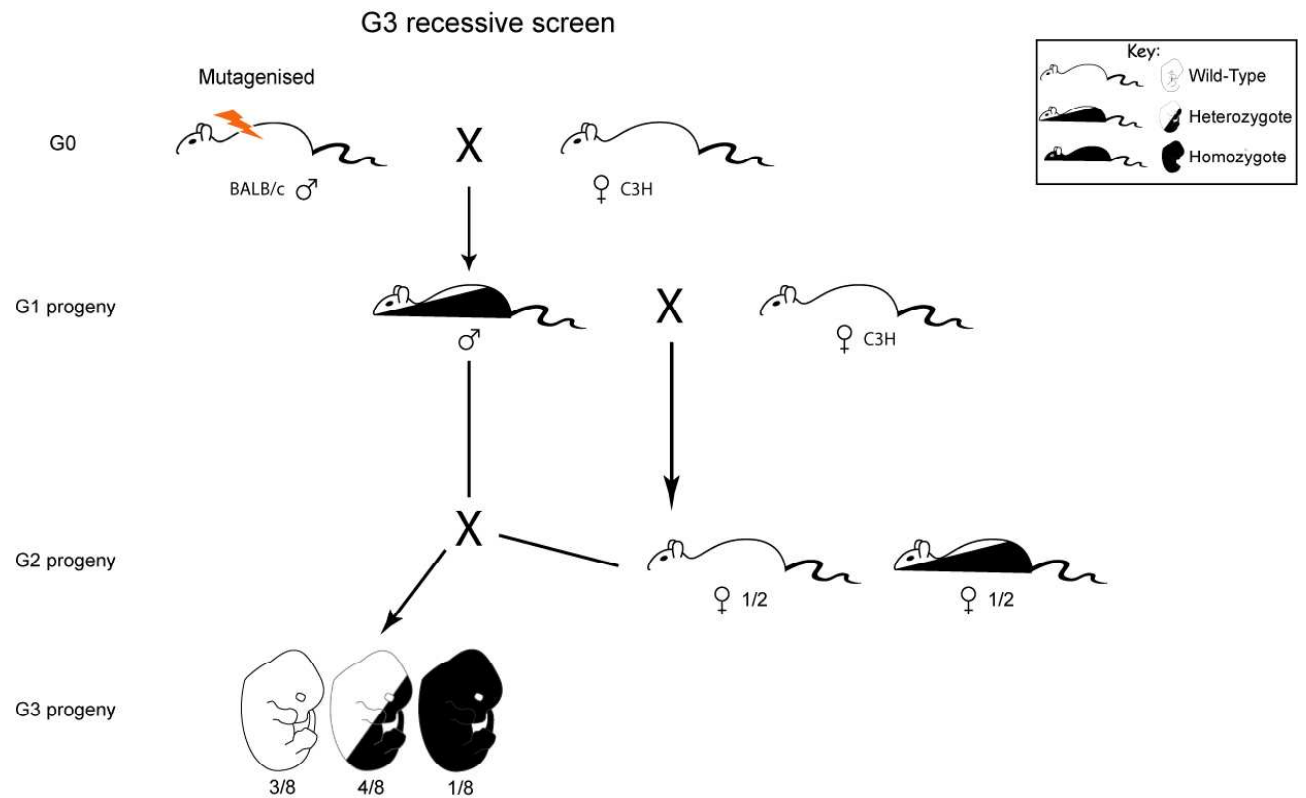


Figure 5. G3 Recessive Screen.

BALB/c males were mutagenised and then crossed to wild type C3H females. Male progeny (Generation 1 (G1)) were used as founder males for screening; they were crossed to wild type C3H females, generating G2 offspring (half of all G2 progeny will carry any specific mutation). G2 females were backcrossed to G1 founder males, generating G3 progeny. 1 in 8 G3 progeny will be homozygous for any particular mutation.

ENU based phenotype-driven screens have been successful in small scale genome wide screens (McDonald et al., 1990; Russell et al., 1979; Symula et al., 1997), suppressor screens on a mutant background (Carpinelli et al., 2004) and regional and chromosome specific screens using balancer chromosomes (Kile et al., 2003) and chromosomal deletions (Bogani et al., 2005). ENU has also been successfully used in large-scale screens of dominant and recessive mutations throughout the genome (Clark et al., 2004; Hrabe de Angelis et al., 2000; Nolan et al., 2000; Sakuraba et al., 2005).

There is currently a major effort, in parallel to KOMP, EUCOMM, NORCOMM and TIGM, to generate ENU derived mutant alleles of all mouse genes under the European Mouse Mutagenesis Consortium (Wilkinson et al., 2010).

1.7.4 Identifying point mutations in ENU derived mutants

The main attraction of using ENU based forward genetic approaches is its unbiased nature; ENU can potentially mutate any gene in the genome. It has been estimated that ENU induces functional point mutations once in every 1.82Mb of coding sequence in the mouse genome (Quwailid et al., 2004). Therefore, an F1 individual generated from a mutagenised male, given the coding region of the mouse genome is 33.5Mb (1.27% of 2.64Gb) (Church et al., 2009) will contain approximately 18 functionally relevant mutations. The possibility of multiple mutations in ENU mutants causes the substantial challenge of identifying the mutation responsible for a phenotype of interest and not an unrelated point mutation. Furthermore, this raises the possibility that different aspects of a phenotype might result

from different mutations. Backcrossing to wildtype helps to mitigate these problems by segregating unlinked mutations from the phenotype of interest (Keays et al., 2006).

Polymorphic genetic markers, including microsatellite markers (DNA repeats that vary in size between different mouse strains) and single nucleotide polymorphisms (SNPs; single base pair differences between strains) allow DNA from different mouse strains to be followed (Wiltshire et al., 2003). In forward genetic screens a defined mouse strain is mutagenised and crossed to wild type individuals of a different strain to facilitate genetic mapping. Initially, DNA from phenotypically abnormal individuals is collected and used to define a region from the mutagenised strain that is genetically linked to the phenotype. Once genetic linkage has been established haplotypic analysis is then carried out to discover sets of linked markers that originate from the mutagenised strain (often called the minimal region) that should contain a mutation.

Additional mutant individuals are then collected to provide additional recombination events that may have occurred within the minimal region. Recombinant mutant individuals will retain the causative mutation but the region originating from the mutagenised strain will be reduced. Ideally, the minimal region needs to be as small as possible to exclude genes that may harbour an unrelated mutation. It has been estimated that the probability of a 5Mb minimal region (assuming 115kb coding DNA in this region) containing more than one mutation is very low ($p < 0.002$) (Keays et al., 2006). Mapping should therefore aim to reduce the minimal region to around 5Mb.

Genes within the refined minimal region are then sequenced to identify any mutations. Given the number of potentially relevant mutations in ENU derived mutants, a mutation found within the minimal region will not necessarily be responsible for the mutant phenotype. Once a mutation has been found two strategies are typically used to test whether the correct mutation has been found. First, genetic rescue of recessive mutations can be attempted. A wild type copy of the affected gene is reintroduced into the original mutant background to assess whether the mutant phenotype is rescued. If the phenotype is rescued then the mutated gene is responsible. The second strategy involves Complementation Analysis; a second mutant allele of the affected gene is found and crossed to the original mutant to generate doubly heterozygous individuals. If the original mutation is not responsible for the mutant phenotype then doubly heterozygous individuals ($A^*B/AB^{\wedge}$) will carry a wild type copy of one gene (A) and a wild type copy of the other gene (B) that “complement” each mutation; these individuals will demonstrate a wild type phenotype. However, if the original mutation is responsible for the phenotype and both alleles of the identified gene disrupt the same function then the alleles will “fail to complement” and doubly heterozygous individuals ($A^*/A^{\wedge}$) will demonstrate the original mutant phenotype. Failure to complement is compelling genetic evidence that the causative mutation has been found.

A second allele is normally generated by disrupting gene function via homologous recombination or by random mutagenesis. However, these approaches are technically challenging and time consuming. A simpler methodology exists that avoids these problems. The generation of parallel sperm and DNA archives of ENU mutagenised G1 males provides a readily available resource of existent mutations. Once a mutation has been found mutant mice can be easily rederived and the phenotype assessed (Quwailid et al., 2004). It has been

estimated that G1 males contain 18 functionally relevant mutations (see above) and since the mouse genome contains approximately 28,851 protein coding regions (Ensembl), on average 1 in 1600 mutagenised males will contain a mutation in a gene of interest.

1.8 RNA interference

RNA interference (RNAi) is a key reverse genetic tool to understand gene function. A key breakthrough that led to the discovery and widespread use of RNAi was the observation that injecting double stranded RNA (dsRNA) into *Caenorhabditis elegans* resulted in gene silencing (Fire et al., 1998). Subsequent work identified RNAi as an endogenous biochemical pathway that controls the levels of mRNA in a cell. A general schema has emerged of how the pathway induces gene silencing: exposure of cells to long dsRNA (greater than 200bp) initiates the RNAi pathway; dsRNAs are processed by the RNase III-like enzyme Dicer into short interfering RNA (siRNA) of between 20-25bp. One strand of an siRNA (called the guide strand) becomes incorporated into the RNA-induced silencing complex (RISC). The RISC then binds to and cleaves mRNA that is complementary to the siRNA. Cleaved mRNA is subsequently degraded by exonucleases (Rana, 2007).

The RNAi pathway can be exploited experimentally to silence a gene by introducing an exogenous dsRNA into a cell (Rana, 2007). During the past 8 years, RNAi has become a key reverse genetic tool to understand gene function; it has been used to silence (or “knockdown”) genes in yeast, *C.elegans*, *Drosophila* and mammalian cells (Rana, 2007). However, the use of long dsRNA in mammalian cells is limited due to a strong, non-specific, antiviral response. This limitation has been overcome by introducing siRNAs (21-22bp in length) into mammalian cells, bypassing the antiviral response but still inducing RNAi (Elbashir et al., 2001).

1.8.1 Experimental Approaches to induce RNAi in Mammalian Cells

There are two main approaches to induce RNAi in mammalian cells; (i) exogenous siRNAs introduced into cells and (ii) expression of short hairpin RNAs (shRNAs). In the first approach, siRNAs are made outside the cell by chemical synthesis or *in vitro* transcription and subsequently transfected into the cell (Elbashir et al., 2001). In contrast, shRNAs are expressed within a cell, subsequently liberating an siRNA via Dicer (Brummelkamp et al., 2002). Exogenous siRNAs only silence a specific gene for a limited time (Elbashir et al., 2001) but shRNAs can be either transiently or stably expressed (Brummelkamp et al., 2002). Stable expression requires cells to have successfully integrated the shRNA-expressing plasmid into their genomes. This requires medium to long term selection of the integration event (Brummelkamp et al., 2002).

The success of RNAi, like other reverse genetic approaches, is dependent on the ability to correctly assess the effect of successful siRNA-mediated knockdown. It is therefore crucial to choose a relevant phenotype to measure following knockdown. For example, measuring the presence or absence of cilia can assess the severe ciliogenic effect of knockdown, whereas more subtle effects can be detected by measuring the length of cilia. It is of equal importance to validate whether the siRNA is targeting the gene of interest. It is possible that the siRNA is acting on another mRNA and that the phenotype that is seen is due to an “off-target” effect. This can be controlled for by validating the expression levels of the gene of interest following knockdown by Real Time PCR or Western Blot (Kim et al., 2010). Another concern is the possibility that the siRNA is affecting the gene of interest and also an off-target mRNA. However, if another siRNA, designed to target a different region of the

gene of interest, causes a similar phenotype it is unlikely to be due to the same off target effect.

Aims and Objectives

A novel embryonic lethal mouse mutant, called *gpg6*, was previously identified during an ENU mutagenesis screen (Ermakov et al, 2009). The aim of this thesis was to characterise *gpg6*, including:

1. Analysing the morphological and molecular phenotype of *gpg6* (Chapter 3).
2. Identifying the causative mutation in *gpg6* (Chapter 4).
3. Investigating the consequences of the mutation that is identified to uncover the underlying cause of the *gpg6* phenotype (Chapter 5 and 6).

Chapter 2

Materials and Methods

2.1 Archive Scan of Harwell ENU DNA

(carried out by Dr Deen Quwailid, MRC Harwell)

Exon 2 and Exon 3 of *Atmin* were screened in the Harwell ENU DNA Archive using an Idaho Technology Light Scanner (with Idaho HotShot PCR mastermix and Framestar 96 well plates). The Light Scanner utilises High Resolution DNA Melting Analysis to detect the presence of point mutations. Exon 2 and 3 were PCR amplified, in the presence of the double stranded DNA binding fluorescent dye LCGreen, using DNA from F1 heterozygous males bred from ENU mutagenised males. PCR products from regions without mutations will form a homoduplex, whereas heteroduplexes, containing a base pair mismatch at the position of the point mutation, will be amplified from carrier males. Homoduplexes will bind more LCGreen than heteroduplexes and the difference in fluorescence, indicative of the presence of a point mutation, is detected by the Light Scanner.

PCR primers were designed using Idaho Technology LightScanner Primer Design Software to generate PCR fragments between 150-550bp. 5ng of archive DNA pooled from 4 mice was used in a PCR reaction mix containing 1x LCGreen plus dye, 1x HotShot Mastermix, 0.2µM forward and reverse primers in a total volume of 20µl. Each reaction was overlaid with 20µl of mineral oil and run on a GRI G-Storm GS4 Thermal Cycler with the following program: 95°C for 2 minutes, 44 cycles of 95°C for 30 seconds, primer annealing for 30 seconds and 72°C for 30 seconds. PCR products were then allowed to form homoduplexes and heteroduplexes by heating to 95°C and then rapidly cooled to 15°C, followed by

centrifugation at 3000rpm for 3 minutes. Cooled duplexes were then run on the Light Scanner according to manufacturers instructions. Following identification of interesting DNA pools, DNA from individual pool members were PCR amplified, purified using Qiagen PCR purification kit and sent to GATC Biotech for sequencing to confirm presence of a mutation.

DNA samples from 6624 mice were tested for each *Atmin* exon analysed. Screening of exon 2 covered 2.11Mb of sequence (with 0.83Mb of coding sequence) and screening of exon 3 covered 1.88Mb of sequencing (with 1.32Mb coding sequence). One mutation, X40D1 T23A, was found in exon 2 and another mutation, X42H8 H210Q, was found in exon 3. The exon 3 mutant mouse was rederived using IVF.

2.2 Cloning

During the project, DNA (either fragments of restriction digested plasmids, cDNA or PCR products) were cloned into pBluescript II KS- (Agilent), TA plasmid made from pBluescript II KS- (pBS KS-) or Myc tagged plasmids (Clontech).

2.2.1 Restriction Digestion

A reaction mix was set up containing either vector or insert DNA, 2µl of various NEB Buffers (1, 2, 3 or 4 depending on the enzyme used), 1µl of BSA, 20 Units of NEB

Restriction Endonuclease and H₂O made up to 20µl. Digestion was carried out for 1 hour at 37°C.

2.2.2 Gel Extraction

DNA for cloning was run on a 1% agarose gel, visualised under long wave UV (Vilber Lourmat, TFP-20L system) and extracted using Qiagen Gel Extraction Kit according to manufacturers instructions.

2.2.3 Making TA Vector

Most *Taq* DNA Polymerases add an extra dATP to the end of a PCR amplicon. Therefore, to clone PCR products with a dATP overhang a complementary dTTP has to be added to a blunt cut pBS KS- vector to generate a TA plasmid. This was achieved by digesting 1µg of pBS KS- with 20 units of EcoRV for 1 hour at 37°C. EcoRV was inactivated by heating the reaction mixture to 80°C for 20 minutes and then cooled on ice. 2mM dTTP and 1 Unit/µg of *Taq* DNA Polymerase was added and incubated at 70°C for 2 hours. The reaction mix was run on a 1% agarose gel and gel extracted using Qiagen Gel Extraction Kit according to manufacturers instructions.

2.2.4 Ligation

Insert and vector were ligated together in the following way: in a reaction volume of 20µl, 50ng of vector was combined with 3x molar excess of insert together with 10µl of T4 Quick Ligase Reaction Buffer (NEB). 1µl of T4 Quick Ligase (NEB) was then added, the ligation reaction was mixed thoroughly and left for 5 minutes at room temperature.

2.2.5 Transformation

To transform ligated plasmids into bacterial cells, 10µl of the ligation reaction was mixed with 100µl of chemically competent DH5α bacterial cells in flat-bottomed tubes and incubated on ice for 30 minutes. Tubes were then placed in a 42°C water bath for 45 seconds. After this time, they were transferred to ice for 2 minutes. 900µl pre-warmed LB without antibiotics (at 37°C) was added to the cells and left in a 37°C shaking incubator for 1 hour. After this time cells were spun down (13,000g for 5 minutes), resuspended in 200µl of LB without antibiotic and plated on 30µg/ml Ampicillin resistant LB plates. Colonies were left to form at 37°C overnight.

The following day colonies were picked and grown overnight at 37°C in LB with 30µg/ml of Ampicillin. Plasmid DNA was extracted from cultured bacteria using Qiagen Mini Prep kit or Qiagen Midi Prep kit according to manufacturers instructions. Diagnostic restriction digests were used to confirm the correct DNA was cloned. Plasmids were also sent to GATC Biotech for sequencing.

2.3 Measuring DNA Concentration

All DNA concentrations were measured on a Thermo Scientific NanoDrop 8000 spectrophotometer according to manufacturers instructions.

2.4 DNA Preparation

2.4.1 Phenol/Chloroform Extraction

DNA was extracted from the liver of *gpg6* embryos and used in mapping and exon scanning. Initially, the liver was digested by adding 500µl lysis buffer and 15µl proteinase K (20mg/ml) and left overnight at 55°C. The following day, 500µl of a 1:1 mix of Phenol/Chloroform was added to the digested liver and gently mixed for 5 minutes. The solution was then spun at 13,000g for 5 minutes and the top layer was moved into a fresh tube. 500µl of Chloroform was added to the decanted solution and the solution was gently mixed for 5 minutes. Again the solution was spun at 13,000g for 5 minutes and the top layer was moved into a fresh tube. 500µl of Isopropanol was added to the DNA solution, mixed and spun at 13,000g for 10 minutes. The supernatant was removed and the pellet was washed in 200µl 70% Ethanol. The tube was spun at 13,000g for 2 minutes. The Ethanol was removed and the pellet air-dried. 100µl of TE (pH8) was added to the DNA pellet and left overnight at room temperature to allow the DNA to be fully dissolved.

2.4.2 Sodium Hydroxide Crude Extraction

DNA from adult ear clips and embryonic yolk sacs was extracted by adding 100µl of 50mM NaOH and heating at 95°C for 1 hour using a PCR machine. The reaction was neutralised by adding 10µl 1M Tris-HCl (pH 5). 2µl of crude DNA solution was used in PCR genotyping assays.

2.5 Gel Electrophoresis

DNA was run on 1% agarose gels made using 10% TBE and contained 0.5mg/ml Ethidium Bromide. Gels were run on BioRad Wide Mini-Sub Cell GT Tanks at 100V according to manufacturers instructions. Gene Ruler DNA ladder was also run to size the DNA bands. Gels were visualised under UV using the BioRad Gel Doc EQ system according to manufacturers instructions.

2.6 Genome Scan, Mapping, Sequencing and Genotyping

14 *gpg6* mutant embryos were used in whole genome linkage analysis (using a panel of 53 SNPs) by PCR based pyrosequencing (2.10). 18 *gpg6* mutants in total and 7 additional SNPs were used in haplotype analysis to map the mutation. *Dynlrb2* and *Atmin* exonic DNA (plus splice sites) was PCR amplified and sent to GATC Biotech for sequencing. The *gpg6 Atmin* mutation and the X42H8 H210Q archive *Atmin* mutant were genotyped by PCR based pyrosequencing. *Smo* mice were genotyped according to Zhang *et al*, 2001.

2.7 Histology

Wild type and *gpg6* 13.5dpc embryos were dissected, embedded in wax and transverse sections were taken and stained with Eosin and Haematoxylin.

2.8 Mutagenesis

N-Ethyl Nitrosourea (ENU) was used to mutagenise BALB/cAnn (BALB/c) males (Ermakov *et al*, 2009). These males were crossed to wild type C3H/HeH (C3H) females to generate a G1 founder male. This male was then crossed to wild type C3H females to generate G2 progeny. Females from G2 were backcrossed to the G1 founder male and G3 embryos were dissected at 13.5dpc. It is expected that 1/8 embryos would be mutant, 4/8 would be heterozygous and 3/8 would be wild type.

2.9 Polymerase Chain Reaction (PCR)

All Polymerase Chain Reactions (PCR) were carried out using the following reagents in a total volume of 25 μ l:

DNA (5ng/ μ l)	2 μ l

Buffer (1.5mM Mg ²⁺)	2.5 μ l

dNTP (10mM/ μ l)	0.5 μ l
Primer L (10pmol/ μ l)	1.0 μ l
Primer R (10pmol/ μ l)	1.0 μ l
Taq polymerase (5 Units)	0.2 μ l
H ₂ O	18.8 μ l

All reactions were carried out using the following PCR program on a G-Storm Thermal Cycler:

- a. 95°C for 5 minutes
- b. Denature at 95°C for 30 seconds, Anneal at X°C (specific for each primer pair) for 30 seconds, Extension at 72°C for 45 seconds, 35 cycles
- c. Extension at 72°C for 5 minutes and stored at 10°C.

2.10 Pyrosequencing

(carried out by GEMs, MRC Harwell)

Pyrosequencing is a sequencing-by-synthesis method that was used to genotype *gpg6* mutants and the exon 3 archive mutant of *Atmin*. The pyrosequencing reaction involves, initially, a standard PCR using a biotinylated forward primer, a normal reverse primer and DNA extracted from adult ear clips or embryonic yolk sacs. PCR products containing biotin

were purified using Streptavidin Sepharose beads and the Qiagen Q96 vacuum workstation according to manufacturers instructions. Then an internal sequencing primer, purified PCR products and Qiagen pyrosequencing reaction mix were used to run the pyrosequencing reaction. This was carried out on a Qiagen Biotage PSQ HS 96A Pyrosequencing machine according to manufacturers instructions.

The pyrosequencing reaction involves the binding of a sequencing primer to the biotinylated PCR amplicon. This is followed by the addition of each nucleotide in turn to the reaction. DNA polymerase adds a complementary nucleotide to the sequencing primer and releases pyrophosphate (PPi). ATP sulfurylase, in the presence of adenosine 5' phosphosulfate (APS), converts PPi to ATP. ATP then drives the conversion of luciferin to oxyluciferin in a luciferase-dependent manner to generate light. The light signal is detected and is proportional to the number of nucleotides added to the sequencing primer. The presence of the nucleotide-degrading enzyme Apyrase removes any unincorporated nucleotides and ATP after each nucleotide addition. Alfa-thio triphosphate (dATP-S), which is not recognised by luciferase, is used instead of natural deoxyadenosine triphosphate (dATP) in the reaction. In this way a short sequence read is generated that includes the base pair of interest (e.g. the *Atmin* point mutations).

2.11 RNA Preparation and Reverse Transcriptase PCR (RT-PCR)

Wild type embryos were dissected at 7.5-8.5dpc under PBS and snap frozen using liquid nitrogen. RNA was extracted using Qiagen RNeasy and Qiagen Shredder kits according to manufacturers instructions. cDNA was generated using Invitrogen Superscript III and random primer according to manufacturers instructions.

2.12 Scanning Electron Microscopy

Embryos were dissected at 7.5-8.5dpc under PBS and fixed in 0.1M Sodium Cacodylate with 2.5% Glutaraldehyde for 1 hour at room temperature. After this time embryos were washed twice in 0.1M Sodium Cacodylate for 5 minutes each at room temperature and then dehydrated in an ethanol series (25%, 50%, 75% in H₂O and 100%). Following this, embryos were washed twice in glass vials with Acetone and stored in Acetone at 4°C.

Embryos were critically point dried using the Critical point dryer EMI-Tech K850 and scatter coated using Polaron SEM Coating Unit E5100. Images were taken with a Hitachi S-530 SEM.

2.13 Real Time PCR

2.13.1 Principle

Real Time PCR allows the monitoring (in “real time”) of PCR amplification to enable the relative quantification of template DNA. ABI Real Time PCR Taqman assays were used. These assays involve two standard primers that amplify a region of cDNA template and a fluorescently labelled Taqman probe (containing a reporter label and a quencher label) that binds to a region between the two standard primers. Normally, the fluorescent reporter is silenced by the quencher and does not emit a signal. However, when the primers and

Taqman probe are allowed to bind to the cDNA template during the annealing and extension phase of the PCR cycle, Taq DNA Polymerase extends the primers until it reaches the Taqman probe and with its 5' exo-nuclease activity cleaves the Taqman probe. In the act of digesting and removing the Taqman probe, the fluorescent label is liberated from the quencher and emits a signal that is detected. As the PCR reaction continues over time more signal is detected. The signal is proportional to the amount of product made, which is directly related to the amount of template initially added to the reaction.

2.13.2 Reaction Conditions

RNA was extracted from 11.5dpc C3H wild type and *gpg6* mutants using Qiagen RNeasy and Qiagen Shredder kits according to manufacturers instructions. cDNA was generated using an ABI High Capacity cDNA reverse transcription kit according to manufacturers instructions. The amount of cDNA that was generated was measured using a Nanodrop 8000 Spectrophotometer. Taqman probes were used for *Dynl1l*, *Dynl1l*, *Cetn4* and endogenous control *β -actin*. All reactions (per well) contained 10 μ l Taqman Fast Universal PCR mastermix, 1 μ l of TaqMan probe, 5 μ l of cDNA (at the desired concentration) and 4 μ l of RNase free H₂O. Reactions were performed in Microamp Fast Optical 96 well plates, sealed with Microamp Optical Adhesive Film and carried out using the 7500 Fast Real Time PCR System.

2.13.3 Standard Curve

To determine the optimum cDNA concentration for each TaqMan probe, a Standard Curve was generated. cDNA was diluted to 100ng/μl, 10ng/μl, 1ng/μl, 0.1ng/μl and 0.01ng/μl and used in the Real Time Reaction mix. Reactions were carried out in triplicate for each concentration with a non-template control (NTC) triplicate. The Standard Curve determines the Cycling Threshold (C_T) or the cycle number when the fluorescent signal is first detected above a defined threshold. The mean C_T and standard deviation were calculated for each concentration. The ΔC_T is a measure of the efficiency of the Real Time reaction and is calculated in the following way:

$$[1] \quad \Delta C_T = C_{T(\text{Target})} - C_{T(\text{Endogenous control})}$$

The standard deviation (s) of the ΔC_T is calculated in the following way:

$$[2] \quad s = \sqrt{(s_1^2 + s_2^2)}$$

The ΔC_T values for each reaction were then plotted against the log input amount of cDNA added to the reaction and the slope of the line was calculated. If the slope of ΔC_T vs log input was <0.1 then the amount of cDNA was optimal and used in the subsequent relative quantification reaction. The optimum cDNA concentration was 100ng/μl for *Dynl1l* and *Dynlt1* and 10ng/μl for *Cetn4* and β -actin.

2.13.4 Relative Quantification

Reactions were carried out at the optimum cDNA concentration in triplicate for both wild type and mutant cDNA samples, including a triplicate NTC. The experimental TaqMan probes (*Dynl1l*, *Dynl1* or *Cetn4*) and the endogenous control (*β -actin*) were used to determine the relative levels of transcript in wild type and mutant cells. Relative levels are calculated using ΔC_T for wild type and mutant as follows:

$$[3] \quad \Delta\Delta C_T = \Delta C_{T \text{ Mutant}} - \Delta C_{T \text{ Wild type}}$$

The standard deviation of $\Delta\Delta C_T$ is the same as that calculated for ΔC_T for wild type and mutant (see [1] and [2]). The fold change between wild type and mutant was calculated in the following way:

$$[4] \quad \text{Fold Change} = 2^{-\Delta\Delta C_T}$$

All calculations were carried out automatically using ABI software supplied with the ABI 7500 Fast Real Time System.

2.14 Tissue Culture

All standard tissue culture experiments used Dulbecco modified eagle media (DMEM) supplemented with 10% Fetal Calf serum (FCS), 1% Pen/strep and 1% L-Glutamine. 10cm tissue culture plates were used.

2.14.1 Making MEFs

gpg6 mutant and C3H wild type embryos were dissected at 11.5dpc under PBS; the hindbrain and visceral organs were removed. The remaining embryonic tissue was disassociated using 1ml Trypsin and a 21g needle attached to a 5ml syringe. 10ml of supplemented DMEM was added to the resultant cell suspension and plated on a 10cm plate. Cells were grown to confluency in a Sanyo MCO-15AC incubator (37°C, 5% CO₂) over 3-4 days.

2.14.2 Splitting Cells

To split cells on to new plates, confluent plates were washed twice in PBS (-MgCl₂). To detach cells, 3ml of Trypsin was added to plates and incubated for 6 minutes (37°C, 5% CO₂). 3ml of supplemented DMEM media was used to inhibit the trypsin and the cell suspension was added to 17ml tubes. Tubes were spun at 1000g for 5 minutes to pellet the cells. Supernatant was removed and 12ml of supplemented DMEM media was used to resuspend the cells. Cells were split 1:3 on 10cm plates and grown to confluency.

2.14.3 Freezing Cells

Freezing media (60% DMEM, 20% DMSO and 20% FCS) was used to freeze cells. Cells were trypsinised as for splitting cells and resuspended in 0.5ml freezing solution containing

50% supplemented DMEM media and 50% freezing media. Cells were frozen in cryovials and placed at -80°C. Cells were stored long term in liquid nitrogen.

2.14.4 Defrosting Cells

Cryovials were removed from storage and kept at -80°C on frozen carbon dioxide. Cells were then quickly thawed in a 37°C water bath and added to 10ml of supplemented DMEM in a 17ml tube. Tubes were then centrifuged (1000g for 5 minutes) and the supernatant removed. Cells were resuspended in 12ml of supplemented DMEM and added to 10cm plates. Plates were grown to confluency (37°C, 5% CO₂).

2.14.5 Plating Cells on slides

2 well slides were used to grow wild type and mutant cells for antibody staining. To plate cells on 2 well slides, confluent 10cm plates were trypsinised and cells counted using a haemocytometer according to manufacturers instructions. 2×10^5 cells were diluted in 2ml of supplemented DMEM per well, added to the wells and slides were incubated (37°C, 5% CO₂) for 24 hours. Supplemented DMEM media without FCS was then added and the slides incubated (37°C, 5% CO₂) for a further 4 days. Slides were briefly washed twice in PBS (-MgCl₂) and processed for antibody staining.

2.15 IFT88 Antibody Staining

Cells on the 2 well slide were processed for antibody staining by fixing and permeabilizing cells using 4% PFA and 0.2% Triton X100 in PBS for 5 minutes. Cells were washed twice in PBS for 5 minutes each and then blocked in PBS +1% BSA for 30 minutes. IFT88 (anti-goat) primary antibody and acetylated tubulin (anti-mouse) primary antibody were diluted 1:1000 in PBS + 1% BSA and added to cells for 60 minutes. Cells were then washed twice in PBS for 5 minutes and then washed in PBS overnight. Alexoflour 594 Donkey anti-goat secondary antibody and Alexoflour 488 Donkey anti-mouse secondary antibody were diluted 1:1000 in PBS + 1% BSA and added to cells for 60 minutes. Cells were then washed twice in PBS for 5 minutes and then washed in PBS overnight. Slides were processed by removing the plastic well dividers, adding vector shield (H100 Vector Lab) to cover slips and inverting the cover slips onto the slide. Cover slips were sealed with nail varnish.

All MEFs cilia pictures were taken using a 60x lens on an Olympus BX44 microscope with an Olympus CFW-1312M camera.

2.16 siRNA Knockdown

All knockdown experiments were carried out in IMCD3 ciliated cells. Defrosting, expanding and freezing of IMCD3 cells was done as described for MEFs but culture flasks and supplemented DMEM/F12 media (10% FCS, 1% Pen/strep and 1% L-Glutamine) was used. All knockdown experiments used IMCD3 cells grown on coverslips. Initially, new

coverslips were washed in acetone and then in 100% ethanol and allowed to dry in a 6-well plate. 1.5×10^5 cells per well were resuspended in 2ml of supplemented DMEM/F12 media and applied to the well containing the coverslip. Knockdown cells harvested for RNA or protein were grown without coverslips. Plates were incubated (37°C, 5% CO₂) overnight until approximately 60% confluent.

Next day, the siRNA/lipofectamine complex mixture for 1 well was prepared by adding 4µl of RNAi MAX lipofectamine and 196µl of OptiMEM reduced serum media in an RNase free eppendorf tube. This was mixed and incubated for 5 minutes at room temperature. Then 195µl of OptiMEM reduced serum media was added along with 100pmol of siRNA duplex (5µl of a 20µM working solution). This was mixed and incubated for 20 minutes at room temperature to allow siRNA/lipofectamine complexes to form. During this time, DMEM/F12 was removed from the IMCD3 cells and replaced with 1ml per well of OptiMEM reduced serum media. After the incubation period, the siRNA/lipofectamine complexes were added to the cells and swirled to mix. Cells were incubated (37°C, 5% CO₂) for 4 hours. Then, 1ml DMEM/F12 media containing 20% FCS was added and cells were incubated (37°C, 5% CO₂) for 24 hours. After this time, media was removed and replaced by DMEM/F12 media containing no FCS (to encourage cilia growth) for the required period (e.g. an additional 24 hours for 48 hour knockdown, an additional 48 hours for 72 hour knockdown or an additional 72 hours for 96 hour knockdown). After the required time, cells were harvested for RNA and protein or processed with antibodies to stain cilia.

2.17 Antibody Staining of Coverslips

After the required period of knockdown, coverslips were carefully removed from the 6-well plates using jeweller's forceps and placed (*cell side up*) into a clean 6-well plate containing 2ml of PBS per well. The coverslips were then wash three times in PBS. Cells were then fixed by removing the PBS and adding 2ml ice cold 100% methanol per well. Plates were incubated at -20°C for a maximum of 5 minutes. On removing the cells from -20°C, the methanol was discarded and replaced by 2ml per well of PBS with 2 drops of blocking solution (1% skimmed milk powder (Marvel) in PBS and spun at 13000rpm for 5 minutes to pellet undissolved milk powder). Cells were then washed once in PBS. 2ml of blocking solution replaced the PBS and cells were left to block at room temperature for 5 minutes.

During this time a humidity chamber was prepared (a sealable eppendorf box with parafilm on top of water soaked tissue paper). Primary antibody was also prepared (1 in 1000 dilution in blocking solution). 100µl of primary antibody per cover slip was carefully pipetted onto the parafilm. After blocking, coverslips were inverted and placed (*cell side down*) onto the primary antibody. The humidity chamber was closed and left for 1 hour at room temperature. After this time, coverslips were returned (*cell side up*) to a 6-well plate containing 2ml of fresh PBS. Cells were washed three times in PBS.

The parafilm in the humidity chamber was replaced and 200µl of secondary antibody (1 in 1000 dilution in blocking solution) per coverslip was carefully pipetted onto the new parafilm. Coverslips were then inverted (*cell side down*) onto the secondary antibody and left in the dark (covered in tin foil) for 1 and half hours, at room temperature. Coverslips

were then returned (*cell side up*) to a 6-well plate containing 2ml PBS. Cells were then washed in PBS four times. The parafilm was replaced as before and coverslips were inverted (*cell side down*) onto 100µl of DAPI solution (1 in 2000 in blocking solution) per well and left in the dark for 5 minutes at room temperature. After this time, coverslips were placed (*cell side up*) into a 6-well plate containing 2ml PBS per slide. Cells were washed four times in PBS and once in PCR grade water. Finally, coverslips were mounted by inverting them (*cell side down*) onto microscope slides with 60µl of Mowiol and left flat overnight in the dark to set. Pictures were taken using a 60x lens on an Olympus BX44 microscope using an Olympus CFW-1312M camera. Z-stacks were taken with a Nikon Eclipse TE2000-E scanning confocal microscope and the associated Nikon EZ-C1 software using a x100 lens. One image was taken every 0.15µm through the cell from just below the cell to just above the cilium, each image was scanned 4 times, and the average of the four scans was taken to be assembled to form a Z stack. A 10µm scale bar was added, and this was used to measure the cilia in each field of view.

2.18 Western Blotting

(Conducted by Rosario Romero and Helen Hilton, Proteomics, MRC Harwell)

2.18.1 Protein Preparation

Protein from embryonic total cell lysate was prepared using Nonidet P40 (NP40) lysis buffer (150mM NaCl, 50mM Tris-HCl (pH8) and 1% NP40 in PBS). Initially, 9.5dpc and 11.5dpc embryos were dissected under PBS and placed in eppendorf tubes. Embryos were weighed and the appropriate volume of NP40 lysis buffer (3ml of NP40 lysis buffer per gram of tissue containing 50µl per gram of 25x stock Proteinase Inhibitor). Tissue was broken up

using a 21g needle attached to a 5ml syringe and incubated on ice for 30 minutes. After this time, cell debris was pelleted by centrifugation at 10,000g for 10 minutes at 4°C. The resulting protein supernatant was either stored at -20°C or used in Western blots.

2.18.2 Protein Gel Electrophoresis

Protein was run on Invitrogen NuPAGE gels (3-8% Tris-acetate for Gli3 and Atmin and 4-12% Bis-Tris for Dynll) according to manufacturers instruction.

2.18.3 Blotting

Following electrophoresis gels were transferred to PVDF membrane using Biorad Transblot SD system according to manufacturers instructions.

2.18.4 Antibody Incubation and Development of Signal

Following blotting, membranes were placed in blocking solution (5% Marvel skimmed milk powder and 0.1% Tween 20 in PBS) for 1 hour at room temperature. During this time primary antibody was diluted in blocking solution (Anti-Atmin 1:500, Anti-Gli3 1:500, Anti- β tubulin 1:5000, Anti-Dynll1 1:5000, Anti-Actin 1:5000). Primary antibody was added to the membrane and incubated at 4°C overnight. Next day, membranes were washed three times in PBS + 0.1% Tween 20 for 10 minutes each. Secondary antibody conjugated to horseradish peroxidase (HRP) was diluted in blocking solution (1:10000; Anti-mouse for

Gli3 and Anti-rabbit for Atmin, Actin, β tubulin and Dynl11) and added to the membrane for 1 hour at room temperature. Then membranes were washed three times in PBS + 0.1% Tween 20 for 10 minutes each. After this time membranes were briefly blotted on blue roll and transferred to a clean Petri dish (care was taken not to allow the membrane to dry out). ECL solution was then added to cover the membrane and incubated for 5 minutes at room temperature. The membrane was then briefly blotted on blue roll and placed in a hypercassette. The membrane was then exposed to ECL hyperfilm and developed in a Xograph developer.

2.19 Whole Mount In Situ Hybridisation (WISH)

Prior to use all solutions were treated with 0.1% DEPC (mixed overnight and then autocleaved) to destroy any RNase. All eppendorf tubes and tips were also RNase free.

2.19.1 Plasmid Details to Synthesize Probes

All plasmids (excluding the *Nodal* plasmid which was a gift from Dr Liz Robertson) were cloned from PCR products generated from 8.5dpc embryonic cDNA.

<i>Gene</i>	<i>Linearisation</i>	<i>RNA Polymerase</i>
<i>Nodal</i>	HindIII	T3
Cdy	BamHI	T3
C15	BamHI	T3
J17	XbaI	T3
Atmin	HincII	T7

Gsch	HindIII	T7
CO3	XbaI	T3
NO5	HindIII	T7
MPhos	BamHI	T3
Dynll1	HindIII	T7
J22	BamHI	T3
HSD1	BamHI	T3
BCM	HindIII	T7
073	BamHI	T3
068	HindIII	T7
Pkd1l2	XhoI	T3

2.19.2 Plasmid Linearisation

10µg of plasmid DNA was digested at the 5' end of the insert using specific restriction endonucleases (NEB). 5µl of Reaction Buffer (NEB Buffer 1, 2, 3 or 4), 5µl of BSA (NEB) and 1µl Restriction endonuclease (20 units/µl) was used in a reaction volume of 50µl. Digests were left at 37°C for 1 hour. After this time 0.5µl (100ng) of reaction mixture was run on a 1% agarose gel to check for complete digestion.

The remaining reaction was extracted using 50µl Phenol/Chloroform (1:1 mix) followed by 50µl Chloroform, removing the top layer into an RNase free eppendorf each time. The extracted, linearised plasmid DNA was precipitated by adding 1µl of glycogen, 5µl of 3M Sodium acetate and 110µl of ice cold 100% Ethanol and leaving the mixture at -20°C for

over 1 hour. After this time, the mixture was spun at 13,000g for 20 minutes at 4°C. The resulting pellet was air-dried, resuspended in 10µl TE (pH8) and stored at -20°C.

2.19.3 Probe Synthesis

Probe was synthesised from linearised plasmid using the following reaction mix (total volume 40µl): 1µg of linearised plasmid, 4µl of 10X transcription buffer, 4µl of DIG dNTP (2mM each), 80 units of RNase Out, 40 units of RNA Polymerase (T7 or T3) and H₂O.

Reactions were incubated at 37°C for 2 hours. 2µl of reaction was run on a 1% agarose gel to check for the presence of probe. The remaining RNA was extracted with 100µl H₂O, 15µl 3M Sodium acetate (pH 5.2) and 300µl of ice cold 100% Ethanol and left at -20°C for over 1 hour. After this time, the mixture was spun at 13,000g for 20 minutes at 4°C. The resulting pellet was air-dried, resuspended in 200µl TE (pH8), aliquoted in 50µl and stored at -80°C.

2.19.4 Embryo Collection

Embryos were dissected under PBS from 6.5dpc – 12.5dpc and fixed using 4% PFA in PBS overnight at 4°C. Embryos were genotyped and then moved into 12 well plates containing Netwell baskets. Embryos were dehydrated through a methanol series (25%, 50%, 75% with PBT and 100%) and stored in 100% methanol at -20°C.

2.19.5 Embryo Bleaching, Digestion and Application of Probe

Embryos were rehydrated through a methanol series (100%, 75%, 50%, 25% in PBT), for 5 minutes each at room temperature. Embryos were washed twice in PBT for 5 minutes each and then washed in 6% Hydrogen Peroxide (H_2O_2) for 1 hour to bleach the embryos. After this time, residual Hydrogen Peroxide was removed by washing in PBT three times for 5 minutes each.

Embryos were digested with Proteinase K (PK; $10\mu g/\mu l$) in PBT, for five and a half minutes for 8.5dpc embryos to allow the probe access to the embryo. The PK reaction was stopped by washing embryos in 2mg/ml Glycine for 5 minutes. Embryos were then washed in PBT twice for 5 minutes each and then fixed in 4% PFA/0.2% Glutaraldehyde in PBS for 20 minutes. Residual fix was removed by washing in PBT twice for 5 minutes each.

A PBT:Hybridisation (1:1) solution mix was prepared and applied to the embryos for 10 minutes at room temperature. Then the embryos were washed in Hybridisation (Hyb) solution twice (once for 10 minutes at room temperature and once at $70^\circ C$ for two and a half hours). After this time old Hyb solution was replaced by fresh Hyb solution, 50-100ng RNA probe was added and embryos were left overnight at $70^\circ C$.

2.19.6 Washing the Probe and Preparation of the DIG Antibody

Following the overnight incubation, embryos were washed in fresh Hyb solution for 5 minutes at 70°C and then washed in prewarmed Solution 1 four times (once for 5 minutes at 70°C and three times for 30 minutes each at 70°C). During the final wash the temperature was lowered to 65°C. Embryos were then washed three times in prewarmed Solution 2 for 30 minutes each at 65°C. After these series of washes the embryos were left at room temperature to cool.

During Solution 1 and Solution 2 washes, MAB buffer was made and used to make a stock solution of 2% Boehringer Mannheim Blocking Reagent (BBR/MAB). Using this stock BBR/MAB solution, 10% serum BBR/MAB was made by adding 9ml stock BBR/MAB to 1ml heat inactivated (65°C for 30 minutes) Sheep serum and 100µl levamisole. In addition, 1% serum BBR/MAB was made by adding 3ml stock BBR/MAB to 40µl sheep serum and 40µl levamisole. The DIG antibody was then pre-absorbed to block the antibody: 1ml of stock BBR/MAB and 6mg embryo powder was added to an RNase free eppendorf (1 tube per 2 wells used) and left at 70°C for 30 minutes. The tube was cooled on ice and vortexed for 5 minutes. 5µl of heat inactivated sheep serum and 1µl DIG antibody was then added and the tube was mixed at 4°C for over 1 hour. After this time the tube was centrifuged for 10 minutes at 13000g. The resulting supernatant was added to 1% serum BBR/MAB.

Once the embryos were cooled following Solution 1 and Solution 2 washes, they were washed three times in MAB buffer for 5 minutes each at room temperature. 10% serum BBR/MAB was added to the embryos and incubated for 1 and a half hours at room

temperature. Embryos were then incubated overnight at 4°C in 2ml of 1% serum BBR/MAB containing the preabsorbed antibody.

2.19.7 MAB Buffer Washes to Reduce Background Signal

Next day, embryos were washed three times in MAB buffer for 5 minutes each at room temperature and then five times in MAB buffer for 1 and a half hours each at room temperature. Embryos were then incubated in MAB buffer overnight at 4°C.

2.19.8 Development of Signal

Following overnight incubation in MAB buffer embryos were washed in AP Buffer three times at room temperature for 10 minutes each. Embryos were then incubated in 2ml BM Purple per well (with 20µl of levamisole and 20µl of 10% Tween 20) until the signal developed. The alkaline phosphatase reaction was stopped by washing in 2mM EDTA/PBT.

Embryos were posted fixed in 4% PFA/0.1% glutaraldehyde in PBS for 1 hour at room temperature, washed in PBS twice for 5 minutes each and stored in 1mM EDTA/PBT. Embryos were then washed through a glycerol series (25%, 50%, 75% in PBT and 100%) and pictures were taken with a Leica DFC 300 camera attached to a Leica MZ 125 microscope.

2.19.9 Solutions for WISH

Hybridisation (Hyb) solution:

Formamide	250ml
20x SSC	125ml
Tween 20	0.5ml
20% SDS	2.5ml
Heparin (50mg/ml in 4x SSC)	0.5ml
Yeast tRNA (10mg/ml) (Sigma)	2.5ml
1M Citric Acid	30ml
H ₂ O	89ml

Solution 1:

Formamide	25ml
20x SSC	12.5ml
1M Citric Acid	3ml
H ₂ O	7ml

Solution 2:

Formamide	25ml
20x SSC	5ml

1M Citric Acid	1.2ml
20% SDS	0.5ml
10% Tween 20	0.5ml
H ₂ O	18.3ml

MAB Buffer (pH 7.5):

Maleic Acid	5.805g
5M NaCl	17ml
NaOH pellets	3.5g
Tween 20	0.5ml

DEPC H₂O to 500ml

(0.5ml of 100% Levamisole was added for every 50ml aliquot of MAB buffer)

Alkaline Phosphatase (AP) Buffer:

1M Tris-HCl (pH 9.5)	5ml
5M NaCl	1ml
1M MgCl ₂	2.5ml
10% Tween 20	0.5ml
100% Levamisole	0.5ml
DEPC H ₂ O	41.5ml

2.20 General Solutions

2.20.1 Stock Solutions

ddH₂O (Gibco)

20% SDS (Amresco)

10x TBE (Biorad)

EDTA (Ethylenediaminetetraacetic Acid, Sigma). Stock solution 0.5M, pH8.0

DMSO (Dimethyl sulphoxide, Sigma)

Sodium Chloride (NaCl) 5M:

292.2g of NaCl was diluted in 1 litre of H₂O.

Sodium Hydroxide (NaOH) 10M:

100g of NaOH was diluted on ice and in a plastic container in 250ml of H₂O.

Sodium Hydroxide (NaOH) 50mM:

5ml of 10M NaOH was added to 1 litre of H₂O.

Tris- HCl 1M:

121.1g Tris base (Sigma) was added to 800ml H₂O and the pH was adjusted by adding concentrated HCl (Sigma). The volume was made up to 1 litre with H₂O.

TE:

10mM Tris HCl (pH8), 1mM EDTA in H₂O.

PBS (Phosphate buffered Saline):

137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄ in H₂O.

20xSSC (pH 4.5):

3M NaCl, 0.5M NaCitrate in H₂O. pH was adjusted using concentrated HCl.

DNA Extraction Lysis Buffer:

50mM Tris-HCl (pH8), 100mM EDTA, 100mM NaCl, 1% SDS in 500ml of H₂O

2.20.2 Gel Electrophoresis

Orange G loading Buffer:

30% Glycerol (Fisher), H₂O and Orange G (Sigma) to desired colour. 5µl was used per PCR or Restriction digest.

Ethidium bromide stock:

10mg/ml Ethidium bromide (Sigma) in H₂O. 5µl was used in 100ml agarose/TBE solution.

2.20.3 Microbiological Solutions

Luria Bertani (LB) medium:

10% tryptone, 5% yeast extract, 10% NaCl in 950 ml H₂O. pH was adjusted to 7.0 using NaOH and the volume was made up to 1 litre with H₂O.

LB + Amp :

LB containing 30µg/ml Ampicilin (Sigma)

Agar plates:

1 litre of LB Agar was melted in a microwave and cooled to 50°C. Ampicillin was added to a final concentration of 30 µg/ml. LB Agar was poured into 10cm plates, allowed to cool and stored at 4°C.

2.21 Materials

2.21.1 Archive Scan

Framestar 96 Well Plate	4TI0961	Framestar
Idaho Light Scanner		Cadama
Idaho HotShot PCR Mastermix	HRLS-ASY0003	Cadama
LC Green Dye	BCHM-ASY-0006	Cadama

2.21.2 Cloning

17ml polypropylene tubes	TIS5406	SLS
Agar	A/1080/53	Fisher
Ampicillin	A-9518	Sigma
Atmin image clone	6847850	IMAGE
BamHI	R0136S	NEB
BseRI	R0581S	NEB
DH5 α cells		
EcoRV	R0195S	NEB
Gene clean spin kit	1101-400	Qbiogene

HincII	R0103S	NEB
HindIII	R0104S	NEB
Myc-tag plasmid	631604	Clontech
NotI	R01895	NEB
dNTPs	NTACG100	Q-biogene
pBluescript KS- plasmid	212206	Agilent
QIAfilter Plasmid Midi Kit (25)	12243	Qiagen
Qiagen Plasmid Mini Kit	27106	Qiagen
QIAquick Gel extraction kit (50)	28704	Qiagen
Quick ligation kit	M2200L	NEB
Sall	R01385	NEB
UV (Long Wave) light box	TFP-20L	Vilber Lourmat
XbaI	R0145S	NEB
XhoI	R0146S	NEB

2.21.3 DNA Concentration

Nano Drop 8000	Thermo Scientific

2.21.4 Gel Electrophoresis

10x TBE	161-0770	BioRad
3% agarose gels	161-3040	Biorad
Agarose Molecular Biology grade	BPE1356-500	Fisher
Gel Doc EQ system		BioRad
Gene Ruler	SM1331	Fermentas
SeaKem GTG Agarose (125g)	50070	Cambrex Bio
Science		
Wide Mini-Sub Cell GT Tanks and Combs	170-4405	BioRad

2.21.5 Immunostaining

Acetylated Tubulin antibody	T6793	Sigma
Alexa Fluor 488 chicken antigoat IgG H	A21467[4812860]	Invitrogen
Alexa Fluor 488 donkey antimouse IgG H	A21202[47209580]	Invitrogen
Alexa Fluor 594 donkey antigoat IgG H	A11058[48126860]	Invitrogen
Anti GFP fluorescent protein rabbit Ig	A21311[47774139]	Invitrogen
Atmin Antibody (anti-rabbit)	ab3271	Chemicon
BSA	B90015	NEB

Coverslips	MNJ-350-110G	Fisher
DAPI	D9564	Sigma
IFT88 goat antibody	AB42497	ABCAM
Microscope	BX44	Olympus
Camera	CFW-1312M	Olympus
Pasteur Pipettes	FB50253	Fisher
PBS	14080089	Invitrogen
Slides	57011/2	Thermo Scientific
Vectashield	H1000	Vector Lab
Vectashield with dapi	H1200	Vector Lab

2.21.6 In situ Probes

Dig labelled dNTPs	1277073	Roche
Ethanol	E/0650DF/17	Fisher
RNAse OUT	10777019	Invitrogen
Sodium Acetate	102363P	BDH
T3 RNA polymerase	11031163001	Roche
T7 RNA polymerase	10881767001	Roche

2.21.7 Whole mount In situ Hybridisation

12 well plates	673-225	Jencons
20% SDS	0837-500ml	Amresco
BM purple 100ml	11442074001	Roche
Boehinger Mannheim Blocking Reagent	1175041	Roche
Citric Acid	C/6160/53	Fisher
Diethyl pyrocarbonate (DEPC)	40718	Sigma
DIG Antibody	11093274910	Roche
EDTA	E5134	Sigma
Formamide	F/1551/PB17	Fisher
Glutaraldehyde solution 25%	360802F	VWR
Glycerol	G/0650/17	Fisher
Glycine	G8898	Sigma
Heparin	H3400	Sigma
Camera	DFC 300	Leica
Microscope	MZ125	Leica
Levamisole	L9756	Sigma

Maleic Acid	M/1255/53	Fisher
Methanol	M/4000/17	Fisher
NaCl	CAS 7647-14-5	Fisher
NaOH	S/4920/53	Fisher
Netwells - Costar	TKT-540-010L	Fisher
PBS	D5773-10L	Sigma
PFA	P6148	Sigma
Proteinase K	3115836001	Roche
Vacuum Pump	FB70155	Fisher
Sheep Serum	52263-100ml	Sigma
SSC	S6639	Sigma
Tween 20	P9416-100ml	Sigma
Yeast tRNA	R-6750	Sigma

2.21.8 Mapping/Genotyping/Pyrosequencing

Archive SNP - biotinylated and normal primer	MWG
Biotage PSQ HS 96A Pyrosequencing machine	Qiagen
Gsp6 SNP - biotinylated and normal primer	MWG

Q96 vacuum workstation		Qiagen
Sepharose Beads	17-5113-01	GE Healthcare

2.21.9 PCR

Adhesive PCR Film	AB-0558	VWR
Distilled Water	15230097[4773979]	Invitrogen
dNTPs	NTACG100	Q-biogene
ProofStart High Fidelity Taq	202203	Qiagen
Ice bucket	FB51477	Fisher
Moltag	p-010-1000	VH Bio
G Storm Thermal Cycler	GS00004	GRI
Primers	Various	MWG
TE8	327345000	Acros organics
Thermofast PCR plates	AB-0600	VWR

2.21.10 Real Time PCR

7500 Fast Real Time PCR system		ABI
β -actin Taqman probe	4352933E	ABI

cDNA synthesis kit	4368814	ABI
Centrin 4 Taqman probe	Mm00782119	ABI
Dynll1 Taqman probe	Mm00850282	ABI
Dynlt1 Taqman probe	Mm00839582	ABI
Microamp fast optical 96 well plate	4346906	ABI
Microamp optical adhesive film	4311971	ABI
Taqman Fast Universal PCR mastermix	4352042	ABI

2.21.11 RNA Preparation and Reverse Transcription

β-mecaptoethanol	M3148	Sigma
Centrifuge Rotor	CFA-114-510J	Fisher
Filter tips 1000ul RNase free	PQP-729-010A	Fisher
Filter tips 20ul RNase free	PQP-709-010X	Fisher
Filter tips 300ul RNase free	PQP-725-010B	Fisher
Filter tips 10ul RNase free	PQP-703-010E	Fisher
QIAshredder	79656	Qiagen
RNase free tubes	TUL-918-016C	Fisher
RNase OUT	10777019	Invitrogen

Rneasy mini kit	74104	Qiagen
Superscript III	180800093	Invitrogen

2.21.12 Scanning Electron Microscopy

Acetone	A/0600/17	Fisher
Critical point drier	K850	EMI-Tech
Glass bottle caps	G3966	Agar
Glass bottles	VC0105155D	Adelphi/Agar
Glutaraldehyde solution for SEM 25%	1.04239.0250	VWR
SEM	S-530	Hitachi
Porous pots	B7895	Agar
Scatter Coater	E5100	Polaron

2.21.13 siRNA Knockdown

25cm Flask	NNC-Bio-655B	Fisher
75cm Flask	658178	Grenier Bio One
Atmin siRNA	ss214922	Invitrogen
Atmin siRNA	ss214923	Invitrogen

Atmin siRNA	ss214924	Invitrogen
Block-it Red Oligo	14750-100	Invitrogen
DMEM/F12 Media	31330-095	Invitrogen
Dynll1 siRNA	s80610	Ambion
Dynll1 siRNA	s80611	Ambion
Dynll1 siRNA	s80612	Ambion
IFT88 siRNA	L-050417-00	Dharmacon
IMCD3		
Marvel Skimmed Milk Powder		Tesco
Mowiol 4-88	475904	Calbiochem
OPTI-MEM	31985-062	Invitrogen
RNAi Max lipofectamine	13778-075	Invitrogen
Scrambled siRNA	D-001810-10-05	Dharmacon

2.21.14 Tissue Culture

10cm tissue culture dish	tkt-110-070a	Fisher
12 well plates	673-225	Jencons
15cm tissue culture dish	tkt-110-130y	Fisher

17ml polypropylene tubes	TIS5406	SLS
2 chamber slides	tkt-210-450f	Fisher
21g needle	SZR-175-530R	Fisher
6cm tissue culture dish	tkt-110-010s	Fisher
8 chamber slides	tkt-210-490q	Fisher
Cell scraper individually wrapped sterile	FB55199	Fisher
Cryotube nunc external thread	CRY-296-020P	Fisher
DMSO	D2650	Sigma
Dulbecco Modified Eagle Media DMEM	31966047[48059689]	Invitrogen
Foetal Bovine Serum	10106185[47894205]	Invitrogen
L-Glutamine	25030024[47353942]	Invitrogen
PBS without Mg and Ca	14080089	Invitrogen
PBS with Mg and Ca	14040174	Invitrogen
Pencilin Streptomycin liquid	15140122[47353942]	Invitrogen
Incubator	MCO-15A	Sanyo
Scalpel swann-morton disposable sterile	SCA-310-070V	Fisher
Trypsin	25300062	Invitrogen

2.21.15 Western Blotting

21g needle	SZR-175-530R	Fisher
Actin Antibody	A2066	Sigma
Atmin Antibody	AB3271	Chemicon
β -tubulin Antibody	T6793	Sigma
Dynll Antibody	AB51603	Abcam
ECL hyperfilm		Amersham
Bioscience		
ECL Solution		Amersham
Bioscience		
Marvel Skimmed Milk Powder		Tesco
Nonidet P40 (NP40)	21-3277	Sigma
NuPAGE 3-8% Tris-Acetate gels		Invitrogen
NuPAGE 4-12% Bis-Tris gels		Invitrogen
Protease Inhibitor	P8340	Sigma
PVDF Hybond P membrane		GE Healthcare
Secondary Mouse Antibody – HRP linked		Sigma
Secondary Rabbit Antibody – HRP linked		Sigma
Transblot SD system		Biorad

Chapter 3

Morphological and Molecular Phenotype of *gpg6*

3.1 Introduction

3.1.1 A G3 recessive embryonic screen identifies the Gasping mutants

A G3 recessive embryonic screen was carried out to identify novel genes involved in L-R patterning (Ermakov et al., 2009). Phenotypic analysis of 13.5dpc embryos identified a novel class of mutants called Gasping (gpg), which demonstrated exencephaly, polydactyly, oedema, situs defects, pulmonary agenesis and hypoplasia, micrognathia and abnormal eye patterning. These phenotypes were argued to be due to defects in cilia (Ermakov et al., 2009).

In this thesis I describe my work analysing, cloning and characterising *gpg6*, one of the gpg mutants identified in the screen. In comparison to the defects identified in other gpg mutants, *gpg6* demonstrated the mildest phenotype since *gpg6* mutant embryos did not show polydactyly and demonstrated normal gross heart and stomach situs (Ermakov et al., 2009).

3.2 Results

3.2.1 Morphological Phenotype of *gpg6*

The original phenotype reported for *gpg6* was on a mixed BALB/C-C3H genetic background (Ermakov et al., 2009), raising the possibility that unlinked mutations contributed to the phenotype. To ensure the correct phenotype was identified, *gpg6* heterozygous mice were backcrossed onto a C3H background. This allowed any unlinked mutations to segregate from the *gpg6* phenotype as well as making *gpg6* congenic for C3H. Intercrossing $+/-gpg6$ heterozygotes generated congenic *gpg6* homozygous mutants (C3.C-*gpg6*) and these were dissected at 13.5dpc (Figure 6). 25 phenotypically abnormal embryos were analysed and DNA collected for subsequent mapping and sequence analysis (Table 6). Externally, 96% of *gpg6* mutants demonstrated exencephaly, 92% had oedema and 100% had a thickening and narrowing of the upper lip, suggestive of an anterior midline defect (Figure 6a-h). Eye patterning was abnormal in at least one eye in 64% of *gpg6* mutants; the most common defect was coloboma (defective closure of the embryonic fissure of the optic cup (OMIM 120200), which was identified by observing an incomplete ring of pigmented epithelium around the iris), with 52% of mutants having two affected eyes (Figure 6i-l). Microphthalmia (small eyes) was also seen in 24% of *gpg6* mutants. Internally, all *gpg6* mutant embryos demonstrated both pulmonary hypoplasia (mutant lungs were markedly smaller than wild type) and pulmonary isomerism. In wild type mice there is one lung lobe on the left and four lobes on the right. In *gpg6* embryos, 60% of mutants demonstrated left isomerism (one lobe on each side) and 40% demonstrated a partial isomerism with one lobe on the left and two lobes on the right (Figure 6m-u).

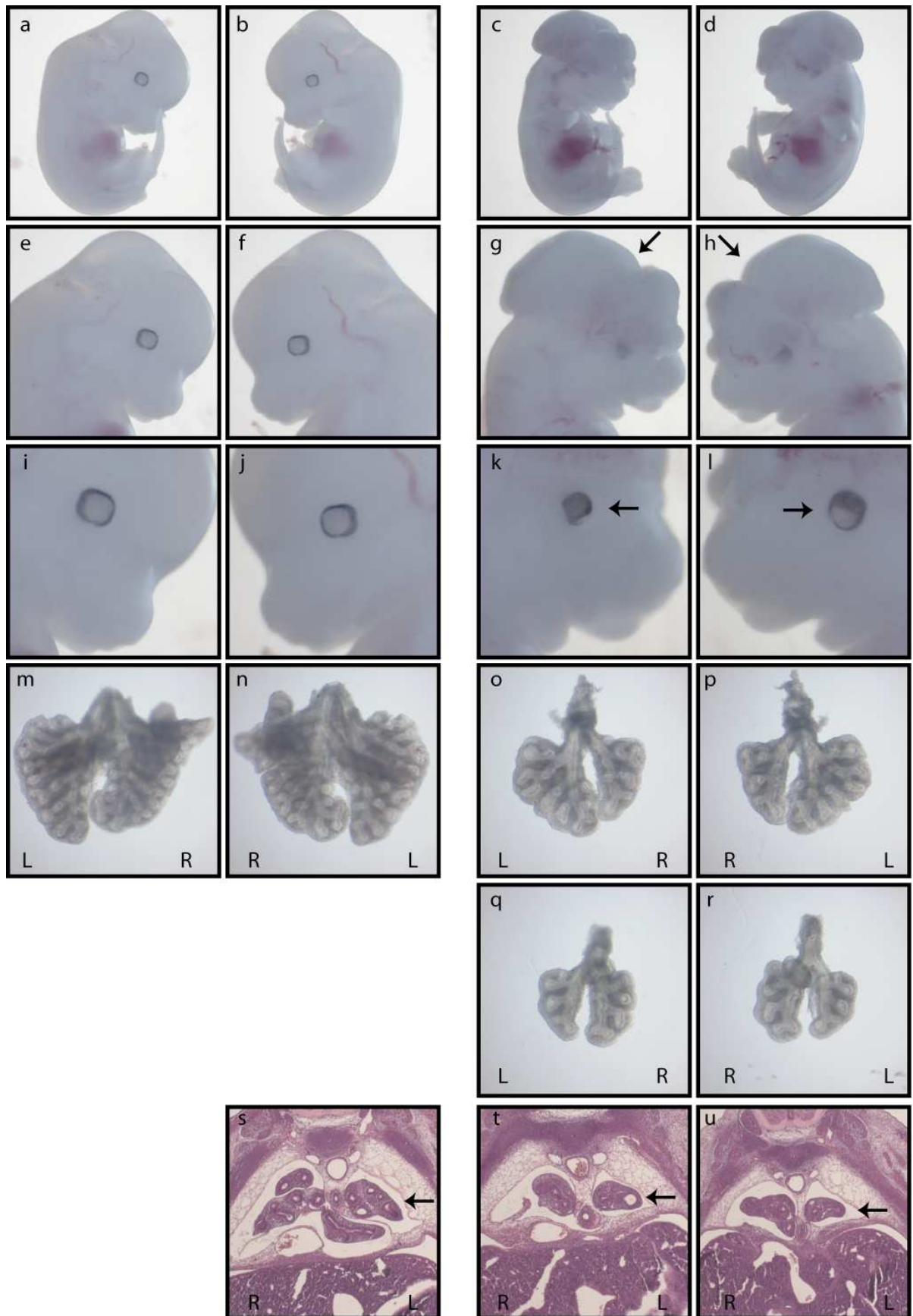


Figure 6. *gpg6* mutant embryos demonstrate a complex phenotype associated with disrupted L-R development and hh signalling.

By 13.5dpc wild type embryos have completed neural tube closure (a-b; e-f) and all major features of the head, including the eyes, have developed (i-j). In contrast, congenic *gpg6* mutant embryos demonstrate exencephaly, a failure to close the anterior neural tube (c-d; g-h). *gpg6* mutants also fail to fully form the iris, a condition called coloboma (k-l). On closer inspection, wild type embryos demonstrate lungs comprising 1 lobe on the left and 4 on the right (m-n). However, *gpg6* mutant lungs are smaller than wild type and can show left pulmonary isomerism (o-p) or partial pulmonary isomerism (q-r). Transverse histological sectioning of wild type (s) and *gpg6* mutant embryos (t-u) also revealed smaller lungs and left pulmonary isomerism in *gpg6* mutants.

(m) Ventral view. (n) Dorsal view (o and q) Ventral view. (p and r) Dorsal view.

Exencephaly	96%	24/25
Oedema	92%	23/25
Polydactyly	0%	0/25
Anterior midline defect	100%	25/25
Normal eyes (both)	28%	7/25
Normal eye (left)	8%	2/25
Normal eye (right)	0%	0/25
Coloboma (both eyes)	52%	13/25
Coloboma (left eye)	12%	3/25
Coloboma (right eye)	4%	1/25
Small eyes (both)	8%	2/25
Small eye (left)	16%	4/25
Small eye (right)	0%	0/25
Small Lungs	100%	25/25
Pulmonary Left Isomerism	60%	15/25
Pulmonary Partial Isomerism	40%	10/25
Heart Situs – Normal/left	100%	25/25
Stomach situs – Normal/left	100%	25/25
Outflow tract – Normal	4%	1/25
Outflow tract – IAA	48%	12/25
Outflow tract - Common trunk	36%	9/25
Outflow tract – Restricted aortic arch	8%	2/25
Outflow tract - Mispatterned aortic arch	4%	1/25

Table 6. *gpg6* mutant phenotype

Despite clear defects in L-R asymmetry in the lungs, gross situs of the heart and stomach was normal (Table 6). However, 96% of mutants demonstrated mispatterning of the cardiac outflow tract (Table 6). The most common defects were interrupted aortic arch (IAA; 48%) and common trunk (36%) with rare incidence of restricted aortic arch (8%) and mispatterned aortic arch (4%).

The phenotype of homozygous congenic C3.C-*gpg6* embryos was highly similar ($p < 0.05$; Chi squared test) to that previously reported (Ermakov et al., 2009). However, differences were evident (Table 7). The major difference is that pulmonary agenesis was no longer seen, while a higher incidence of pulmonary hypoplasia and pulmonary isomerism was seen (Table 7).

3.2.2 The Phenotype of *gpg6* suggests cilia related defects in hh signalling and L-R development

The role of hh signalling in neural tube patterning, eye patterning, lung and craniofacial development is well established (Ingham and McMahon, 2001) while L-R patterning mutants have defects in both heart and lung asymmetry (Brennan et al., 2002; Kitamura et al., 1999; Lin et al., 1999; Meno et al., 1998; Meno et al., 2001; Norris et al., 2002). Cilia are important in both these processes (Huangfu et al., 2003; Nonaka et al., 1998). As the *gpg6* phenotype could be explained to result from a combination of defective hh signalling and L-R patterning, we hypothesised that the primary defect in *gpg6* mutants could result from a defect in ciliogenesis (Ermakov et al., 2009).

<i>Phenotype</i>	<i>Gasping 6</i>	<i>Congenetic gpg6</i>
Oedema	12 (60%)	23 (92%)
Exencephaly	18 (90%)	24 (96%)
Polydactyly	0	0
Coloboma	12 (60%)	17 (68%)
Anterior midline defect	4 (20%)	25 (100%)
<i>Heart</i>		
Left Sided	20 (100%)	25 (100%)
Right Sided	0	0
Central	0	0
<i>Lungs</i>		
Normal Size	4 (20%)	0 (0%)
Small	13 (65%)	25 (100%)
Absent	3 (15%)	0 (0%)
Left Isomerism	5 (25%)	15 (60%)
Right Isomerism	0	0
Partial Isomerism	5 (25%)	10 (40%)
<i>Stomach</i>		
Left Sided	20 (100%)	25 (100%)
Right Sided	0	0
Central	0	0
Total	20 (100%)	25 (100%)

Table 7. Comparing non-congenetic and congenic *gpg6* phenotypes. Non-congenetic phenotyping data from Ermakov *et al*, 2009.

Short stumpy cilia were previously reported to be present in *gpg6* mutant nodes (Ermakov et al., 2009), a ciliated embryonic structure that is crucial for L-R development (Sulik et al., 1994; Nonaka et al., 1998). Such short cilia are characteristic of retrograde IFT mutants (Tran et al., 2008), suggesting that the *gpg6* phenotype has an underlying defect in retrograde IFT.

To ensure the node cilia phenotype originally reported was retained in congenic C3.C-*gpg6* mutant embryos, *gpg6* mutant node cilia were analysed. 4 wild type and 4 mutant embryos were dissected at 8.0dpc and processed for scanning electron microscopy (SEM). SEM images were taken by Dr Paraskevi Goggolidou (Figure 7). In wild type embryos, cilia were evenly distributed across the ventral surface of the node (Figure 7a-b) and were approximately 4µm in length (Figure 7c). In contrast, *gpg6* mutant nodes had fewer cilia (Figure 7d-e) and the cilia that were present were approximately 2µm in length (Figure 7f). This difference was subsequently shown to be statistically significant (Dr Paraskevi Goggolidou, personal communication; data not shown). Surprisingly, congenic *gpg6* mutant cilia did not show a stumpy phenotype as was previously reported (Ermakov et al., 2009) and in addition *gpg6* mutants had reduced numbers of cilia in the node, possibly reflecting the congenic nature of the mutants examined here.

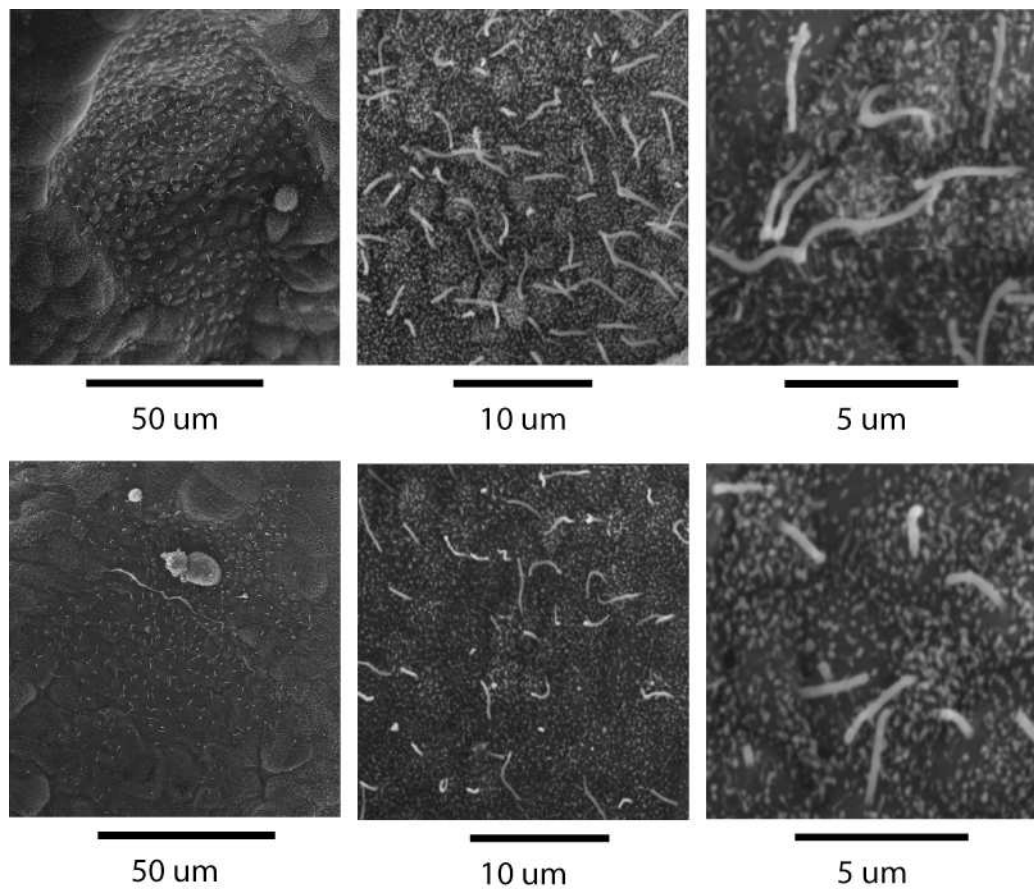


Figure 7. *gpg6* mutant nodes demonstrated defects in node cilia

Embryonic nodes from wild type embryos (a-c) and *gpg6* mutant embryos (d-f) were visualised using a scanning electron microscope (SEM). Cilia in wild type embryos were evenly distributed over the surface of the node (b) and were approximately 3-4 μ m in length (c). However, *gpg6* mutant embryos demonstrated reduced numbers of cilia in the node (e) and those cilia that formed were approximately 1-2 μ m in length (f). This difference was subsequently shown to be statistically significant (Dr Paraskevi Goggolidou, personal communication; data not shown).

3.2.3 Molecular Phenotype of *gpg6*

To understand the underlying cause of the morphological defects seen in *gpg6* mutants, the primary molecular defect was investigated by analysing the signalling output of the L-R pathway and also the hh pathway in *gpg6* mutants.

3.2.3.1 Left-Right Defects in *gpg6*

Nodal is a key gene in the establishment of L-R asymmetry in all vertebrates (Shiratori and Hamada, 2006) and is a good marker of L-R development since it is one of the earliest genes to be asymmetrically expressed at the node (stronger on the left than right side). Strikingly, *Nodal* is also expressed in the left but not right lateral plate mesoderm (Collignon et al., 1996; Lowe et al., 1996; Zhou et al., 1993). *Nodal* expression was therefore examined in *gpg6* mutant embryos. To do this *gpg6* homozygous mutants were generated by intercrossing *+/gpg6* heterozygous individuals. A range of embryos were dissected (from pre-somite to 5 somite stage) and processed for whole mount *in situ* hybridisation using a *Nodal* antisense RNA probe.

As expected from previously published data (Collignon et al., 1996; Lowe et al., 1996; Zhou et al., 1993), wild type and heterozygous embryos demonstrated symmetrical perinodal expression of *Nodal* from late pre-somite - 1 somite (Figure 8a-c, g-h). By 2-4 somites there was a qualitative increase in left sided expression of *Nodal* in the node (Figure 8d-f, i-l). *gpg6* mutants also showed a similar early symmetrical perinodal expression from pre-1

somite (Figure 8o-r). However, the marked increase in *Nodal* expression on the left side of the node from 2-4 somites failed to occur, resulting in symmetrical expression at later stages (Figure 8s-x).

Lateral plate mesoderm expression of *Nodal* was also examined in *gpg6* mutants. As expected (Collignon et al., 1996; Lowe et al., 1996; Zhou et al., 1993), 2-5 somite wild type and *gpg6* heterozygous embryos demonstrated expression of *Nodal* throughout the left lateral plate mesoderm, from the level of the heart tube to the posterior of the embryo (Figure 9a-g). Surprisingly, *gpg6* mutants from 2-3 somites demonstrated an anterior truncation of *Nodal* expression in the left lateral plate mesoderm (i.e. expression did not reach the level of the heart tube) (Figure 9sh-k). By 4-5 somites the extent of *Nodal* expression had recovered to that seen in wild type and heterozygous embryos (Figure 9l-n). This expression data suggests that there is a delay in the propagation of *Nodal* throughout the left lateral plate mesoderm in *gpg6* mutants.

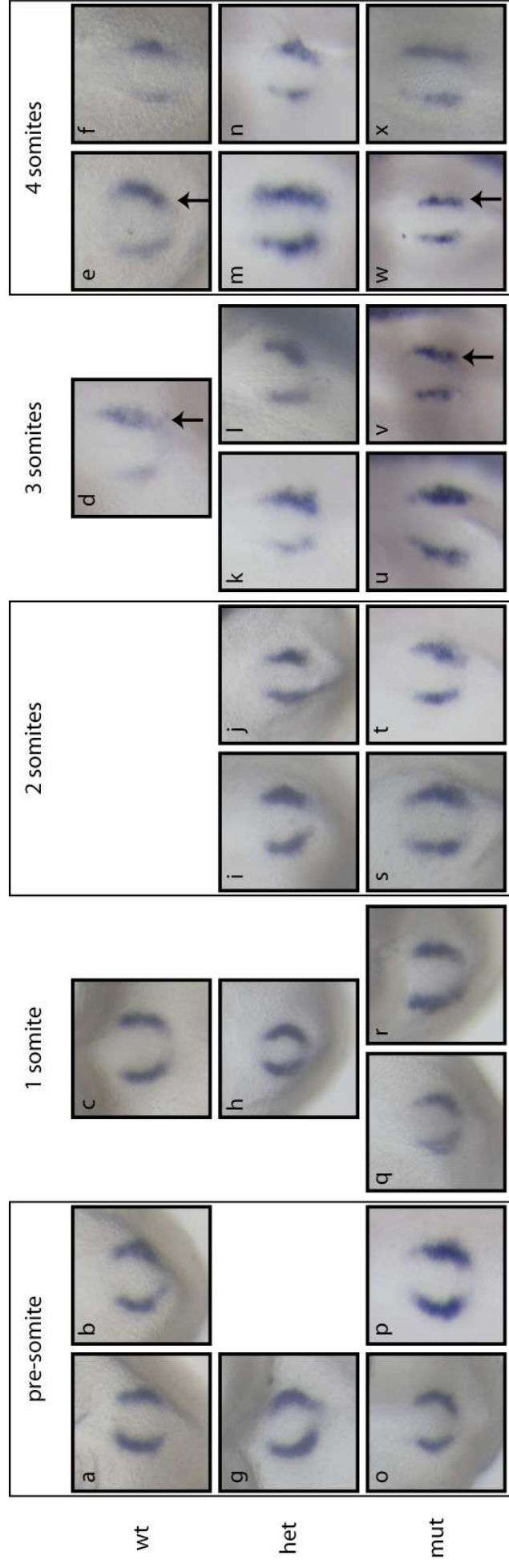


Figure 8. *Nodal* expression at the node fails to become asymmetric in *gpg6* mutants.

Nodal expression was analysed by WISH in 8.0dpc wild type, *gpg6* heterozygous and *gpg6* homozygous mutant embryos. In wild type (a-c), *gpg6* heterozygous (g-h) and *gpg6* homozygous mutant embryos (i-n) *Nodal* was initially expressed in a symmetrical perinodal ring around the node. At later stages, both wild type (d-f) and *gpg6* heterozygous (i-n) demonstrated a qualitative increase in left sided *Nodal* expression. However, similarly aged *gpg6* homozygous mutant embryos (s-x) failed to manifest the increase in left sided *Nodal* expression.

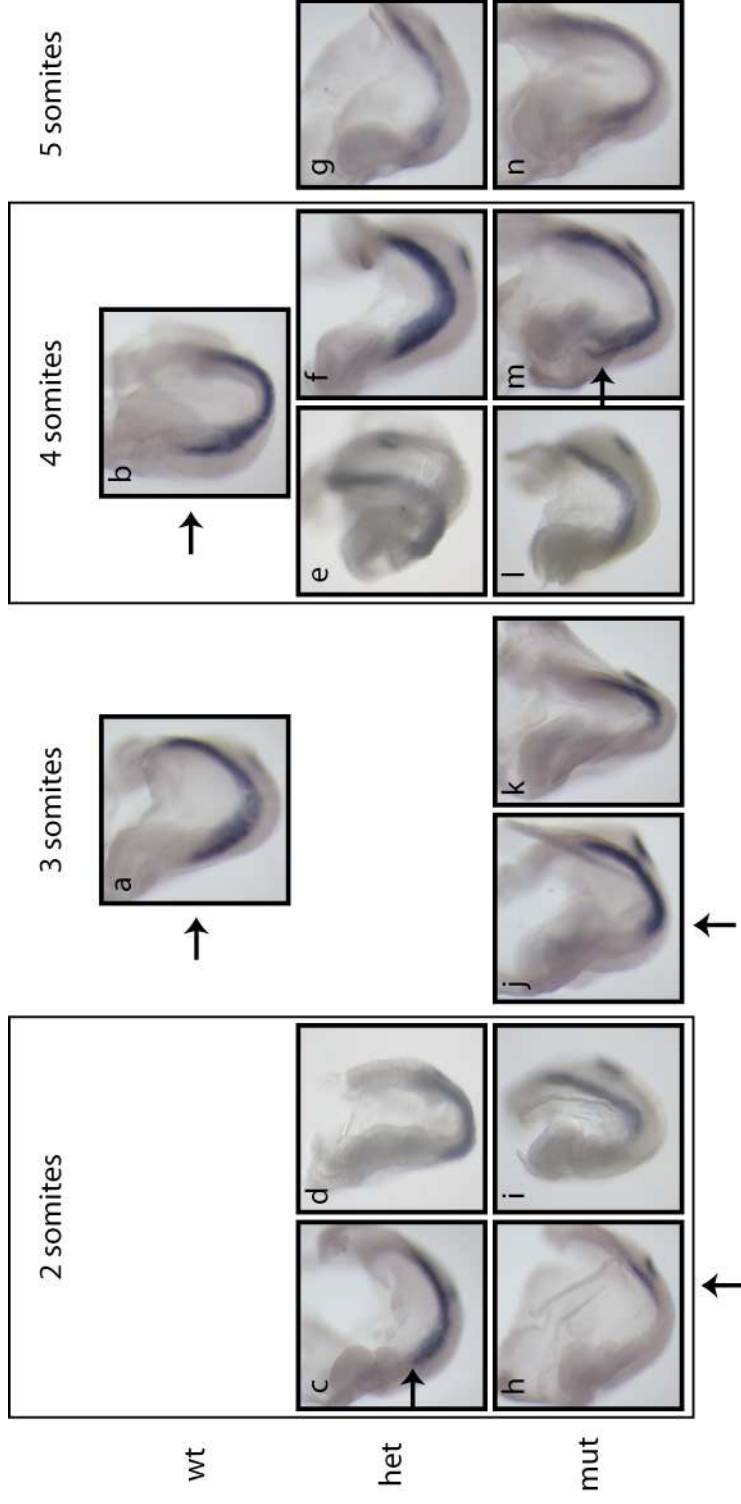


Figure 9. *gpg6* mutant embryos demonstrate anterior truncation of left lateral plate *Nodal* expression.

Nodal expression was analysed by WISH in 8.0-8.5dpc wild type, *gpg6* heterozygous and *gpg6* homozygous mutant embryos. Wild type (a-b) and *gpg6* heterozygous embryos (c-g), demonstrated *Nodal* expression throughout the left lateral plate. However, *gpg6* homozygous mutant embryos (h-k) initially demonstrated an anterior truncation of *Nodal* expression in the left lateral plate. At later stages, *gpg6* homozygous mutant embryos (l-n) demonstrated *Nodal* expression throughout the left lateral plate mesoderm.

3.2.3.2 hh signalling defects in *gpg6*

The role of the hh pathway in *gpg6* mutants was investigated using both genetic and molecular approaches. These included assessing whether *gpg6* genetically interacted with the hh pathway and analysing the downstream consequences of hh signalling such as Gli3 processing, *Ptch1* expression and D-V patterning of the neural tube.

3.2.3.2.1 Genetic Interaction with *Smo*

Smoothened (*Smo*) is a co-receptor and positive regulator of the hh pathway (Zhang et al., 2001). To investigate the relationship between *gpg6* and the hh pathway, genetic interaction with *Smo* was assessed. *Smo* mutants demonstrate a severe embryonic lethal phenotype, they fail to undergo embryonic turning, have a linear heart tube, ventral cyclopia and holoprosencephaly and die around 10.5dpc (Zhang et al., 2001). In contrast, *gpg6* mutants undergo embryonic turning, have a grossly normally patterned heart and demonstrate exencephaly (Table 6). These different phenotypes made it possible to perform epistasis analysis and to assess genetic interaction between *Smo* and *gpg6*.

Double heterozygous *+/gpg6;+/Smo* animals were intercrossed and 68 10.5dpc embryos were dissected, analysed and genotyped (Table 8 and Appendix A). All classes of genotype were represented at Mendelian ratios (Table 8). Most classes of embryo appeared wild type, including all doubly heterozygous embryos that were examined (*+/gpg6;+/Smo*) (Appendix A). However, as expected *gpg6/gpg6;+/+* and *+/+;Smo/Smo* embryos demonstrated *gpg6* and *Smo* mutant phenotypes respectively (Figure 10a-f, Figure 11a-h). Eleven *gpg6/gpg6;+/Smo* embryos were collected, nine of which resembled *gpg6/gpg6;+/+* mutants; one embryo,

however, resembled *Smo* mutants and one embryo resembled a *gpg6* mutant but lacked exencephaly (Appendix A). This suggests that loss of one copy of *Smo* on a *gpg6* mutant background only mildly affects the hh signal in *gpg6* mutants.

Interestingly, *+/gpg6;Smo/Smo* embryos showed two distinct classes of phenotype (Figure 10g-n, Figure 11i-s). Most embryos (9/11) grossly resembled *+/+;Smo/Smo* mutants in that they did not turn, had ventral cyclopia and holoprosencephaly but significantly they exhibited a partially looped heart (Figure 10g-j, Figure 11i-n); heart looping did not occur in any *+/+;Smo/Smo* embryos (Figure 10a-d, Figure 11a-f). Two other embryos (2/11) more closely resembled *+/+;gpg6/gpg6* mutants in that they had turned and had undergone heart looping although they did not show exencephaly (Figure 10k-n, Figure 11o-s). The partial rescue of the *Smo* mutant phenotype in these embryos suggests that one copy of the *gpg6* allele is able to partially activate hh signalling in the absence of the crucial positive regulator *Smo*. Therefore, this data implies that *gpg6* is a negative regulator of the hh pathway.

Four doubly homozygous mutant embryos (*gpg6/gpg6;Smo/Smo*) were collected, allowing epistasis to be assessed. All four embryos failed to undergo embryonic turning but had partially looped hearts (Figure 10o-v, Figure 11t-ei). Three out of the four *gpg6/gpg6;Smo/Smo* embryos had a larger forebrain region compared to *+/+;Smo/Smo* embryos (Figure 11t-u, z-ei) and one had exencephaly (Figure 11w-y). The presence of *gpg6* phenotypes in these embryos (heart looping and exencephaly) suggests that *gpg6* is, in part, epistatic to and therefore downstream of *Smo*. However, lack of a more complete rescue makes this data difficult to interpret.

Date of dissection	+/+ +/+	+/+ +/smo	gpg/+ +/+	gpg/+ +/smo	gpg/gpg +/+	+/+ smo/smo	+gpg smo/smo	gpg/gpg +/smo	gpg/gpg smo/smo	Number of Embryos in litter	Number Genotyped
21/1/08		6		7,8,9		3	1,4	2,5	10	10	10
24/1/08			6,8	4		3		5,7,9		9	8
28/1/08		5		4,8,11		1,2		3,6,9	12	12	10
6/3/08	2	1,5	10,11				9	7	4	11	9
10/3/08		4,6		1,2			7,8	3	5	11	9
11/3/08	2,3,8	6					4,5	1		12	7
12/3/08 2.5c			1		3		4			5	3
12/3/08 1.5e	1	2,5,11	3,8,10		4,6		7,9,12			12	12
Total Embryos	5	10	8	9	3	5	11	11	4	82	68
Expected	4.125	8.25	8.25	16.5	4.125	4.125	8.25	8.25	4.125	-	-

Table 8. Embryos obtained from the intercross between *Smo* and *gpg6*.

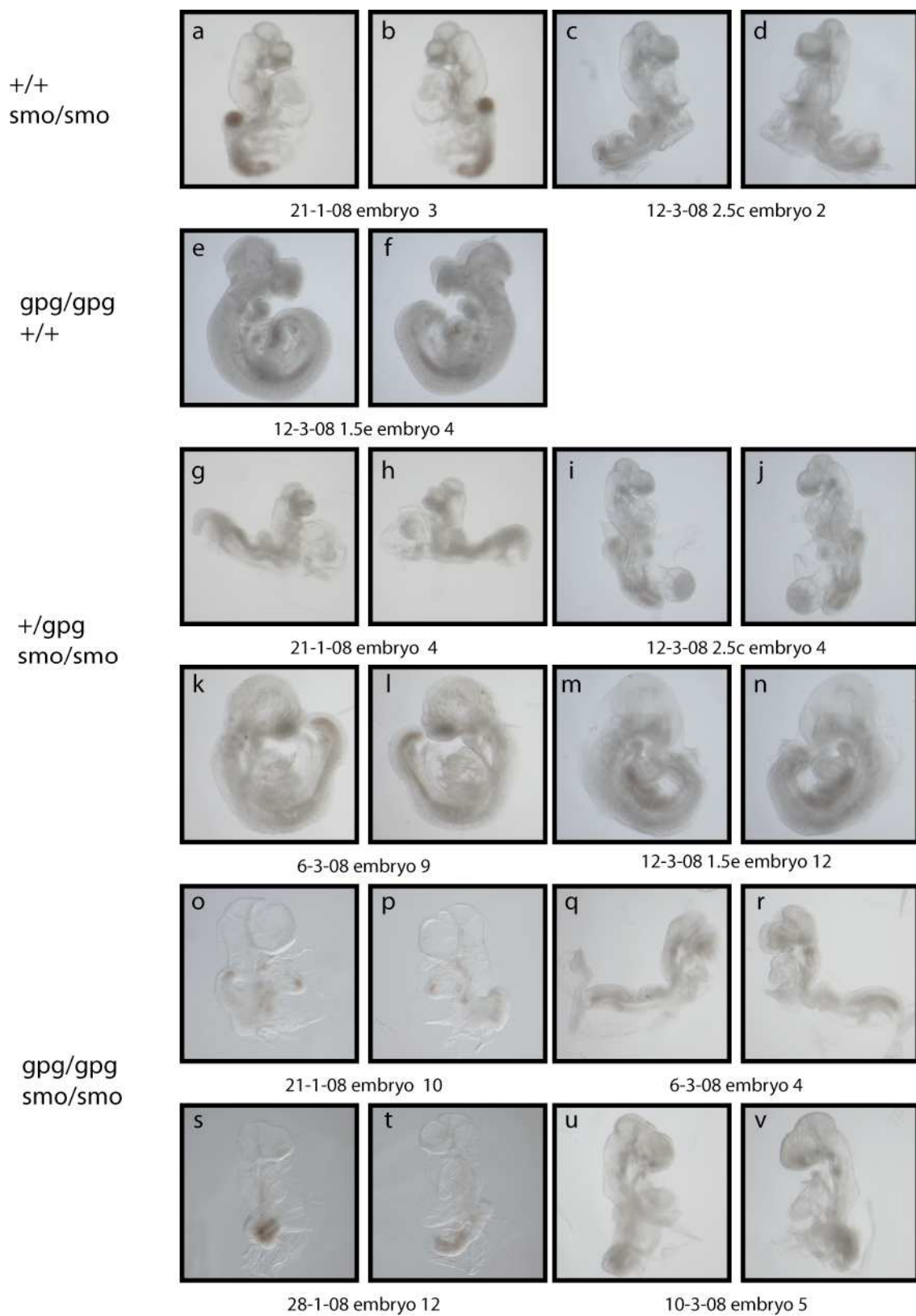


Figure 10. *gpg6* partially rescues the *Smo* mutant phenotype.

(a-d) $+/+$; *Smo/Smo* embryos failed to undergo embryonic turning, had a linear heart tube, ventral cyclopia and holoprosencephaly. In contrast, *gpg6/gpg6* ; $+/+$ embryos underwent embryonic turning, had a normally patterned heart but demonstrated exencephaly (e-f). However, $+/gpg6$; *Smo/Smo* embryos demonstrated two different phenotypes; (g-j) group I showed a mild rescue of the *Smo* phenotype (embryos demonstrated a typical *Smo* mutant phenotype but had a partially looped heart); (k-n) group II demonstrated a more complete rescue of the *Smo* mutant phenotype (embryos underwent embryonic turning, had a grossly patterned head and demonstrated a partially looped heart). *gpg6/gpg6* ; *Smo/Smo* embryos similarly demonstrated a range of phenotypes; (o-p, s-v) most embryos showed a mild rescue of the *Smo* mutant phenotype (embryos demonstrated a typical *Smo* mutant phenotype but had a partially looped heart and an expanded forebrain region). (q-r) One *gpg6/gpg6* ; *Smo/Smo* embryo demonstrated a *gpg6*-like exencephaly in addition to a partially looped heart but, like *Smo* mutants, did not turn.

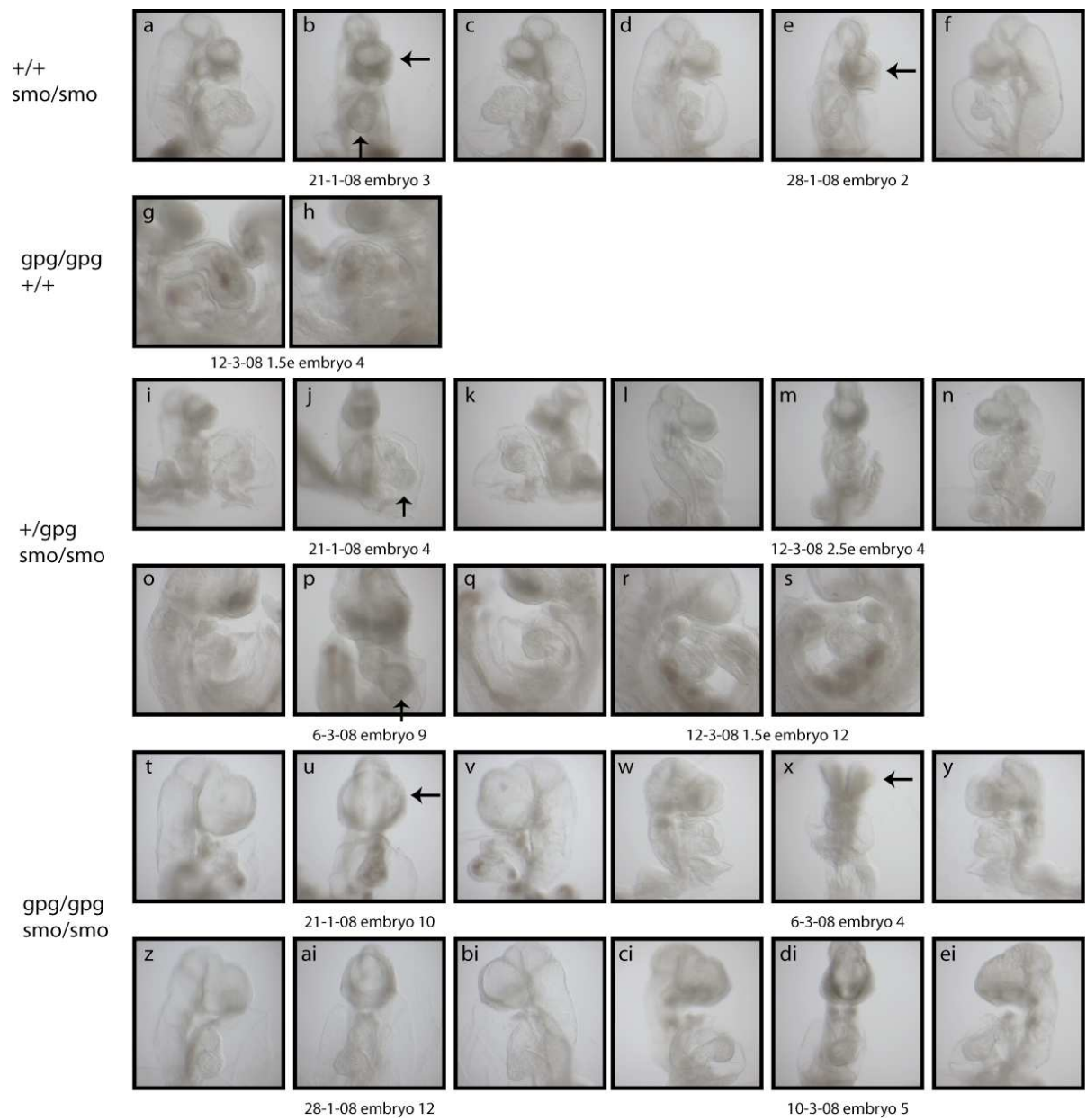


Figure 11. *gpg6* partially rescues the *Smo* mutant phenotype.

(a-f) $+/+$; *Smo/Smo* embryos failed to undergo embryonic turning, had a linear heart tube, ventral cyclopia and holoprosencephaly. In contrast, *gpg6/gpg6* ; $+/+$ embryos underwent embryonic turning, had a normally patterned heart but demonstrated exencephaly (g-h). However, $+/gpg6$; *Smo/Smo* embryos demonstrated two different phenotypes; (i-n) group I showed a mild rescue of the *Smo* phenotype (embryos demonstrated a typical *Smo* mutant phenotype but had a partially looped heart); (o-s) group II demonstrated a more complete rescue of the *Smo* mutant phenotype (embryos underwent embryonic turning, had a grossly patterned head and demonstrated a partially looped heart). *gpg6/gpg6* ; *Smo/Smo* embryos similarly demonstrated a range of phenotypes; (t-v, z-ei) most embryos showed a mild rescue of the *Smo* mutant phenotype (embryos demonstrated a typical *Smo* mutant phenotype but had a partially looped heart and an expanded forebrain region). (w-y) One *gpg6/gpg6* ; *Smo/Smo* embryo demonstrated a *gpg6*-like exencephaly in addition to a partially looped heart but, like *Smo* mutants, did not turn.

Further epistatic and genetic interaction studies with other components of the hh pathway are needed to clarify the role of *gpg6* in the hh pathway.

3.2.3.2.2 Analysing levels of hh signalling in *gpg6*

The neural tube is subdivided across its D-V axis into distinct subpopulations of neurons. Ventral patterning of the neural tube is specified during embryonic development by a gradient of Shh arising from the ventrally positioned notochord and then floor plate of the neural tube. D-V patterning of the neural tube is a sensitive read out of hh signalling (Dessaud et al., 2008). To investigate the levels of hh signalling in *gpg6* mutants, D-V patterning of the neural tube was assessed in James Briscoe's lab (NIMR, London). Olig2 (specific to Motor neurons and activated by Shh), Nkx2.2 (specific to V3 neurons and activated by Shh) and Pax6 (present in the dorsal half of the neural tube and inhibited by Shh) (Briscoe and Ericson, 2001) were analysed by immunofluorescence at 9.5dpc. No differences in the level or domain of expression of these genes were evident between wild type (Figure 12a-c) and *gpg6* mutant embryos (Figure 12d-f). This suggests that abnormal D-V patterning of the neural tube is not the cause of exencephaly in *gpg6* mutants.

The genetic experiments suggested that *gpg6* is a weak negative regulator of the hh pathway. This would suggest that hh target gene expression would be elevated in *gpg6* mutants. The hh receptor *Ptch1* is a known transcriptional target of the hh pathway (Goodrich et al., 1996). Therefore, *Ptch1* expression was analysed in 9.5dpc *gpg6* mutants by whole mount *in situ* hybridisation (Figure 13a-x). In wild type embryos, *Ptch1* expression was present

throughout the neural tube (Figure 13a-l). Similarly, in *gpg6* mutants *Ptch1* expression was also seen throughout the neural tube (Figure 13m-n, q-r, u-v) but, strikingly, was more intense in the mid- and hindbrain region (Figure 13o-p, s-t, w-x). Interestingly, this is the region of the neural tube that fails to close in *gpg6* mutants. Other regions of *gpg6* mutants had levels of *Ptch1* expression similar to wild type (Figure 13m-n, q-r, u-v). This implies a regionalised hh signalling defect in *gpg6* mutants and is consistent with exencephaly in *gpg6* mutants being caused by increased hh signalling.

Gli3 is both a transcriptional activator and repressor of hh target genes and is processed in a hh-dependent manner (Aza-Blanc et al., 1997; Wang et al., 2000). Proteolytic cleavage of Gli3 is dependent on the presence of cilia (Haycraft et al., 2005; Huangfu et al., 2003; Humke et al., 2010) and may be disrupted by the short cilia present in *gpg6* mutants. In order to assess Gli3 processing, *gpg6* mutant embryos were dissected at 9.5dpc and protein was extracted. Western blot analysis was then conducted by the Proteomics facility at MRC Harwell. There was a nearly significant increase in the full length form of Gli3 in *gpg6* (100% wild type compared to 108% in *gpg6* mutants; $p = 0.069$ T-test) and a slight decrease that was not statistically significant in the cleaved repressor form compared to wild type (100% wild type compared to 88% in *gpg6*; $p = 0.52$ T-test) (Figure 14).

In summary, the partial rescue of the *Smo* mutant phenotype seen in *+/gpg6;Smo/Smo* and *gpg6/gpg6;Smo/Smo* embryos suggests that *gpg6* is a weak negative regulator of the hh pathway. Consistent with this, increased *Ptch1* expression in *gpg6* mutants and a tendency towards increased full length Gli3 protein was seen, suggesting that levels of hh signalling are moderately increased in *gpg6* mutants.

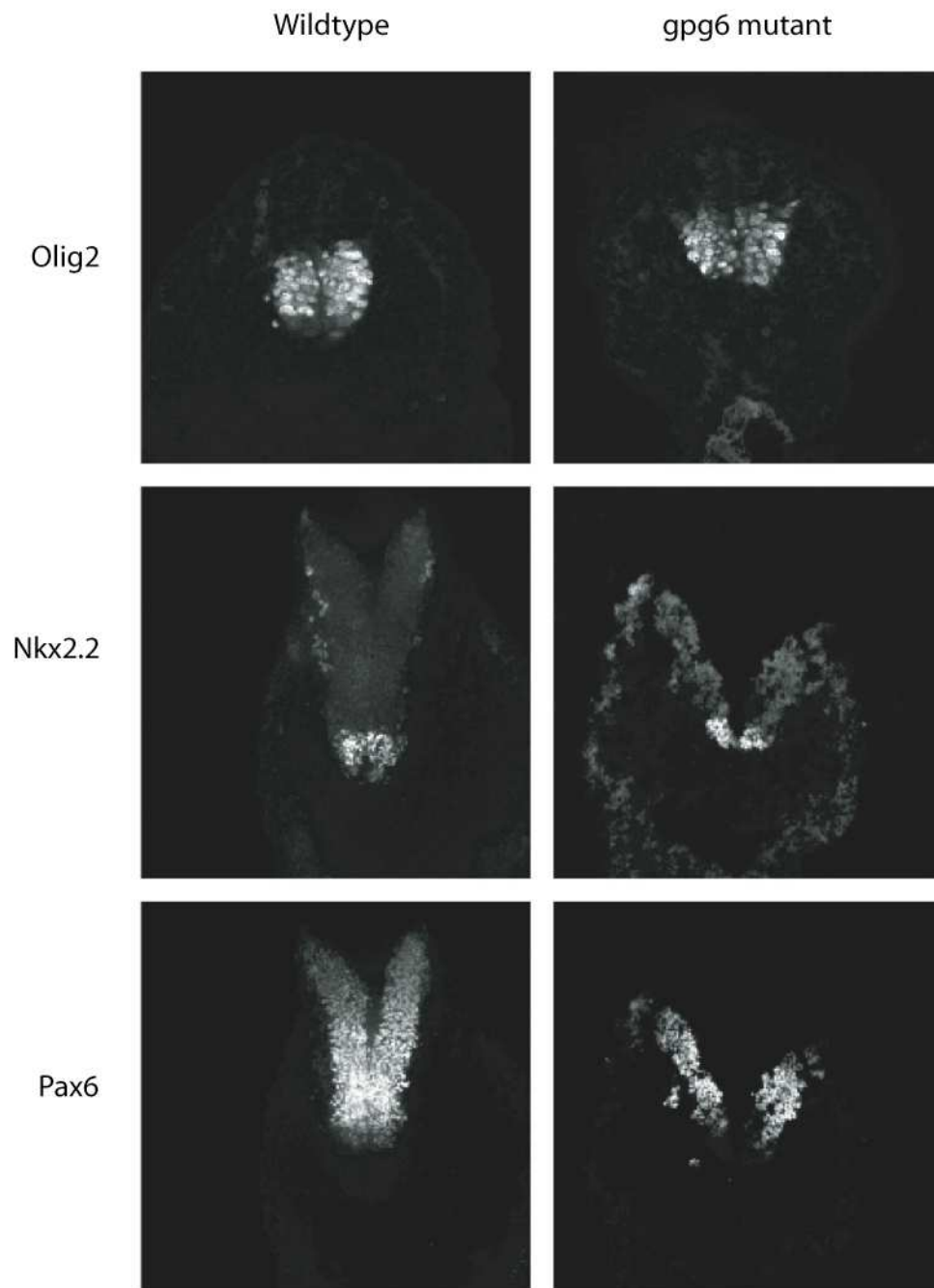
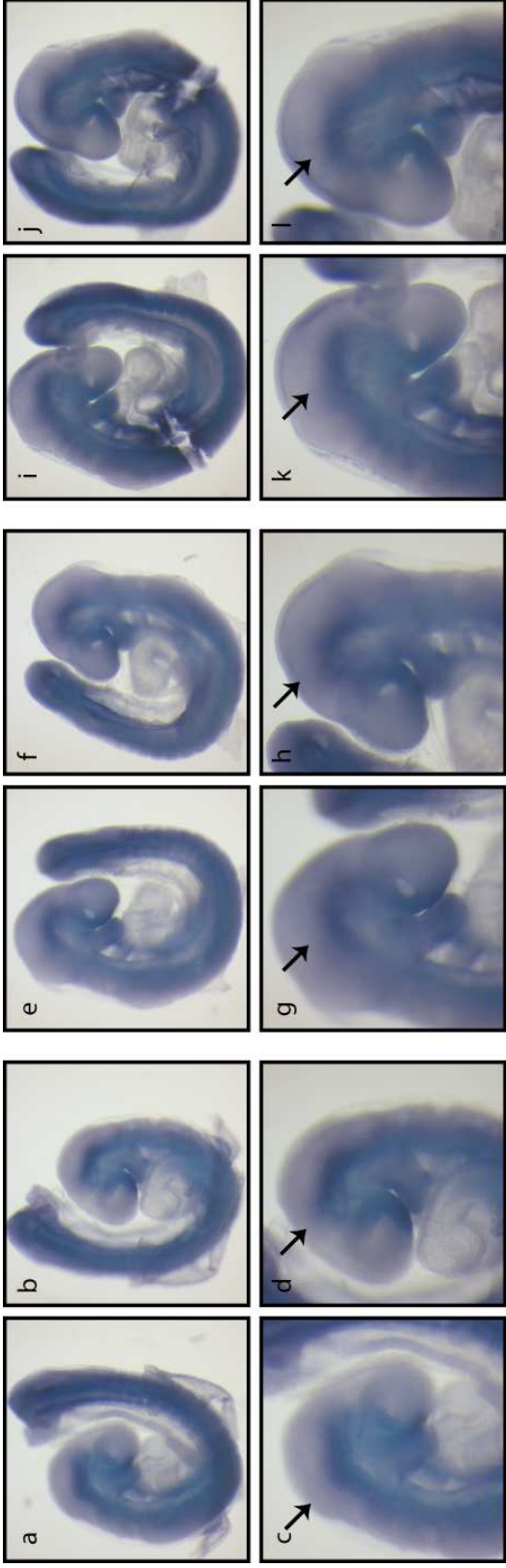
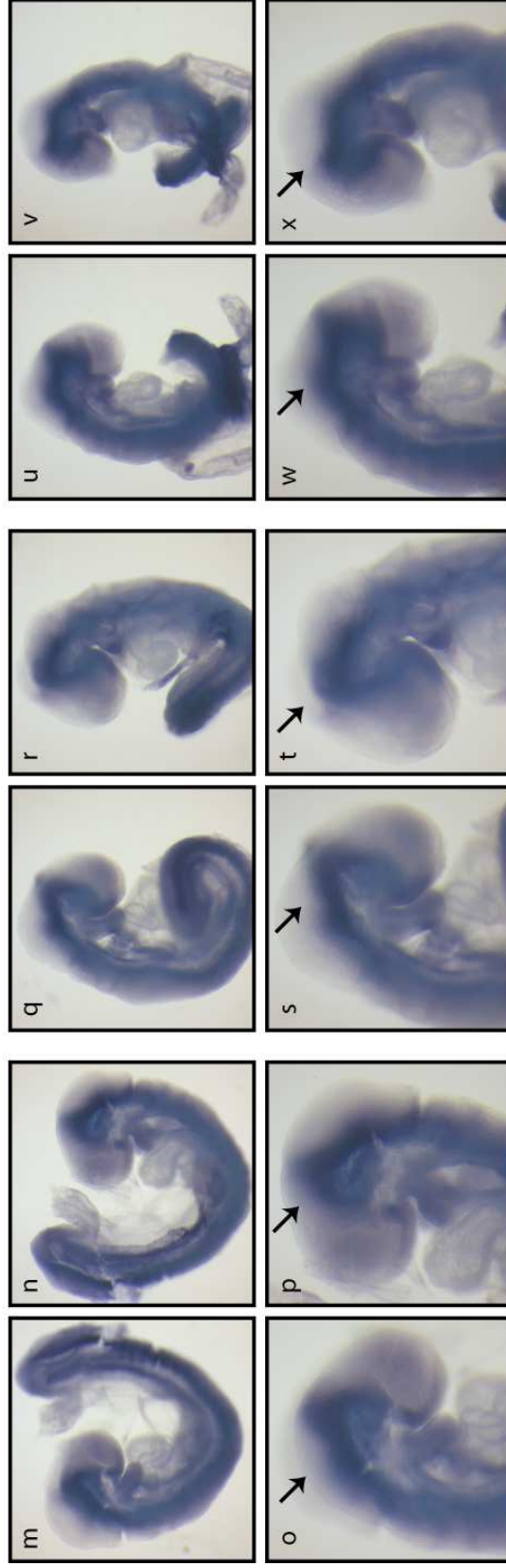


Figure 12. Dorso-ventral patterning of *gpg6* neural tubes appears normal.

Olig2 (specific to Motor neurons), *Nkx2.2* (specific to V3 neurons) and *Pax6* (present in the dorsal half of the neural tube) were expressed in the neural tube of wild type embryos. The same expression was seen in *gpg6* mutant embryos. Experiment conducted by James Briscoe.



gp96 wild types



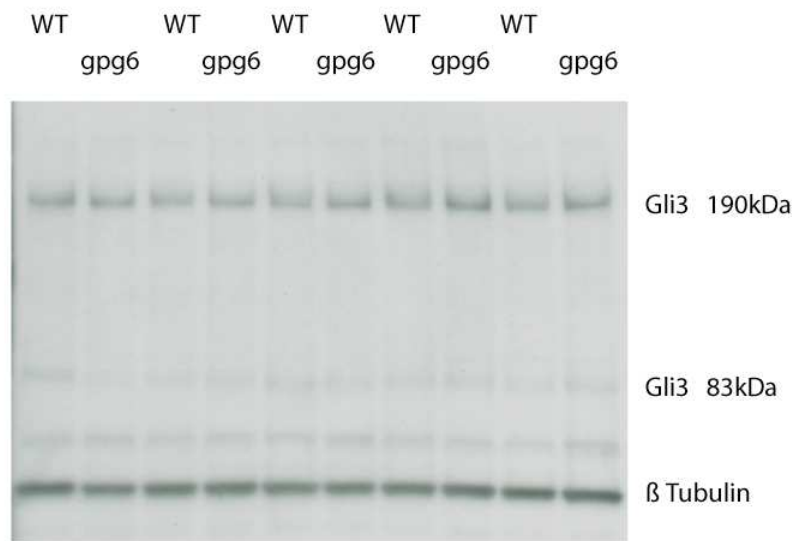
gp96 mutants

Figure 13. *gpg6* mutant embryos show increased *Ptch1* expression.

Ptch1 expression was analysed by WISH in 9.5dpc wild type and *gpg6* mutant embryos.

Wild type (a-b, e-f, i-j) and *gpg6* mutant (m-n, q-r, u-v) embryos demonstrated equivalent *Ptch1* expression throughout the neural tube. However, compared to wild type (c-d, g-h, k-l), *gpg6* mutant embryos (o-p, s-t, w-x) demonstrated increased *Ptch1* expression in the mid-hind brain, implying an increase in hh signalling.

a



b

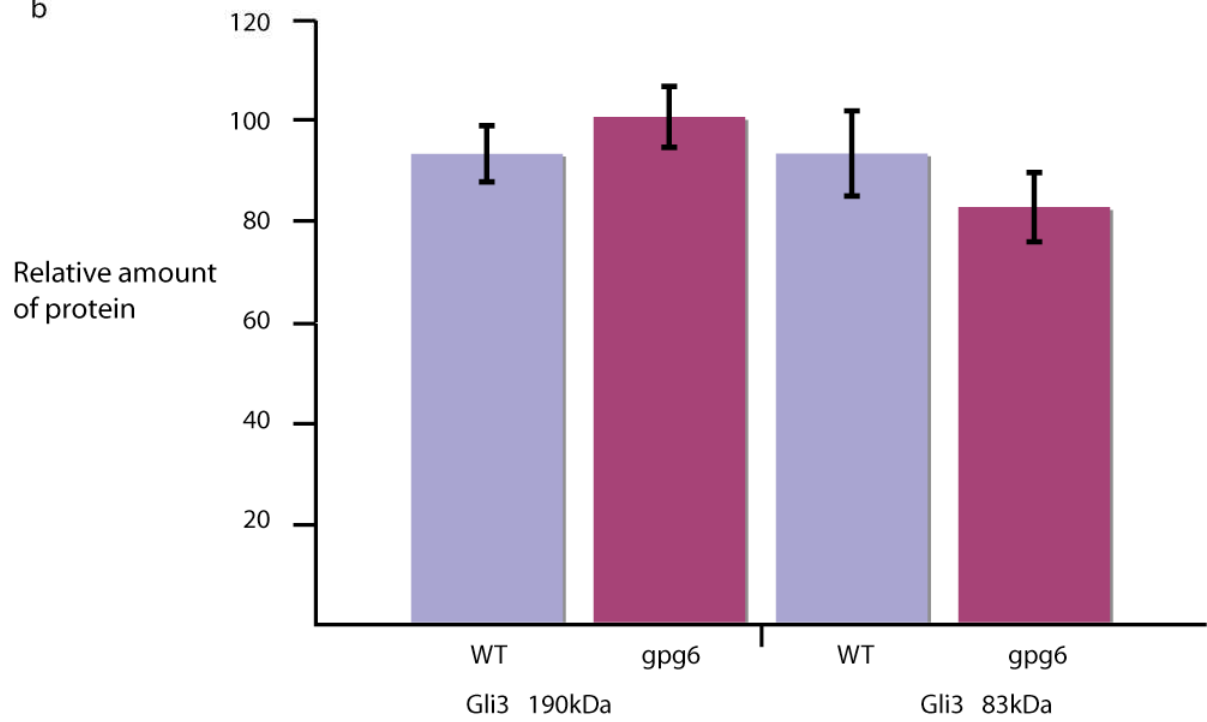


Figure 14. Levels of full length Gli3 show a trend towards being increased in *gpg6* mutants.

Protein from 9.5dpc wild type and *gpg6* mutant embryos were size fractionated on an acrylamide gel, Western blotted and probed with a Gli3 antibody. (a) Image of a developed film showing full length Gli3 (190kDa) and processed, short repressor form Gli3 (83kDa) in wild type and *gpg6* mutant embryos. (b) Quantification of levels of Gli3 in wild type and *gpg6* mutants. In *gpg6* mutants full length Gli3 is increased to 108% compared to wild type. Short, repressor form Gli3 is reduced to 88% compared to wild type. All measurements are normalised to levels of the loading control β tubulin.

3.3 Discussion

Phenotypic analysis of congenic C3.C-*gpg6* mutant embryos revealed morphological defects associated with L-R development (oedema, outflow tract defects and pulmonary isomerism) and hh signalling (exencephaly, small lungs and coloboma). The reduction in node cilia length in *gpg6* mutants suggested a cilia based mechanism linking the defects in L-R development and hh signalling, a hypothesis supported by subsequent molecular phenotyping. In *gpg6* mutants, *Nodal* expression at the node failed to become asymmetric and there was an anterior truncation of *Nodal* in the left lateral plate mesoderm. This suggested that disrupted *Nodal* expression in the left lateral plate, possibly due to abnormal nodal flow, is responsible for the L-R morphological defects in *gpg6* mutants. The hh signalling pathway was also investigated in *gpg6* mutants by testing whether *gpg6* genetically interacted with the hh pathway (crossing *gpg6* to *Smo* mutants) and by assessing the downstream effect on hh signalling (D-V patterning of the neural tube, *Ptch1* expression and Gli3 processing). This work suggested that *gpg6* is a weak negative regulator of the hh pathway.

3.3.1 Left-Right Defects in *gpg6*

It can be argued that L-R mutants fall into two major classes; the first class of mutants affect the node and the second class of mutants affect the lateral plate mesoderm. The node class of mutants can be further subdivided into those that (i) lack node cilia, (ii) mutants in which node cilia are immotile, (iii) mutants where node cilia are motile but nodal flow is disrupted and (iv) mutants that fail to perceive nodal flow. For instance, (i) *Kif3a* and *Kif3b* mutants

lack node cilia and demonstrate randomised heart looping, fail to turn, lack nodal flow and show bilateral expression of *Lefty2* (Marszalek et al., 1999; Nonaka et al., 1998; Takeda et al., 1999). Bilateral *Lefty2* expression is likely due to defective hh signalling in these mutants leading to failure to establish a midline barrier (Chiang et al., 1996; Huangfu et al., 2003; Meno et al., 1998). (ii) *iv* mutants, which have immotile node cilia, also demonstrate randomised heart looping, lack nodal flow and have randomised lateral plate *Nodal* expression (Okada et al., 1999; Supp et al., 1999; Supp et al., 1997). (iii) *inv* mutants have motile cilia but disrupted nodal flow (Mochizuki et al., 1998; Okada et al., 1999), argued to result from abnormal node morphology, delayed development of nodal flow and abnormal anterior tilt to node cilia (Hashimoto et al., 2010; Okada et al., 1999). *inv* mutants demonstrate *situs inversus*, right sided *Nodal* and delayed *Lefty2* expression (Okada et al., 1999). (iv) *Pkd2* mutants demonstrate randomised heart looping, have right pulmonary isomerism, absent or bilateral *Nodal* expression and mostly bilateral *Pitx2* expression (Pennekamp et al., 2002). This was argued to be due to the lack of Ca^{2+} release on the left side of the node, hypothesised to result from a failure to perceive nodal flow (McGrath et al., 2003).

In contrast to these node mutants, *gpg6* mutants do not demonstrate randomised heart looping and express *Nodal* in the left lateral plate mesoderm. However, *gpg6* mutants still demonstrate abnormalities associated with disrupted L-R development (Aylsworth, 2001; Ramsdell, 2005), such as left or partial pulmonary isomerism and outflow tract defects. The absence in *gpg6* mutants of severe L-R defects (failure to turn or randomised heart looping), which are seen in mutants that lack cilia (*Kif3a* and *Kif3b*), lack motile cilia (*iv*) or fail to perceive nodal flow (*Pkd2*), suggests that *gpg6* short cilia still rotate, generate a form of nodal flow and initiate a left sided calcium signal at the node. Therefore, *gpg6* mutants are

likely to be closer in nature to *inv* mutants, which contain motile cilia but have disrupted nodal flow. Nevertheless, *gpg6* mutants demonstrate a milder phenotype than *inv* since they do not demonstrate abnormal node morphology or *situs inversus*. It has been reported that nodal flow develops in stages and is stuck at the uncoordinated stage in *inv* mutants (Okada et al., 1999). Due to the milder phenotype detected in *gpg6* mutants, it can be proposed that fewer, shorter cilia in *gpg6* mutants generate smooth but not laminar nodal flow, a stage beyond the uncoordinated flow detected in *inv* (Okada et al., 1999). This flow could cause inefficient Ca^{2+} signalling at the node (McGrath et al., 2003) and cause the relatively mild L-R defects in *gpg6* mutants. This hypothesis can be tested by analysing nodal flow in *gpg6* mutants using video microscopy and analysing Ca^{2+} signalling in *gpg6* mutants using Ca^{2+} sensitive fluorescent dyes.

The second class of L-R mutants affect signalling events in the lateral plate mesoderm, which are downstream of nodal flow and calcium signalling. This class can be further subdivided into mutants demonstrating right isomerism (*Nodal*, *Fgf8*, *Bmp4*, *Cryptic* and *Pitx2*) and those demonstrating left isomerism (*Lefty1* and *Lefty2*). Various *Nodal* mutants have been published, demonstrating that *Nodal* has roles early in development in the primitive streak and later in L-R development (Brennan et al., 2002; Collignon et al., 1996; Conlon et al., 1994; Norris et al., 2002; Zhou et al., 1993). Deletion of the *Nodal* lateral plate enhancer in *Nodal* $\Delta 600$ mutants resulted in embryos demonstrating randomised heart looping, partial right pulmonary isomerism, very strong downregulation of *Nodal* and anterior truncation of left sided *Pitx2* expression (Norris et al., 2002). In contrast, when the *Nodal* node enhancer was deleted mutant embryos demonstrated ectopic expression of *Nodal* in a few cells of the node, normal lateral plate *Nodal* expression and normal L-R development (Brennan et al., 2002). However, replacing one copy of the *Nodal* node allele

with a *Nodal* null allele (reducing *Nodal* node expression by half) resulted in randomised heart looping, right pulmonary isomerism, outflow tract defects (transposition of the great arteries (TGA)), common atrial chamber and failure to activate expression of *Lefty1*, *Lefty2* and *Pitx2* (Brennan et al., 2002). Similar phenotypes were seen in mutants of *Fgf8* and the *Nodal* co-receptor *Cryptic* and were associated with failure to activate lateral plate expression of *Nodal*, *Lefty1*, *Lefty2* and *Pitx2* (Meyers and Martin, 1999; Yan et al., 1999). *Bmp4* was shown to be required to activate *Cryptic* expression (Fujiwara et al., 2002) and *Shh/Ihh* signalling is required in the left lateral plate to activate *Bmp4* expression (Tsiairis and McMahon, 2009). Similar to mutants that lack *Nodal* expression, *Pitx2* mutants have right pulmonary isomerism and right isomerism of the gut. However, *Pitx2* mutants demonstrate normal heart looping and embryonic turning (Kitamura et al., 1999; Lin et al., 1999).

In contrast to *Nodal* and *Pitx2* mutants, *Lefty1* mutants demonstrate left pulmonary isomerism, TGA, common atrial chamber and bilateral expression of *Nodal*, *Lefty2* and *Pitx2* (Meno et al., 1998). Similarly, *Lefty2* mutants show left pulmonary isomerism, TGA and double outlet right ventricle (DORV) and bilateral *Pitx2* expression (Meno et al., 2001).

gpg6 mutants do not demonstrate randomised heart looping or right pulmonary isomerism, as seen in mutants that lack *Nodal* in the lateral plate mesoderm (Brennan et al., 2002; Fujiwara et al., 2002; Meyers and Martin, 1999; Norris et al., 2002; Yan et al., 1999). It is therefore unlikely that the anterior truncation of *Nodal* expression in the left lateral plate of *gpg6* mutants abolishes all *Nodal* signalling. Furthermore, *Nodal* signalling is likely to be delayed or inefficient since *Nodal* expression in *gpg6* mutants is seen throughout the left

lateral plate only at later stages of development. The *gpg6* mutation is also unlikely to abolish *Pitx2* expression because *gpg6* mutants do not demonstrate right pulmonary isomerism and right isomerism of the gut, phenotypes seen in *Pitx2* mutants (Kitamura et al., 1999; Lin et al., 1999). 60% of *gpg6* mutants have left pulmonary isomerism but, unlike *Lefty1* and *Lefty2* mutants, demonstrate truncated left sided *Nodal* expression. It is a puzzle how a phenotype (left pulmonary isomerism) that is associated with bilateral *Nodal* and *Pitx2* expression can be generated by truncated left lateral plate *Nodal* expression.

Nodal expression in *gpg6* mutants most closely resembles the anterior truncation of *Pitx2* expression seen in *Nodal* $\Delta 600$ mutants. However, *Nodal* $\Delta 600$ mutants demonstrate partial right pulmonary isomerism and randomised heart looping (Norris et al., 2002), phenotypes not seen in *gpg6* mutants. The difference between *Nodal* $\Delta 600$ and *gpg6* mutants is most likely to result from the severe downregulation of *Nodal* expression in *Nodal* $\Delta 600$ mutants (Norris et al., 2002), whereas in *gpg6* mutants *Nodal* expression recovers at later somite stages. This raises the possibility of different strengths of *Nodal* signalling in *gpg6* mutants compared to *Nodal* $\Delta 600$ mutants. For example, very low *Nodal* expression is unable, through failure to activate anterior expression of *Pitx2*, to provide sufficient left identity to lungs and hence causes partial right isomerism in *Nodal* $\Delta 600$ mutants (Norris et al., 2002). In contrast, recovery of *Nodal* expression in *gpg6* mutants is able to impart a greater *Nodal* signal and therefore greater left identity to lungs.

It can therefore be hypothesised that at earlier somite stages in *gpg6* mutants *Nodal* signalling is strong in the posterior left lateral plate but absent in the anterior left lateral plate due to the anterior truncation of *Nodal* expression. At later somite stages *Nodal* signalling is

still strong in the posterior left lateral plate and is gradually becoming stronger in the anterior left lateral plate as *Nodal* expression recovers. Therefore, posterior lateral plate derivatives such as the stomach, which have been exposed to consistent *Nodal* signalling, will develop with normal L-R identity in *gpg6* mutants. Anterior lateral plate derivatives will be exposed to weaker *Nodal* signalling that is still sufficient to prevent randomised heart looping and right pulmonary isomerism but is not strong enough to prevent IAA and common trunk, outflow tract defects associated with abnormal L-R development (Aylsworth, 2001; Ramsdell, 2005).

The above hypothesis also suggests a possible explanation how truncated *Nodal* expression could cause left pulmonary isomerism. Strong and consistent *Nodal* signalling in the posterior left lateral plate will set up the *Lefty1*-dependent midline barrier in the posterior left lateral plate. Weaker *Nodal* signalling in the anterior left lateral plate will set up a weaker anterior midline barrier, allowing low levels of *Nodal* (similar to the low levels detected in *Nodal* $\Delta 600$ mutants that were still able to activate *Pitx2* expression (Norris et al., 2002)) to move to the anterior right lateral plate to activate *Pitx2* expression and give rise to left pulmonary isomerism. This hypothesis can be tested in the future by analysing *Lefty1*, *Lefty2* and *Pitx2* expression in *gpg6* mutants. An alternative explanation that cannot be ruled out is that the hh signalling defects that are also present in *gpg6* mutants may be influencing lung patterning since *Shh* is needed for correct growth and lobation of the lung (Chiang et al., 1996).

The anterior truncation and later recovery of *Nodal* expression detected in *gpg6* mutants is similar to the wave of *Xnr1* and *Xlefty* expression (homologs of *Nodal* and *Lefty2*

respectively) reported in the left lateral plate of *Xenopus* (Ohi and Wright, 2007). *Xnr1* and *Xlefty* is initially expressed in a posterior peri-notochordal domain and subsequently in the posterior left lateral plate mesoderm. *Xnr1* from posterior regions of the lateral plate then move to more anterior regions to activate expression of itself and that of *Xlefty* to cause a rolling wave of *Xnr1* and *Xlefty* expression from posterior to anterior in the left lateral plate. Expression of *Xnr1* is subsequently downregulated by *Xlefty* in a similar posterior to anterior wave (Ohi and Wright, 2007). A wave of *spaw* expression (a homolog of *Nodal*) is also seen in zebrafish (Wang and Yost, 2008). When a *spaw* inhibitor, *charon*, is knocked down at Kupffers vesicle (KV) in zebrafish the wave of expression is initiated earlier than normal, suggesting that signals from KV control the timing of *spaw* propagation in the lateral plate (Wang and Yost, 2008). In light of these studies, it can be proposed that inefficient nodal flow and calcium signalling at the node in *gpg6* mutants results in a delay in the propagation of *Nodal* expression in the left lateral plate, which causes an anterior truncation of *Nodal* expression and subsequent L-R defects in *gpg6* mutants.

3.3.2 hh Signalling Defects in *gpg6*

The combination of exencephaly, coloboma and pulmonary hypoplasia in *gpg6* mutants suggested defects in hh signalling (Chiang et al., 1996; Litingtung et al., 1998; Pepicelli et al., 1998). However, the defects seen in *gpg6* mutants are markedly less severe than loss of function alleles of individual components of the hh pathway. For example, *Shh/Ihh* double mutants and *Ptch1* and *Smo* single mutants demonstrate a severe early embryonic lethal phenotype and die around 9.5dpc (Goodrich et al., 1997; Zhang et al., 2001), whereas *gpg6* mutants die around 13.5dpc. The mild nature of the hh defects seen in *gpg6* mutants is highlighted by the absence of overt defects in D-V patterning of the neural tube. Typically,

loss of positive regulators of the hh pathway (e.g. *Shh* and *Smo*) results in dorsalisation of the neural tube (Chiang et al., 1996; Zhang et al., 2001) whereas loss of negative regulators of the hh pathway (e.g. *Ptch1*, *Rab23*, *Pka* and *Sufu*) results in ventralisation of the neural tube (Cooper et al., 2005; Eggenschwiler et al., 2001; Goodrich et al., 1997; Huang et al., 2002; Svard et al., 2006). This suggests in *gpg6* mutants that hh signalling is neither abolished nor hyperactivated but is instead perturbed. It can be further argued that components of the hh pathway are still processed in short *gpg6* mutant cilia but that processing is inefficient.

One of the downstream consequences of hh signalling is the inhibition of Gli proteolytic cleavage. Therefore, disrupted hh signalling in *gpg6* mutants could be affecting Gli processing. *Gli2* and *Gli3* have overlapping activator functions (Bai et al., 2004) and have important roles in different regions of the neural tube; *Gli2* has a major function in the caudal neural tube whereas *Gli3* is needed throughout the neural tube (Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Motoyama et al., 2003). It is possible that *gpg6* mutants do not demonstrate D-V patterning defects because the *gpg6* mutation only mildly increases levels of full length *Gli3*, which may be compensated for by normal *Gli2* processing (Motoyama et al., 2003). It can be proposed that if *Gli2* was affected in *gpg6* mutants then more severe defects would be seen in the caudal neural tube, where *Gli2* is a major determinant of D-V patterning (Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Motoyama et al., 2003). This hypothesis can be tested by analysing *Gli2* processing in *gpg6* mutants. Alternatively, it may be that neural tube cilia are normal in the neural tube of *gpg6* mutants and the defect in ciliogenesis is regional specific. This may also be relevant in the limb since polydactyly is not detected in *gpg6* mutants.

Most hh pathway mutants that result in increased hh signalling typically have disrupted D-V patterning and neural tube defects (Murdoch and Copp, 2010). In *gpg6* mutants, however, the lack of overt defects in D-V patterning argues that exencephaly in *gpg6* mutants is not related to the D-V axis of the neural tube. Recently, it has been shown that ectopic expression of *Shh* in chick disrupts cell-adhesion to cause neural tube defects (Fournier-Thibault et al., 2009). Further work, including immunofluorescent analysis of cell adhesion markers in the neural tube of *gpg6* mutants, is needed to test if this is the cause of exencephaly in *gpg6* mutants.

A major feature of *gpg6* mutants (and the Gasping class of mutants in general (Ermakov et al., 2009)) is the lung phenotype. The presence of hypoplastic lungs in congenic *gpg6* mutant embryos is reminiscent of *Shh* mutants, *Gli2* mutants and *Gli1*^{-/-}; *Gli2*^{-/-} double mutants (Chiang et al., 1996; Park et al., 2000). This further suggests that Gli processing is affected in *gpg6* mutants, which has been shown to require cilia (Haycraft et al., 2005; Kovacs et al., 2008; Molla-Herman et al., 2008; Ocbina and Anderson, 2008). In the absence of *Smo* increased levels of Gli3R are seen leading to inhibition of hh target genes (Wijgerde et al., 2002). When *gpg6* was crossed to *Smo* mutants, *gpg6/gpg6;Smo/Smo* and *+/gpg6;Smo/Smo* embryos demonstrated a partial rescue of the *Smo* phenotype, suggesting that *gpg6* acts downstream of *Smo* and is a mild negative regulator of the hh pathway. This suggests that the increased levels of Gli3R due to loss of *Smo* is reduced by the presence of *gpg6* alleles, thereby moderately increasing hh signal and partially rescuing the *Smo* mutant phenotype. Most *gpg6/gpg6;Smo/Smo* and *+/gpg6;Smo/Smo* embryos only partially looped their hearts but did not turn, suggesting a region specific rescue of the *Smo* phenotype. This is supported by the observation that *Ptch1* expression was increased only in the head region of 9.5dpc *gpg6* mutants. It can also be argued, however, that *gpg6* is required more globally since

gpg6/gpg6;Smo/Smo and *+gpg6;Smo/Smo* embryos were generated that had a more complete *gpg6*-like phenotype. This argument is supported by the presence of multiple hh related defects in *gpg6* (rostral neural tube, lungs and eyes) that are typically found in mutants of the hh pathway. The issue of whether *gpg6* is required regionally or more globally could be addressed by analysing Gli processing and hh target gene expression in different regions of *gpg6* mutant embryos. Additional epistatic analysis using *Ptch1*, *Gli2* and *Gli3* mutants will also better place *gpg6* in the hh pathway.

Collectively the data presented here, although not conclusive, does suggest that an increase in hh signalling is responsible for some aspects of the *gpg6* phenotype.

3.3.3 *gpg6* is a Cilia Mutant

Evidence based on phenotypic and molecular investigation of *gpg6* mutants suggests the underlying cause of the *gpg6* phenotype is a combination of aberrant L-R development and hh signalling. Cilia have been shown to be crucial for both these processes (Huangfu et al., 2003; Nonaka et al., 1998). When congenic C3.C-*gpg6* mutant node cilia were analysed they were both reduced in number and in length. We therefore hypothesised that defective cilia are the cause of the morphological and molecular defects in *gpg6* mutants.

A class of mouse mutants with cilia abnormalities resulting in defects in L-R development and hh signalling has recently emerged (Table 4). These mutants demonstrate a spectrum of defects, including exencephaly, polydactyly, randomised heart looping and abnormal D-V patterning of the neural tube (Table 4). Within this class of embryonic lethal mutants, *gpg6*

mutants can be seen as relatively mild since they demonstrate normal D-V patterning, heart looping and left sided, albeit truncated, *Nodal* expression. A possible reason for the relatively mild defects seen in *gpg6* mutants could be that the causative mutation disrupts only a limited range of components of the ciliary machinery. For example, a subset of proteins may fail to be targeted to the cilia or the efficiency of Kinesin II or Cytoplasmic Dynein 2 is reduced but not abolished. This limited defect could still reduce the length of cilia, thereby affecting nodal flow and L-R development and altering the processing of the hh signal in cilia but not abolish these processes.

Noticably, the node cilia found in *gpg6* mutants most resembles the retrograde IFT mutants *Dnchc2* (MGI: *Dync2h1*) (May et al., 2005) and *Thm1* (Tran et al., 2008), since both have short cilia. In contrast, anterograde IFT mutants (Nonaka et al., 1998) and IFT particle complex B mutants (Houde et al., 2006; Huangfu et al., 2003) lack node cilia. This suggests that *gpg6* mutants may have defects in retrograde IFT. However, until the causative mutation is found the role of *gpg6* in ciliogenesis can only be the subject of speculation.

Chapter 4

gpg6 is an *Atmin* mutant

4.1 Introduction

4.1.1 Ethylnitrosourea (ENU)

Ethylnitrosourea (ENU) was first identified as a potent mutagen for use in the mouse in 1979 (Russell et al., 1979) and since then has been used extensively in phenotype driven screens to understand gene function (Acevedo-Arozena et al., 2008). ENU is an alkylating agent that adds ethyl adducts onto DNA (Beranek et al., 1983; Heflich et al., 1982). Alkyl DNA alkyltransferase (AGT) has evolved to remove these adducts but if this repair mechanism fails a single base pair mutation will result. Ethyl adducts cause mutations by inducing aberrant base pairing during replication and can affect any nucleotide (Barbaric et al., 2007; Takahasi et al., 2007). For example, O²-ethyl T is misread by the DNA replication machinery and becomes base paired with a T. This results in a T to A point mutation (O'Neill, 2000). In rare incidences ENU can also induce insertion and deletion mutations (Barbaric et al., 2007). Analysis of ENU mutants has shown a non-random distribution of possible mutations with the most frequent point mutation found to occur at T/A base pairs, most likely resulting in a missense amino acid change (Barbaric et al., 2007; Takahasi et al., 2007).

4.2 Results

4.2.1 Mapping the *gpg6* mutation

To identify the *gpg6* mutation, 13 phenotypically abnormal 13.5dpc *gpg6* embryos (generated from intercrossing *+/-gpg6* heterozygotes) were collected. Embryonic liver was harvested and DNA extracted. The GEMs facility at MRC Harwell then used this mutant DNA to assess genome wide linkage using a panel of single nucleotide polymorphisms (SNPs). This assessed markers that are polymorphic between BALB/c (the mutagenised strain) and C3H (the wild type strain). The *gpg6* phenotype showed the highest linkage to chromosome 8 (data not shown). Initial haplotypic analysis using markers on chromosome 8 was then performed and this demonstrated a minimal region between 95-123Mb (data not shown). To allow breeding of a *gpg6* mouse colony, genotyping of this region was performed with polymorphic markers; animals heterozygous across this region were assumed to be *+/-gpg6*. These were outcrossed to C3H to maintain the colony.

The initial minimal region contains 345 genes (Ensembl). To refine the minimal region and reduce the number of potential candidate genes that needed to be sequenced, further mapping was performed. 7 additional polymorphic markers between 95-123Mb (Table 9) were verified to be polymorphic between BALB/C and C3H (data not shown). A further five phenotypically abnormal *gpg6* embryos were collected and all 18 mutant DNAs were typed using the new markers. This additional haplotypic analysis refined the minimal region to 117.8-120.9Mb on chromosome 8 (Table 9). The refined minimal region contains 21 genes, comprising 38.91Kb of coding sequence over 174 exons (Table 10). This additional mapping therefore excluded 324 genes.

<i>gpg6</i> DNA	D8mit139 117Mb	rs13480001 117.8Mb	D8mit321 118.62Mb	D8mit272 118.9Mb	D8mit167 120.1Mb	rs13480012 120.9Mb	rs4227432 126.2Mb
1	BALB/c		BALB/c	BALB/c	BALB/c		F1
2	BALB/c		BALB/c	BALB/c	BALB/c		C3H
3	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
4	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
5	BALB/c		BALB/c	BALB/c	BALB/c	F1	F1
6	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
7	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
8	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
9	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
10	F1	F1	BALB/c	BALB/c	BALB/c	BALB/c	F1
11	BALB/c		BALB/c	BALB/c			BALB/c
12	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
13	BALB/c		BALB/c	BALB/c	BALB/c		BALB/c
14	BALB/c			BALB/c	BALB/c		BALB/c
15	F1	BALB/c		BALB/c	BALB/c		
16	BALB/c			BALB/c	BALB/c		
17	BALB/c			BALB/c	BALB/c		
18	BALB/c			BALB/c	BALB/c		

Table 9. Genetic mapping of *gpg6* identified a 3Mb minimal region on chromosome 8.

DNA from 18 phenotypically abnormal *gpg6* mutants were genotyped using polymorphic markers (microsatellite and single nucleotide polymorphisms (SNPs)) on chromosome 8.

BALB/c is shown in yellow and F1 BALB/c/C3H in grey. BALB/c was the originally mutated strain and is therefore likely to carry the *gpg6* mutation. As *gpg6* is recessive, any mutation in heterozygous regions (grey) is unlikely to contribute to the *gpg6* phenotype and can therefore be excluded. Mapping reduced the *gpg6* minimal region to 117.8-120.9Mb on chromosome 8.

	Ensembl Gene ID (ensmusg000000)	GO Terms	Position in region (Mb)	Sequence	Previous knock out	Phenotype of knock out
snRNA	65090	?	118.5			
Maf	55435	Regulation of transcription	118.6		Yes	Microphthalmia, impaired IL4
Novel gene 073	74073	?	119.3			
Dynlrb2	34467	Dynein complex	119.3	Yes – no change		
Cdyl2	31758	?	119.5		Yes	Not published
Pseudogene	74072	?	119.6			
2310061C15Rik	14633	?	119.7			
2610510J17Rik (Cenpn)	31756	Centrosome	119.8			
Atmin	47388	Zinc finger	119.8	Yes - change		
1700030J22Rik	31847	?	119.8			
Gcsh	34424	Glycine catabolism	119.8			
pkd112	34416	Cation channel	119.8			
Bcmo1	31845	Oxidation reduction	119.9			
Gan	52557	Cytoskeletal organisation	120		Yes	Giant axon neuropathy
Novel gene 068	74068	?	120.1			
4933407C03Rik	34390	?	120.2			
Plcg2	34330	Lipid metabolism	120.4		Yes	Immune, renal, hematopoietic, skeletal
4632417N05Rik (Sdr42e1)	34308	Steroid biosyntheses	120.5			
Hsd17b2	31844	Lipid	120.6			

Table 10. Candidate genes within the *gpg6* minimal region

4.2.2 Analysis of Candidate Genes

Although it was possible to sequence all 21 candidate genes it would have proved to be time consuming and inefficient. Therefore, several strategies were followed to prioritise which candidate genes were most likely to contain the mutation. Initially, previously published data on each gene was gathered from the MGI database (<http://www.informatics.jax.org/>). Out of the 21 candidate genes, 3 have been previously mutated but did not have a ciliogenic phenotype, 2 were pseudogenes and 1 was a novel small nuclear RNA (snRNA). The remaining 15 genes had no known mouse mutant and these were given priority in the subsequent analysis (Table 10).

To further narrow down the number of candidate genes for sequencing, expression of the 15 candidates was analysed. Given the cilia phenotype discovered in the node of *gpg6* mutant embryos (Chapter 3), it was likely that the mutated gene would be expressed between 7.5-8.5dpc, a period of development when node cilia form (Sulik et al., 1994). RT-PCR was used to amplify regions of candidate genes from cDNA generated from whole 7.5-8.5dpc wild type embryos. All 15 genes were expressed at this stage (data not shown). This approach does not take into account where a gene is expressed, which is relevant to its function in the embryo. Therefore, to determine whether the candidate genes were expressed in the node, their temporospatial expression was investigated by WISH. To do this, candidate gene RT-PCR products were cloned and used to generate antisense RNA probes. WISH analysis revealed that 11 genes were broadly expressed throughout the embryo (Figure 15 a-z), 3 were expressed only in extraembryonic tissue (Figure 15 ai-ii) and that *Dynlrb2* was expressed specifically in the node (Figure 15 ji-li).

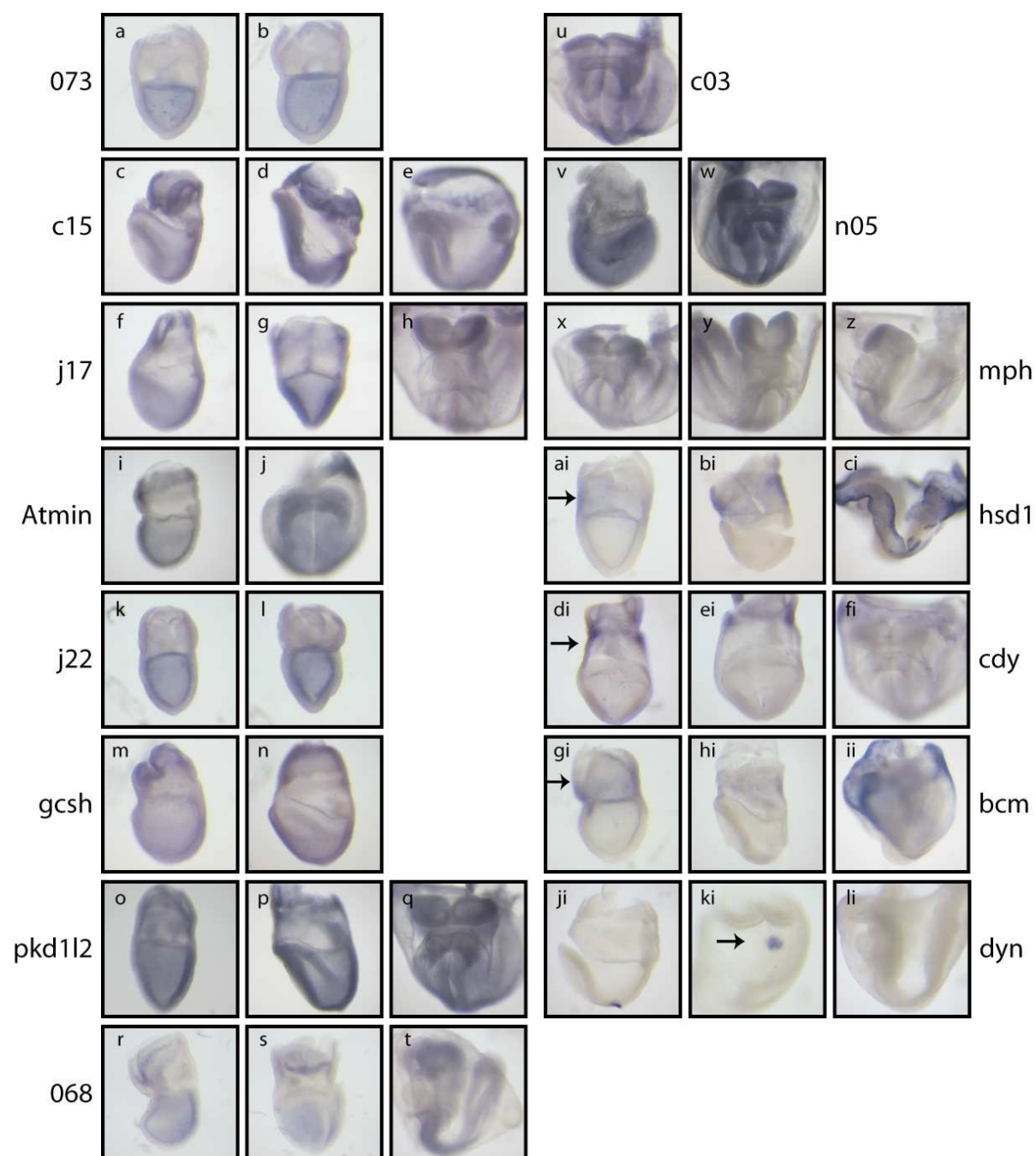


Figure 15. Temporospatial expression analysis of candidate genes in 7.5-8.5dpc wild type embryos

WISH analysis excluded genes that were not expressed at the node (*Hsd17b2*, *Cdyl2*, *Bcmo1*).

(a-z) Genes expressed in the node and other embryonic tissues.

(a-b) Novel gene ensmusg00000074073

(c-e) 2310061C15 Rik

(f-h) 26105J17 Rik

(i-j) Atmin

(k-l) 1700030J22 Rik

(m-n) Gcsh

(o-q) Pkd112

(r-t) Novel gene ensmusg00000074068

(u) 4933407C03 Rik

(v-w) 4632417N05 Rik

(x-z) Mphosph6

(ai-ii) Genes expressed in extra-embryonic tissues

(ai-ci) Hsd17b2

(di-fi) Cdyl2

(gi-ii) Bcmo1

(ji-li) Dynlrb2 – expressed in the node

Underlined letters and numbers correspond to the abbreviations used in the figure.

It seemed unlikely that the 3 genes expressed only in extraembryonic tissue could be responsible for the node cilia phenotype and so they were excluded, reducing the list of candidates to 12 genes.

To understand the potential function of the remaining 12 candidate genes expressed in embryonic tissue at 7.5-8.5dpc, Gene Ontology (GO) terms were examined for each gene (Table 10). GO terms give an indication of gene function through the use of standardised functional terms (www.geneontology.org). In the context of the cilia defects found in *gpg6* mutants, the most interesting GO terms were for *Dynlrb2* (“dynein complex”), *Atmin* (“zinc finger”) and *Pkd1l2* (“cation channel”). *Dynlrb2* is an LC7/roadblock family member (Jiang et al., 2001) and Dynein light chains are essential to bind cargo to the motor complex of Dynein (Tai et al., 1999); Cytoplasmic Dynein has been shown to be critical for ciliogenesis (Pazour et al., 1998). It seemed reasonable that a mutant form of *Dynlrb2* could explain the cilia defects in *gpg6* mutants. The potential function of *Atmin* as a zinc finger transcriptional activator raised the interesting possibility that the *gpg6* phenotype results from transcriptional defects; downregulation of genes involved in ciliogenesis could lead to short cilia and subsequent hh and L-R signalling defects. *Pkd1l2* belongs to the *Pkd* family of genes (Li et al., 2003; Yuasa et al., 2004) and *Pkd* family members have been shown to be involved in cilia related calcium signalling (Hanaoka et al., 2000), polycystic kidney disease (Nauli et al., 2003) and L-R development (McGrath et al., 2003). Therefore, a *Pkd1l2* mutant might explain at least the L-R defects in *gpg6* mutants. Subsequent to this analysis, it was demonstrated that overexpression of *Pkd1l2* causes muscle atrophy and chronic neuromuscular impairment (Mackenzie et al., 2009).

Based on the mapping data, WISH embryonic expression data at 7.5-8.5dpc and their potential function as indicated by GO terms, *Dynlrb2*, *Atmin* and *Pkd1l2* seemed most likely to be mutated in *gpg6* mutants and were therefore sequenced.

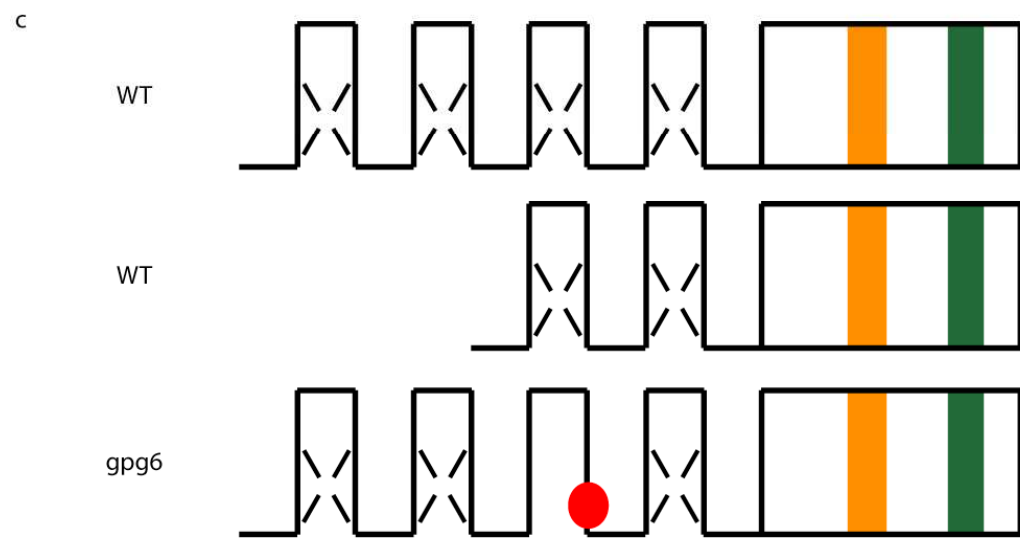
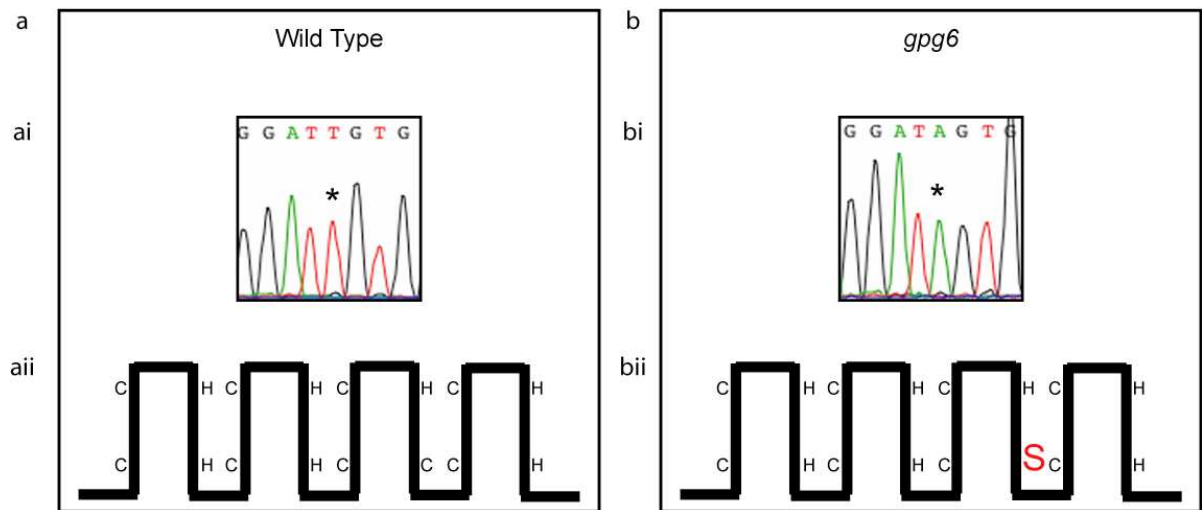
4.2.3 Mutation identified in *Atmin*

Exons and adjacent splice sites of the 3 most likely candidate genes were PCR amplified from genomic DNA from two *gpg6* mutants and wild type controls (BALB/c and C3H wild type). The resultant PCR products were then sequenced. All four exons of *Dynlrb2* were sequenced but no exonic or splice site mutation was found (data not shown). Only six exons of *Pkd1l2* were sequenced before a mutation was found in another gene (see below); no mutation was found in these exons (data not shown).

Sequencing of *Atmin* revealed a T to A change within exon 3 at nucleotide 566 of the *Atmin* transcript, corresponding to amino acid 183 (Figure 16). This base pair change is predicted to cause a cysteine to serine amino acid substitution. The N-terminal region of Atmin contains four zinc finger domains (Kanu and Behrens, 2007). Amino acids 80-105, 127-156, 161-183 and 187-210 respectively encode the four zinc fingers found in Atmin (InterProSite web site). Zinc fingers have been shown to interact with DNA (Wolfe et al., 2003), RNA (Lu et al., 2003) and protein (Mackay and Crossley, 1998). They were first identified in *Xenopus* transcription factor IIIA and are classically thought of as being responsible for binding DNA as a crucial initial step to activate transcription (Miller et al., 1985). C2H2 zinc fingers, which are present in Atmin, are made up of the following consensus sequence: [*C* - x (2 or 4) - *C* - x (11) - *H* - x (3 or 5) - *H/C*], where x is any amino acid, C is a critical cysteine and H

is a critical histidine that chelates the Zn^{2+} atom to stabilise the finger (Nietfeld et al., 1989). The amino acid substitution identified in *gpg6* mutants changes the last residue, a critical cysteine, in the third zinc finger of *Atmin* (Figure 16a-c). Strikingly, this amino acid is conserved in vertebrates, suggesting its function has been evolutionarily conserved (Figure 16d).

The *gpg6* phenotype is complex with multiple regions of mutant embryos demonstrating defects (Chapter 3). It can be argued that a gene responsible for the *gpg6* phenotype should be expressed in those regions of the embryo that are affected. To determine whether *Atmin* fulfils this criterion the initial WISH analysis of *Atmin* expression at 7.5-8.5dpc (Figure 15) was expanded to examine expression from 6.5-12.5dpc. This analysis demonstrated that *Atmin* was broadly expressed in embryonic tissue during this time with an apparently reduced level of expression in the heart, supporting a role for *Atmin* in the complex *gpg6* phenotype (Figure 17).



d

Mouse Atmin	KRHEEDCGKTFQCTCGCPYASRTA
Human Atmin	KRHAEDCGKTFRCTCGCPYASRTA
Chicken Atmin	KRHIEDCGKTFRCTCGCPYASRPA
Frog Atmin	KRHLGYCGKTFHCTCGCPYASRTA
Zebrafish Atmin	RRHVEDCGRTYSCTCGCPYASRAA
<i>gpg6</i> Atmin	KRHEEDSGKTFQCTCGCPYASRTA

Figure 16. *Atmin* is mutated in *gpg6*.

Atmin was sequenced from wild type (a) and *gpg6* mutant (b) DNA. Exon 3 sequencing revealed the expected wild type sequence (ai). However, a T to A (bi*) base pair change was detected in *gpg6* homozygous mutants. This mutation results in a Cysteine (aii) to Serine (bii) amino acid substitution in a critical residue in the third zinc finger of *Atmin*. The amino acid substitution in *gpg6* (red circle) is predicted to disrupt the third zinc finger (c); yellow box is a PEST domain and green box is an ATM binding domain, both predicted to be unaffected in *gpg6*. The residue that is affected in *gpg6* mutants is highly conserved in vertebrates (d).

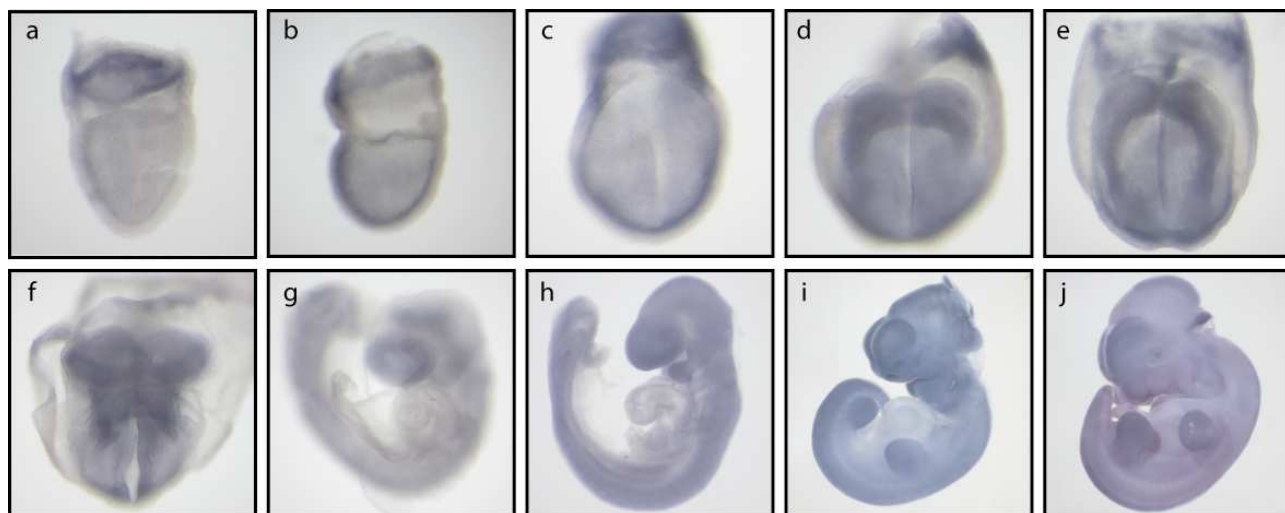


Figure 17. *Atmin* is expressed in mouse from 6.5-12.5dpc.

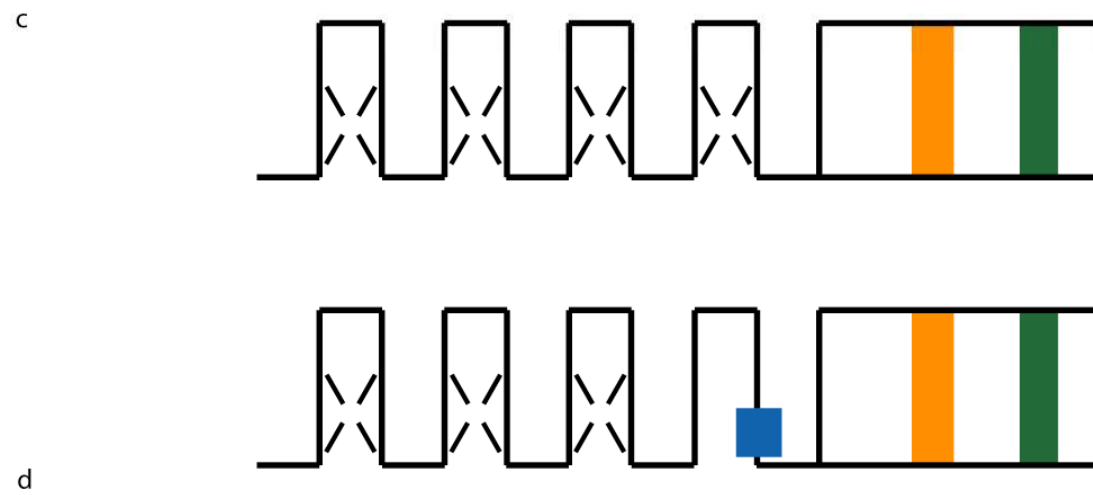
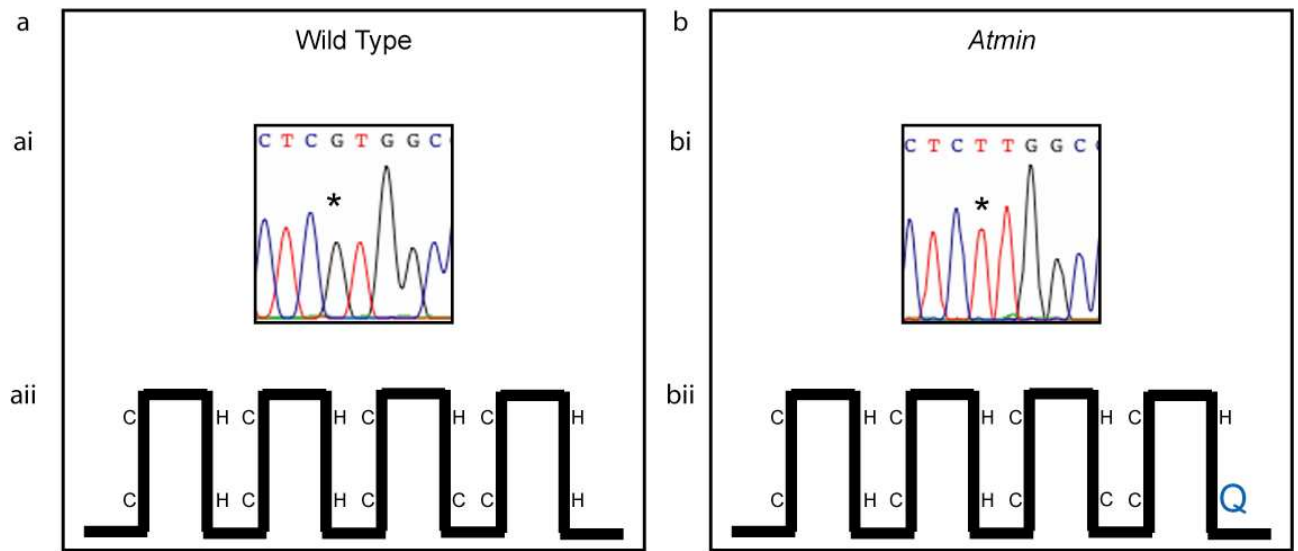
Atmin expression was analysed by WISH in 6.5-12.5dpc wild type embryos. *Atmin* is expressed at low levels in both embryonic and extra embryonic tissue from 6.5-7.5dpc (a-c). Expression is upregulated in embryonic tissue from 8.0-9.5dpc (c-g). From 10.5-12.5dpc *Atmin* continues to be broadly expressed (h-j) but is weaker in the heart (h-i) and stronger in the limb buds (i-j).

4.2.4 A Second Allele of *Atmin* fails to complement *gpg6*

While the mutation identified in *Atmin* shows high linkage to the *gpg6* phenotype and is predicted to disrupt the function of the third zinc finger, there still remained a possibility it was not responsible for the *gpg6* phenotype. To test if the causative mutation had been found, a second allele of *Atmin* was sought. To identify additional alleles of *Atmin*, the MRC Harwell ENU DNA Archive (Quwailid et al., 2004) was screened for mutations by Dr Deen Quwailid. Exon 2 and 3 of *Atmin* were screened in an attempt to identify functionally equivalent mutations, since these exons encode the zinc finger domain and the *gpg6* mutation affects this domain. 2 mutations were identified; the first (*Atmin*^{T132A}), in exon 2, resulted in a threonine to alanine amino acid substitution at a non-conserved residue in mouse. This mutation did not appear to have a striking functional consequence and was not pursued (Appendix B). The second mutation (*Atmin*^{H210Q}) was in exon 3 of *Atmin* at nucleotide position 649. Remarkably, this C to A base pair change was predicted to result in a histidine to glutamine amino acid substitution affecting the critical, final canonical histidine in the fourth zinc finger (Figure 18a-c). This histidine is conserved in all vertebrates (Figure 18d). It is reasonable to postulate that *Atmin*^{gpg6} and *Atmin*^{H210Q}, since they affect canonical residues that stabilise the structure of their respective zinc finger, will disrupt the same function.

Mice carrying the *Atmin*^{H210Q} mutation were rederived from frozen sperm by IVF (FESA, MRC Harwell) and a breeding colony was established (data not shown). It should be noted that *Atmin*^{H210Q} animals were not extensively backcrossed to C3H and as a result had a mixed genetic background. Homozygous *Atmin*^{H210Q} mutant embryos were compared to non-

congenic *Atmin*^{gpg6} homozygous mutant embryos. This analysis demonstrated that both mutants had a very similar phenotype (Figure 19 and Table 11). For instance, 90% of *gpg6* mutants and 93% of *Atmin*^{H210Q} mutants demonstrated exencephaly. Similarly, 65% of *gpg6* mutants had small lungs whereas 75% of *Atmin*^{H210Q} mutant embryos showed this phenotype (Table 11 for further details). This phenocopy supports the prediction that both alleles of *Atmin* disrupt the same function. *Atmin*^{gpg6} and *Atmin*^{H210Q} were then used in Complementation Analysis. Crucially, doubly heterozygous individuals (+/*Atmin*^{H210Q};+/*Atmin*^{gpg6}) demonstrated an embryonic lethal, *gpg6* mutant phenotype (Figure 20 and Table 11). This demonstrates that the two alleles of *Atmin* fail to complement, providing compelling genetic evidence that *Atmin* is responsible for the *gpg6* phenotype.



Mouse Atmin	TALQSHIYRTGHEIPAEHRD
Human Atmin	TALQSHIYRTGHEIPAEHRD
Chicken Atmin	PALLSHIYRTGHEIPAEHRD
Frog Atmin	TALLSHINRTGHEIPVEHRE
Zebrafish Atmin	AALLSHIYRTGHEVPKEHRY
H210Q Atmin	TALQSHIYRTGQEIPAEHRD

Figure 18. Identification of *Atmin*^{H210Q}, a second allele of *Atmin*.

A second allele of *Atmin* (*Atmin*^{H210Q}) was identified in the MRC Harwell ENU DNA archive. Sequencing of Exon 3 from wild type (a) DNA revealed the expected wild type sequence (ai). However, sequencing *Atmin*^{H210Q} mutants (b) revealed a G to T (bi*) base pair change. This mutation results in a Histidine (aii) to Glutamine (bii) amino acid substitution in a critical residue in the fourth zinc finger of Atmin. The amino acid substitution in *Atmin*^{H210Q} (blue square) is predicted to disrupt the fourth zinc finger (c); yellow box is a PEST domain and green box is an ATM binding domain, both predicted to be unaffected in *gpg6*. The residue that is affected in *Atmin*^{H210Q} mutants is highly conserved in vertebrates (d).

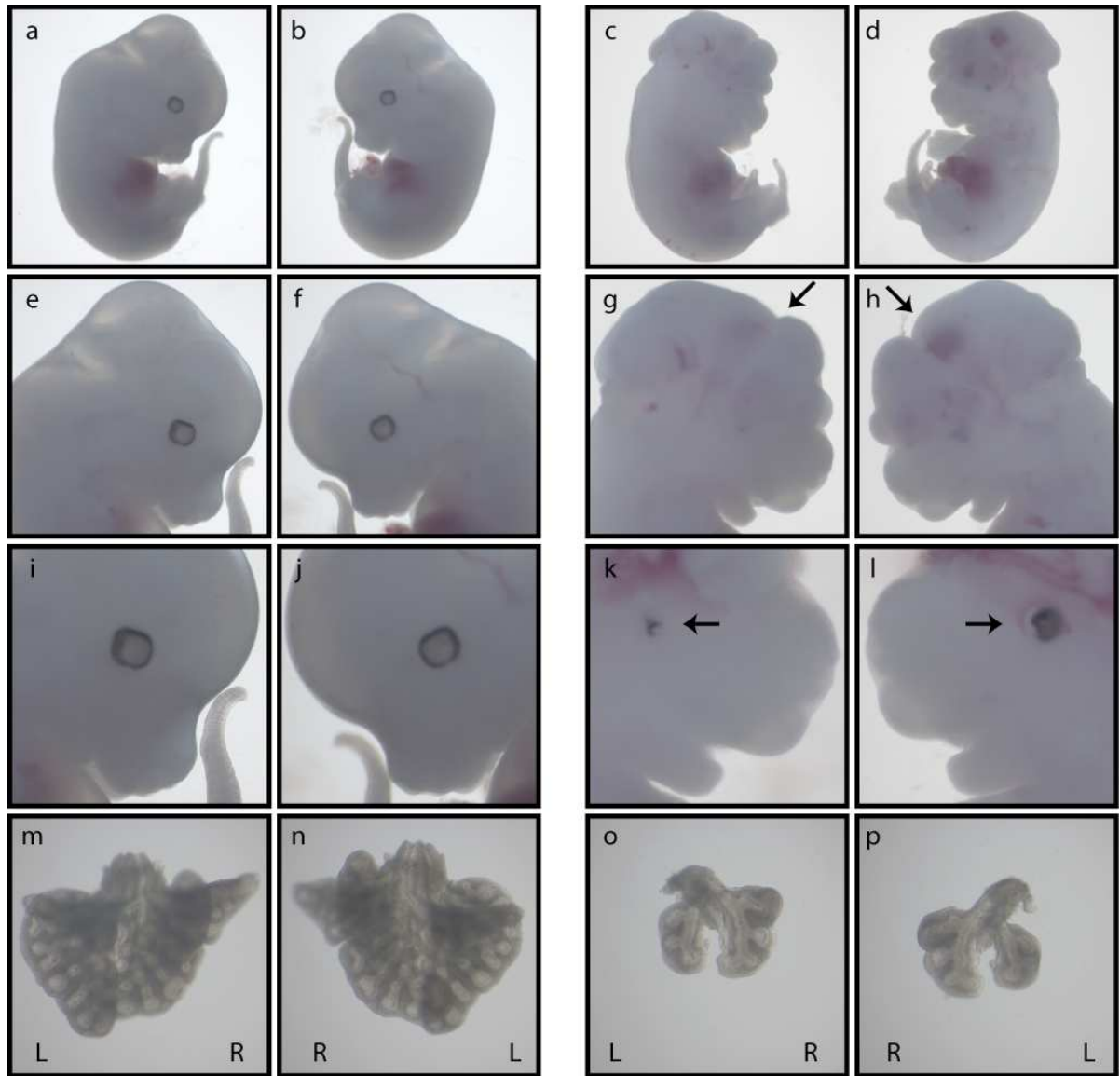
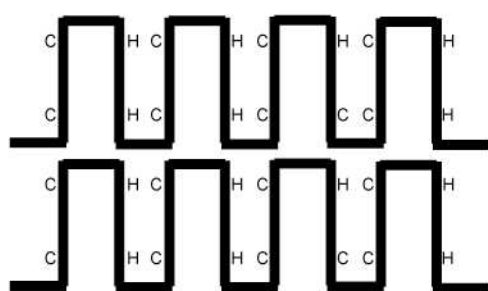


Figure 19. Phenotype of *Atmin*^{H210Q} mutant embryos

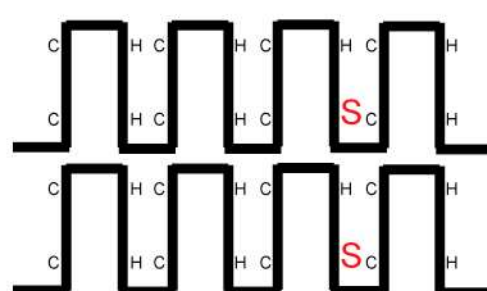
By 13.5dpc wild type embryos have completed neural tube closure (a-b; e-f) and all major features of the head, including the eyes, have developed (i-j). In contrast, *Atmin*^{H210Q} mutant embryos demonstrate exencephaly, a failure to close the anterior neural tube (c-d; g-h). *Atmin*^{H210Q} mutants also demonstrate microphthalmia (reduced eyes) (k) and fail to fully form the iris, a condition called coloboma (l). On closer inspection, wild type embryos demonstrate lungs comprising 1 lobe on the left and 4 on the right (m-n). However, *Atmin*^{H210Q} mutant lungs are smaller than wild type and can show left pulmonary isomerism (o-p). (m) Ventral view. (n) Dorsal view (o) Ventral view. (p) Dorsal view.

Phenotype	Gasping 6	Congenetic gpg6	<i>Atmin</i>^{H210}	<i>gpg6/+</i> <i>Atmin</i>^{H210/+}
Oedema	12 (60%)	23 (92%)	4 (25%)	2 (40%)
Exencephaly	18 (90%)	24 (96%)	15 (93%)	5 (100%)
Polydactyly				
Coloboma	12 (60%)	17 (68%)	14 (87%)	5 (100%)
Micrognathia	4 (20%)	25 (100%)	9 (56%)	4 (80%)
Heart				
Left Sided	20 (100%)	25 (100%)	16 (100%)	5 (100%)
Right Sided				
Central				
Lungs				
Normal Size	4 (20%)			
Small	13 (65%)	25 (100%)	12 (75%)	3 (60%)
Absent	3 (15%)	0 (0%)	4 (25%)	1 (20%)
Left Isomerism	5 (25%)	15 (60%)	9 (56%)	3 (60%)
Right Isomerism				
Partial Isomerism	5 (25%)	10 (40%)	3 (18%)	
Stomach				
Left Sided	20 (100%)	25 (100%)		
Right Sided				
Central				
Total	20 (100%)	25 (100%)	16 (100%)	5 (100%)

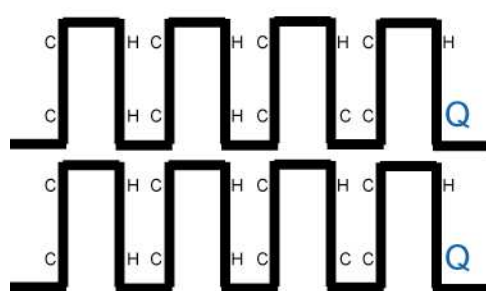
Table 11. Comparison of non-congenic *Atmin*^{gpg6}, congenic *Atmin*^{gpg6}, *Atmin*^{H210Q} and *+/Atmin*^{gpg6}; *+/Atmin*^{H210Q} double heterozygous embryos at 13.5dpc



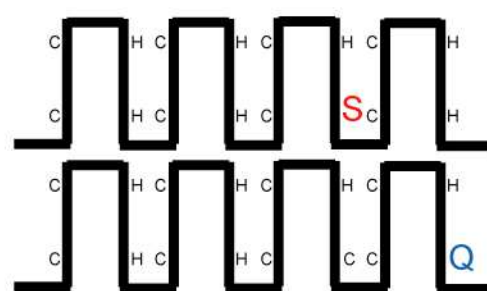
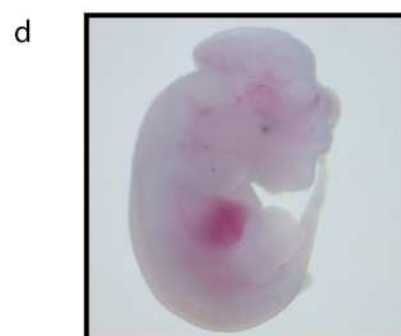
Wild type



gpg6



Atmin



gpg6 / Atmin

Figure 20. *Atmin*^{gpg6} and *Atmin*^{H210Q} double heterozygotes fail to complement: *Atmin* is the gene mutated in *gpg6*

In contrast to wild type (a), *Atmin*^{gpg6} (b) and *Atmin*^{H210Q} (c) mutants demonstrated similar, abnormal phenotypes. This is predicted to result from the disruption of *Atmin* zinc finger function in both mutants (b-c). Importantly, *+/Atmin*^{gpg6}; *+/Atmin*^{H210Q} embryos demonstrated a *gpg6*-like phenotype (d), fail to complement and thereby demonstrate that *Atmin* is the gene mutated in *gpg6*.

4.3 Discussion

4.3.1 Mutation of *Atmin* causes the *gpg6* phenotype

It was crucial in the analysis of the *gpg6* phenotype to establish the underlying genetic lesion. This involved mapping, expression analysis and ultimately sequencing of candidate genes. Throughout this analysis *Atmin* emerged as the best candidate. It was present within the minimal region on chromosome 8 at 119.8Mb and was found to be ubiquitously expressed from 6.5-12.5dpc, consistent with it being responsible for the multiple defects seen in *gpg6* mutants. Crucially, a mutation was detected in *Atmin* that was predicted to cause an amino acid substitution in a highly conserved cysteine in the third zinc finger of *Atmin*; this cysteine is predicted to bind a zinc ion and stabilise the zinc finger (Miller et al., 1985). This mutation was confirmed to be responsible for the *gpg6* phenotype by its failure to complement a second allele of *Atmin* affecting the fourth zinc finger. Collectively, these data demonstrate that *gpg6* is an *Atmin* mutant.

4.3.2 Known functions of *Atmin*

Atmin (ATM interacting; original name *Asciz*) was first identified as a Checkpoint kinase 2 (*Chk2*) interacting protein in a yeast-two hybrid screen (McNees et al., 2005). *Atmin* has an N-terminal region comprising four zinc fingers, a PEST domain (involved in ubiquitin mediated protein degradation (Rogers et al., 1986)) and a C terminal ATM-binding domain (Kanu and Behrens, 2007; McNees et al., 2005). *Atmin* has multiple SQ and TQ motifs that can be phosphorylated, suggesting it can be post-translationally modified (Kanu and

Behrens, 2007; McNees et al., 2005). The *Atmin* locus encodes at least two transcripts from different in-frame start sites encoding a protein with either four zinc fingers (Kanu and Behrens, 2007) or two zinc fingers (McNees et al., 2005).

The interaction between Atmin and Chek2 suggested a role for *Atmin* in DNA repair since *Chek2* is required to prevent cell cycle progression in the presence of DNA damage (Matsuoka et al., 1998). McNees and colleagues (2005) observed that Atmin colocalised in the nucleus with Rad51-containing DNA repair foci following methylation induced DNA damage. These foci form at damaged chromatin in the early phases of the DNA repair response and act as checkpoint signalling and repair centres (Lisby et al., 2004).

Interestingly, foci still formed when truncated forms of Atmin lacking the zinc finger domain (and tagged with GFP) were expressed in U2OS cells (McNees et al., 2005). This demonstrates that the zinc fingers are dispensable for the formation of DNA repair foci following DNA damage.

To understand the function of Atmin at these foci, siRNA-mediated knockdown of *Atmin* was carried out in U2OS cells. In cells where *Atmin* was knocked down, Rad51 localisation to foci following methylation DNA damage was reduced, suggesting that Atmin recruits Rad51 to DNA repair foci. These cells also showed an increase in apoptosis, suggesting a cell survival role for *Atmin*. This increased apoptosis in knockdown cells is Mhl1 dependent since *Atmin* knockdown in *Mhl1* mutant cells did not show the same increase in apoptosis (McNees et al., 2005). A model of *Atmin* function was therefore proposed: methylation DNA damage results in abasic sites on DNA, which are recognised and processed by Mhl1. Atmin foci form in response to persistent single strand DNA gaps generated by Mhl1 processing

and Atmin then recruits Rad51 to foci to repair single stranded DNA gaps and prevent apoptosis (McNees et al., 2005).

Kanu and Behrens (2007) extended this analysis by investigating the role of the ATM-binding domain of *Atmin*. *Atm* (Ataxia telangiectasia mutated) is a crucial phosphatidylinositol kinase that responds to DNA damage by activating cell cycle checkpoints and promoting DNA repair (Bakkenist and Kastan, 2004; Shiloh, 2003). In the absence of DNA damage *Atm* forms a dimer with itself and is inactive. In response to double strand breaks, UV light, hypotonic stress and hydroxyurea a serine residue at amino acid 1981 of *Atm* becomes autophosphorylated, resulting in the release of active *Atm* monomers (Bakkenist and Kastan, 2003). Activated *Atm* then phosphorylates p53, 53bp1, Chk2, Brca1, Smc1, Nbs1 and Fancd2 to set up checkpoints throughout the cell cycle (Bakkenist and Kastan, 2004; Shiloh, 2003). In cells, *Atmin* present within repair foci colocalise with active S1981-phosphorylated *Atm* following hydroxyurea and hypotonic stress (Kanu and Behrens, 2007). It was shown by coimmunoprecipitation that *Atm* and *Atmin* interact via the *Atm*-binding domain of *Atmin* (Kanu and Behrens, 2007). To understand the function of this interaction, siRNA mediated knockdown of *Atmin* and also *Atm* was carried out. Following knock down of *Atmin* (in the presence of hydroxyurea and hypotonic stress) cells showed a reduction in the levels of S1981 phosphorylated *Atm*, suggesting that *Atmin* contributes to *Atm* autophosphorylation and activation. When *Atm* was knocked down *Atmin* levels were reduced. This effect was reversed by the use of protease inhibitors, suggesting that *Atm* stabilises *Atmin* by inhibiting the PEST domain of *Atmin* (Kanu and Behrens, 2007).

Kanu and Behrens (2007) generated a null allele of *Atmin* (see below) and cells derived from mutant embryos showed reduced S1981 Atm autophosphorylation and reduced p53 and Chek2 phosphorylation following hypotonic stress. This suggests that Atmin and Atm reciprocally stabilise each other at DNA repair foci to activate cell cycle arrest and DNA repair via p53 and Chek2 (Kanu and Behrens, 2007). Further analysis of *Atmin* null mutant MEFs demonstrated persistent DNA damage associated with a failure to recruit Atm to DNA repair foci, consistent with Atmin having a role at DNA repair foci (Kanu et al., 2010). Another null allele of *Atmin* has been reported and MEFs derived from mutant embryos demonstrate DNA damage associated with oxidative stress (Jurado et al., 2010). Jurado and colleagues (2010) argue that their mutant allele, in contrast to the allele reported by Kanu and colleagues (Kanu and Behrens, 2007; Kanu et al., 2010), does not show a reduction in levels of Atm and conclude that Atmin and Atm do not reciprocally stabilise each other (Jurado et al., 2010). However, in contrast to Kanu and colleagues (2007), they only analyse gross levels of Atm and do not consider the levels of S1981 autophosphorylated Atm in mutant MEFs. They also fail to detect the reduced Atm recruitment to DNA repair foci in the absence of Atmin (Kanu et al., 2010). The precise role of Atmin in DNA repair remains uncertain although on current evidence it appears likely that Atmin has a role in supporting Atm activation.

Despite the lack of detailed phenotypic analysis, the recently reported *Atmin* mutant phenotype is similar to the *gpg6* phenotype: embryonic lethality, exencephaly and pulmonary agenesis (Jurado et al., 2010; Kanu et al., 2010). This further supports the identification of *gpg6* as an *Atmin* mutant. Interestingly, *Atmin* mutant embryos fail to induce a trachea-oesophageal septum during foregut development, suggesting this is the cause of pulmonary agenesis in mutants (Jurado et al., 2010). Kanu and colleagues (2010)

conditionally reduced levels of *Atmin* in the adult mouse brain and demonstrated an age-related accumulation of DNA damage and premature death due to neuronal loss. This demonstrates a role for *Atmin* in adult tissue in addition to its role in embryonic development.

4.3.3 A Novel Transcriptional Function of *Atmin* is revealed by *gpg6* mutants?

The identification of *Atmin* as the gene responsible for the *gpg6* phenotype raises the important question of whether there is a link between DNA repair and ciliogenesis. The DNA repair function of *Atmin* is, in part, mediated by *Atm*, *Chek2*, *Rad51* and *p53* (Kanu and Behrens, 2007; McNees et al., 2005); if the known DNA repair function of *Atmin* is involved in ciliogenesis, then mouse mutants of these genes would be likely to demonstrate a ciliogenic, *gpg6*-like phenotype. However, this is not the case since *Atm*, *Chek2* and *p53* homozygous mutants survive to birth, manifesting a susceptibility to neoplasia during adulthood (Barlow et al., 1996; Donehower et al., 1992; Takai et al., 2002). *Rad51* mutants demonstrate a very early pre-implantation lethality, a phenotype unlike *gpg6* mutants and other cilia mutants (Tsuzuki et al., 1996). These data argue against DNA repair having a direct role in ciliogenesis.

The mutation identified in *gpg6* mutants disrupts a single amino acid in the N-terminal zinc finger domain of *Atmin*. It seems reasonable that this change will not affect the C-terminal ATM-binding domain. As previously noted, truncated forms of *Atmin* lacking the zinc finger domain still form DNA repair foci, a crucial early step in DNA repair (McNees et al., 2005). These data suggest that in *gpg6* mutants *Atmin* can still bind *Atm* at repair foci and

mediate the DNA repair response. Furthermore, it argues, along with the phenotype and nature of the mutation identified in *gpg6* mutants, that the zinc finger domain of *Atmin* has a previously unappreciated, DNA repair-independent function in ciliogenesis.

Zinc fingers have been shown to interact with DNA (Wolfe et al., 2003), RNA (Lu et al., 2003) and protein (Mackay and Crossley, 1998). It is therefore possible that *Atmin* is binding to any of these to facilitate its role in ciliogenesis. Zinc fingers were first discovered in *Xenopus* transcription factor IIIA (Miller et al., 1985) and have since been strongly associated with binding DNA to control transcription of target genes (Klug, 2010). It can be argued that the simplest explanation for the role of the zinc finger domain of *Atmin* is the activation of genes required for ciliogenesis. This predicts that *Atmin*, in addition to its role in mediating DNA repair, is a transcription factor and this function is abolished in *gpg6* mutants. It follows that downregulation of *Atmin* target genes involved in ciliogenesis may be responsible for the *gpg6* phenotype.

To determine the possible role of the zinc finger domain of *Atmin*, the hypothesis that it binds to DNA to activate transcription needs to be tested and only then can downstream transcriptional targets be sought.

Chapter 5

Atmin regulates expression of the ciliogenic
locus *Dynll1*

5.1 Introduction

Zinc fingers were first described in the *Xenopus* transcription factor IIIA (Miller et al., 1985) and are now considered to be classical features of a class of transcription factors. Within a zinc finger, a zinc ion chelates four canonical cysteine and histidine residues to stabilise its structure (Miller et al., 1985). Not only do zinc fingers bind to DNA (Wolfe et al., 2003) but also to RNA (Lu et al., 2003) and protein (Mackay and Crossley, 1998). It remains unclear which of these functional interactions is relevant to *Atmin* and the *gpg6* phenotype. To begin to understand the functional consequence of the *gpg6* mutation, it would be useful to determine whether a similar change has been reported in other zinc finger proteins.

Remarkably, the same zinc finger cysteine to serine amino acid substitution found in *gpg6* mutants has been described in mutants of the transcription factor *Kruppel* in *Drosophila*; this change results in a null phenotype, suggesting the mutated cysteine is crucial for *Kruppel* function (Redemann et al., 1988). In the mouse, a point mutation in the transcription factor *Zic2* has been identified that also results in a zinc finger associated cysteine to serine substitution (Elms et al., 2003). *In vitro* analysis of this change demonstrated that it abolishes transcriptional activation of a *Zic2* target gene, likely due to mutant *Zic2* failing to bind to DNA (Brown et al., 2005). Collectively, this suggests the following hypothesis: the *gpg6* mutation in the third zinc finger of *Atmin* disrupts transcription of *Atmin* target genes by failing to bind DNA or RNA. This is supported by the observation that *Atmin* activates expression of *ATF3* (Axel Behrens, personal communication). If the *gpg6* mutation does affect the transcriptional function of *Atmin*, target genes can then be sought and tested for their role in ciliogenesis.

5.2 Results

5.2.1 Activation of *Atmin* target genes is abolished in *gpg6* mutants

Before the problem of the functional consequence of the *gpg6* mutation could be addressed experimentally, it was crucial to generate a full length *Atmin* cDNA clone containing the *gpg6* point mutation. This was achieved by designing PCR primers containing the *Atmin*^{*gpg6*} point mutation (equivalent to base pair 566 of the *Atmin* cDNA, Chapter 4). These primers were used to generate two PCR amplicons, which were subsequently cloned: containing base pair 130-580 (P1) and 550-1100 (P2) of the *Atmin* transcript. A *XhoI* restriction site at base pair 422 and a unique *BseRI* restriction site at base pair 557 was then used to combine P1 and P2, giving a cloned fragment of *Atmin*^{*gpg6*} from base pair 422-1100. *XhoI* (restriction site at base pair 422) and *StuI* (restriction site at base pair 1080) were used to clone the *Atmin*^{*gpg6*} point mutation into the full length *Atmin* cDNA clone (Figure 21). The wild type and mutant *Atmin* cDNA clones, together with *Sall* and *NotI*-containing primers, were then used to PCR amplify the coding region of wild type and mutant *Atmin*; these amplicons were cloned, in-frame, into a *Sall* and *NotI* digested pCMV-Myc expression vector (Figure 22). Both constructs were confirmed to be correct by sequencing then provided to Nnenna Kanu (London Research Institute) to test whether the mutant form of *Atmin* could activate expression of *ATF3*, a known target of *Atmin* (Axel Behrens, personal communication). *ATF3* and *Atmin* constructs were used in a luciferase reporter assay. Co-transfection of *ATF3::Luciferase* plasmid with wild type *Atmin* resulted in strong activation of *ATF3*. In contrast, when *Atmin*^{*gpg6*} was analysed, transcriptional activation of *ATF3* was not seen (Figure 23 and Table 12).

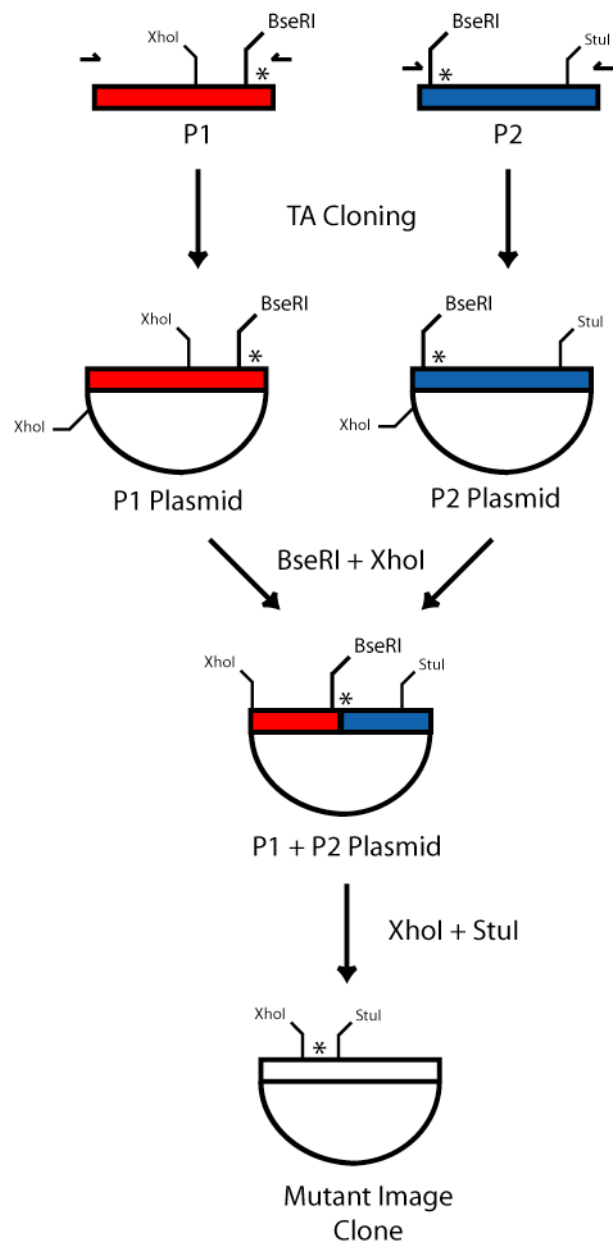


Figure 21. Generating a cDNA clone of *Atmin* containing the *gpg6* mutation

Two sets of PCR primers (p1 and p2) were designed to amplify the region of *Atmin* containing the *gpg6* mutation. Two PCR products were generated (which included the *gpg6* mutated base pair) from a full length *Atmin* IMAGE clone and cloned into bluescript plasmid (P1 and P2 plasmid). P1 plasmid contained 130-580bp of *Atmin* (XhoI site at 422bp and a BseRI site at 557bp). P2 plasmid contained 550-1100bp of *Atmin* (BseRI site at 557bp and a StuI site at 1080bp). The mutated base pair is at 566bp (*).

A XhoI-BseRI fragment from P1 plasmid was excised and cloned into the corresponding sites in P2 plasmid, utilising the XhoI site in the bluescript backbone of P2 plasmid. This formed an insert from 422-1100bp (P1+P2). XhoI and StuI sites were then used to clone a fragment containing the mutated base pair into the corresponding sites in the *Atmin* IMAGE clone.

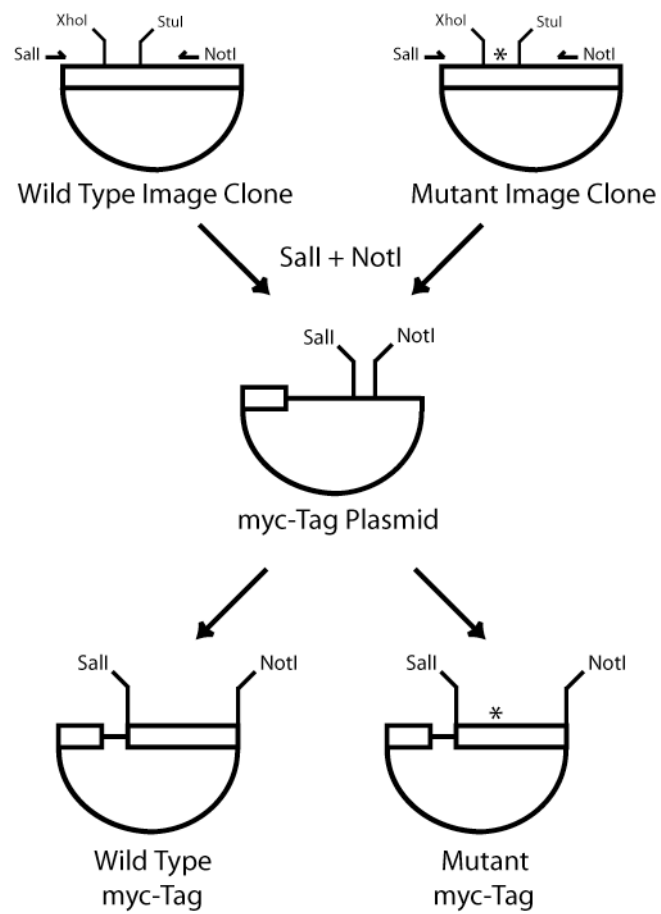


Figure 22. Generating a wild type and mutant *Atmin* myc-tag plasmid

A Sall containing primer and a NotI containing primer were used to PCR and clone the wild type and *Atmin*^{pg6} coding region of *Atmin* into the corresponding Sall and NotI sites in a N-myc tag plasmid.

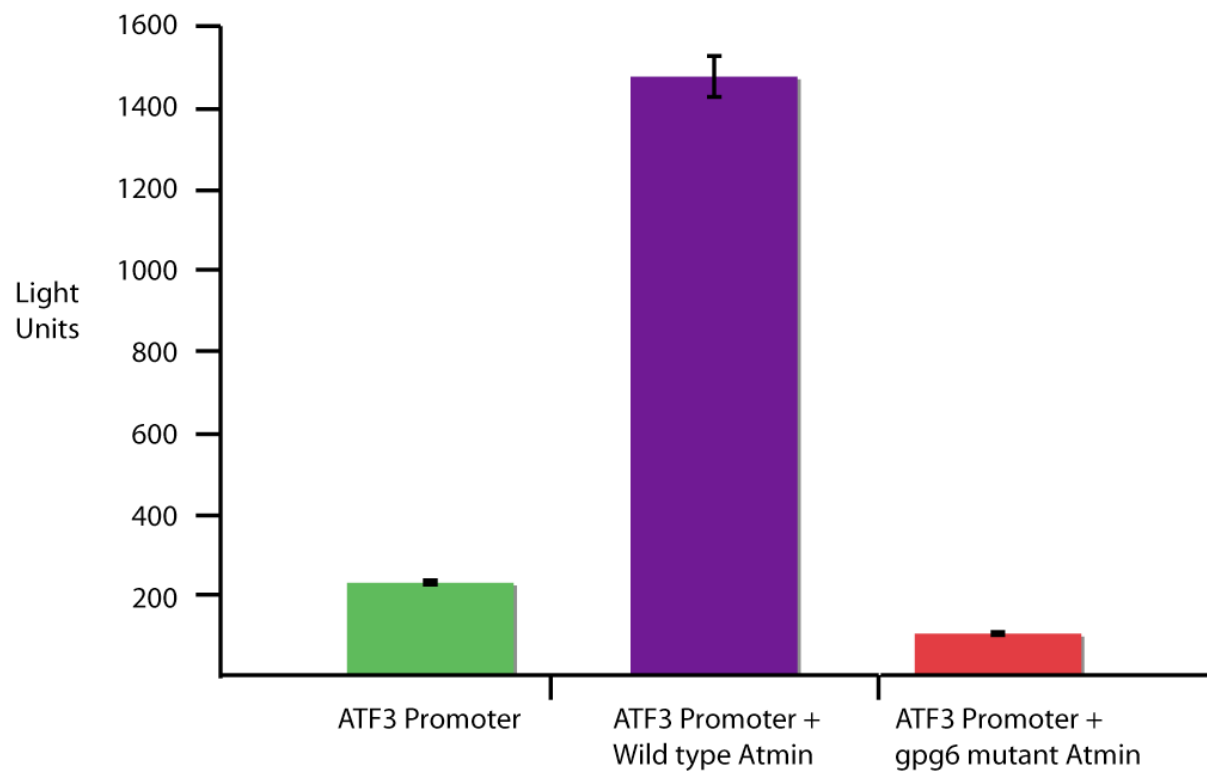


Figure 23. Transcriptional activation by *Atmin* is abolished by the *gpg6* mutation

ATF3 is a known transcriptional target of *Atmin*; when the *ATF3* promoter was transfected in COS7 cells in the presence of wild type *Atmin* there was strong induction of luciferase activity, compared to minimal activity with *ATF3* promoter alone (compare “ATF3 promoter” with “ATF3 promoter + wild type *Atmin*”). However, when *ATF3* promoter was transfected with mutant *Atmin*^{*gpg6*} there was a strong inhibition of luciferase activity (“ATF3 promoter + *gpg6* mutant *Atmin*”). This demonstrates that transcription of an *Atmin* target gene is severely affected by *Atmin*^{*gpg6*}. Experiment carried out by Nnenna Kanu.

<i>Vectors</i>	<i>Mean Light Units</i>
ATF3	224.74
ATF3 + Wild type <i>Atmin</i>	1447.36
ATF3 + <i>Atmin</i> ^{gpg6}	101.55

Table 12. Transcriptional activation by *Atmin* is abolished by the *gpg6* mutation.

Compared to wild type *Atmin*, there was an approximately 12-fold inhibition of luciferase activity when *Atmin*^{gpg6} was used to activate *ATF3*. Experiment carried out by Nnenna Kanu.

It should be noted that these data do not exclude the possibility that the *gpg6* point mutation results in loss of Atmin protein and absence of transcriptional activation is not due to specific disruption of the zinc finger domain. Despite this, these data strongly suggest that the functional consequence of the point mutation identified in *gpg6* mutants is to disrupt transcription of *Atmin* target genes. This is likely due to *Atmin*^{*gpg6*} failing to bind DNA.

5.2.2 Identifying transcriptional targets of *Atmin*

It was hypothesised, based on the observation that *Atmin*^{*gpg6*} failed to activate transcription of *ATF3* (Figure 23 and Table 12), that transcription of other *Atmin* target genes would be disrupted in *gpg6* mutants. Therefore, to further understand the underlying cause of the *gpg6* phenotype, transcriptional targets of *Atmin* were sought. Expression analysis comparing primary MEFs derived from 11.5dpc wild type and *Atmin* null embryos (Kanu and Behrens, 2007) had been carried out by Nnenna Kanu and Axel Behrens (London Research Institute). This generated a list of 368 differentially expressed genes that was kindly provided to us by Axel Behrens (data not shown).

Given the cilia related phenotype seen in *gpg6* mutants the list of genes identified in the microarray analysis was compared to the cilia proteome (Gherman et al., 2006) and 48 genes were identified as important in ciliogenesis (data not shown). 21 of these have been previously knocked out but did not demonstrate a ciliogenic phenotype at 13.5dpc; they were excluded from further analysis (MGI; data not shown). The remaining 27 candidates were ranked according to the difference of expression between wild type and *Atmin*^{-/-} MEFs (Table 13).

Gene	Fold difference in microarray (wt vs mut)	GO Terms
Pleckstrin	-8.00	Signalling
dynein light chain LC8-type 1	-5.66	Dynein complex
cancer susceptibility candidate 4	-3.39	?
Tripartite motif protein 30	-3.32	?
dynein light chain Tctex-type 1	-3.24	Dynein complex
ring finger protein 17	-3.09	?
calmodulin-like 4	-3.04	?
mitochondrial ribosomal protein L47	-2.70	?
Rho GTPase activating protein 30	-2.61	Signalling
ankyrin repeat and SOCS box-containing protein 4	-2.56	?
gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 4	-2.44	?
Dynamin 1-like	-2.29	?
glutaminyl-peptide cyclotransferase	-2.29	?
ring finger protein 180	-2.16	?
tyrosine 3-monooxygenase	-2.12	?
centrin 4	-2.09	Basal body assembly
RAB14, member RAS oncogene family	-2.06	?
protocadherin beta 22	-2.05	Cell adhesion
C1q domain containing 2	+2.11	?
septin 14	+2.16	Cell cycle
guanine nucleotide binding protein, beta 4	+2.22	Signalling
OTU domain containing 7A	+2.38	Ubiquitin
ELAV-like 1	+2.5	mRNA stabilization
ribosomal protein S4, Y-linked 2	+2.82	Translation
fibronectin type III domain containing 1	+3.25	?
Prohibitin	+3.27	?
Mannose receptor C type 1	+5.2	?

Table 13. Microarray candidate genes differentially expressed in *Atmin* mutant MEFs compared to wild type MEFs

Mutants that disrupt components of the retrograde IFT motor and IFT particle complex A have short or malformed node cilia. In contrast, mutants affecting IFT particle complex B and anterograde IFT motor components lack node cilia (Goetz and Anderson, 2010). Since *gpg6* mutants demonstrate short node cilia, it was hypothesized that retrograde IFT or IFT particle complex A defects likely underlie the *gpg6* phenotype. Given this hypothesis, the most obvious candidates to test further were the Cytoplasmic Dynein components *Dynein light chain LC8-type 1* (King and Patel-King, 1995) (*Dynl1l*; 5.6-fold downregulation in *Atmin*^{-/-} MEFs) and *Dynein light chain Tctex-type 1* (DiBella et al., 2001) (*Dynlt1*; 3.2-fold downregulation in *Atmin*^{-/-} MEFs). The basal body protein *Centrin 4* (*Cetn4*; 2.09-fold downregulation in *Atmin*^{-/-} MEFs) is enriched in ciliated cells (Gavet et al., 2003) and was therefore analysed further (Table 13).

5.2.3 *Dynl1l* is downregulated in *gpg6* mutant embryos

To test whether *Dynl1l*, *Dynlt1* and *Cetn4* were downregulated in *gpg6* mutants, relative expression levels of each gene were analysed in wild type and *gpg6* mutant embryos by Real Time PCR. 5 mutant and 6 wild type 11.5dpc embryos were collected and RNA was extracted. cDNA was then generated and analysed by Real Time PCR Taqman assays for *Dynl1l*, *Dynlt1* and *Cetn4*; β -actin was used as an endogenous control. When expression levels of *Dynlt1* and *Cetn4* were analysed no statistically significant difference between wild type and *gpg6* mutant 11.5dpc embryos was detected (data not shown). In contrast, analysis of *Dynl1l* levels demonstrated a mean cycling threshold (mean ΔC_T) in 5 mutant samples of 7.97 ± 0.064 , whereas in 6 wild type samples this was 3.838 ± 1.008 ; this shows that it took longer to amplify *Dynl1l* cDNA to the same threshold level in mutant compared to wild type samples, calculated as a $\Delta \Delta C_T$ of 4.1383 ± 0.064 . The fold change difference between wild

and mutants is therefore calculated as 0.057 or a striking 17.5-fold downregulation in *Dynll1* transcript levels in *gpg6* mutant embryos (Figure 24 and Table 14).

The similarity of *Dynll1* to its paralogue *Dynll2* (Naisbitt et al., 2000; Puthalakath et al., 2001; Wilson et al., 2001) raised the possibility that *Dynll2* may also be downregulated in *gpg6* mutants. However, Dr Paraskevi Goggliodou, utilising the same cDNA samples used to analyse levels of *Dynll1*, carried out similar Real time PCR analysis of *Dynll2* and demonstrated that levels of *Dynll2* are not reduced in *gpg6* mutants (Figure 24 and Table 14). This suggests that *Dynll1* is a specific target of *Atmin* and that *Dynll1* may contribute to the *gpg6* phenotype.

5.2.4 *gpg6* MEFs demonstrate defects in IFT88 staining

The observations that *gpg6* mutants have short node cilia and show severe downregulation of *Dynll1*, a component of Cytoplasmic Dynein (King and Patel-King, 1995), suggested that IFT is disrupted in *gpg6* mutants. Recent advances in live cell imaging has allowed accurate measurements of the rate of IFT (Tran et al., 2008). However, this approach is technically demanding and could not be attempted during this project. Therefore, an alternative experimental approach was chosen to address the possibility of disrupted IFT in *gpg6* mutants.

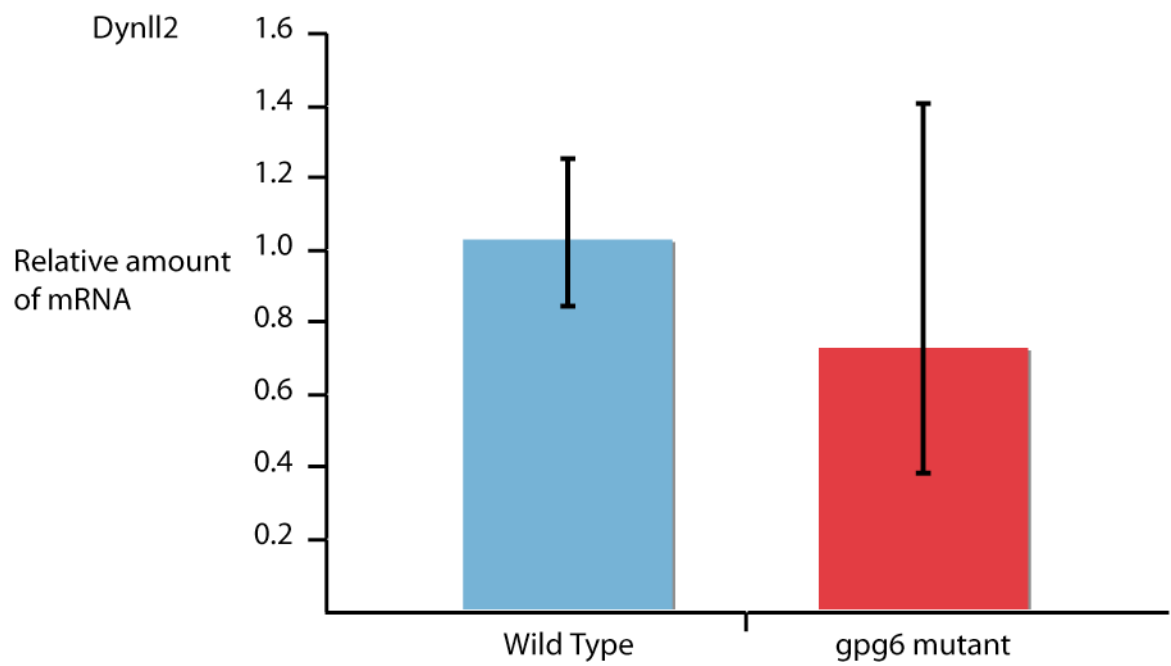
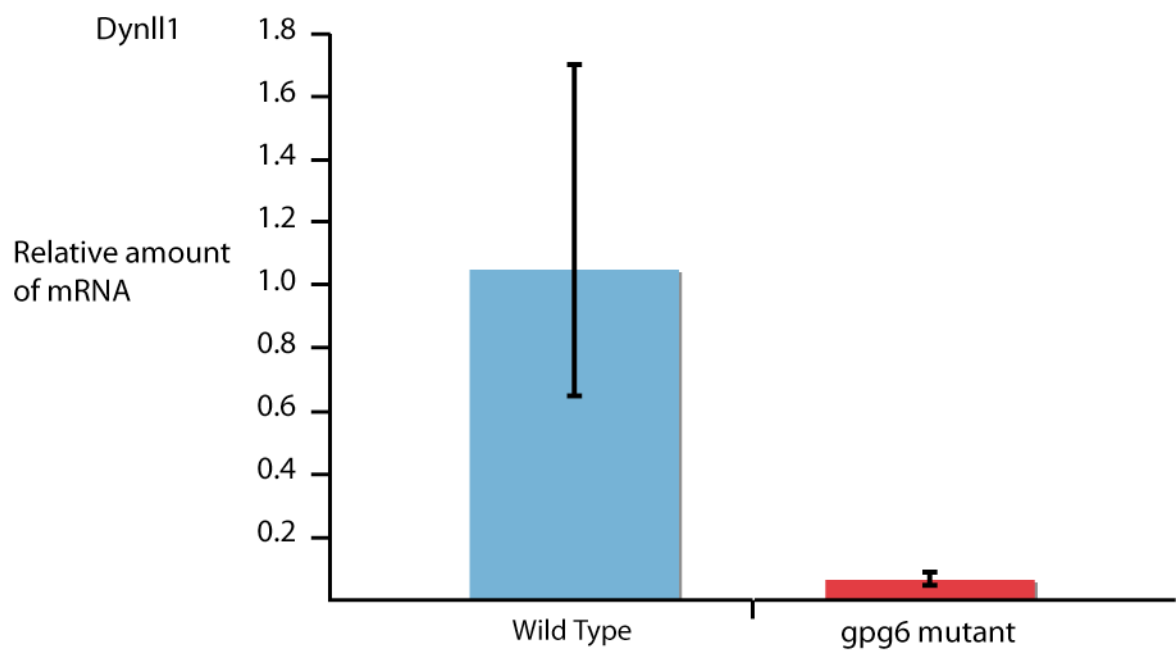


Figure 24. *Dynll1* is downregulated in *gpg6* mutant embryos

cDNA from wild type and *gpg6* mutant embryos was used in *Dynll1* and *Dynll2* Real Time PCR analysis. All reactions were normalised to levels of β -actin. (a) There was a striking 17.5 fold downregulation of *Dynll1* in *gpg6* mutants compared to wild type. (b) However, there was no statistically significant change in *Dynll2* transcript levels in *gpg6* mutants compared to wild type.

	<i>Dynll1</i>	<i>Dynll2</i>
Mutant 1 Av ΔCT	7.185	3.527
Mutant 2 Av ΔCT	7.553	4.97
Mutant 3 Av ΔCT	8.073	4.318
Mutant 4 Av ΔCT	8.169	3.605
Mutant 5 Av ΔCT	8.38	3.264
Wild type 1 Av ΔCT	3.703	3.996
Wild type 2 Av ΔCT	4.327	3.968
Wild type 3 Av ΔCT	4.895	3.658
Wild type 4 Av ΔCT	4.185	3.714
Wild type 5 Av ΔCT	3.874	3.003
Wild type 6 Av ΔCT	2.045	2.29
Mean Mutant ΔCT	7.9763	3.9368
Mean Wild type ΔCT	3.838	3.438
$\Delta\Delta$CT	4.1383	0.4988
Relative Quantification (RQ)	0.057	0.707
Standard Error of Mean – WT RQmax	1.624	1.219
Standard Error of Mean – WT RQmin	0.616	0.82
Standard Error of Mean – Mut RQmax	0.081	1.6
Standard Error of Mean – Mut RQmin	0.017	0.6

Table 14. Raw Real Time PCR data presented in Figure 20

An antibody against Ift88, an IFT particle component transported into and out of cilia, was used to stain primary MEFs derived from 11.5dpc wild type and *gpg6* mutant embryos. Following preliminary analysis of these cells it became clear that cilia phenotypes could be separated into 5 different classes: “abnormal morphology”, “spotty staining”, “gaps in staining”, “stained at one end” and “staining throughout cilia” (Figure 24 presents typical examples of each class). It was observed that the majority of wild type cilia could be classed as “staining throughout cilia”, whereas the proportion of cilia showing this staining was reduced in *gpg6* mutant MEFs. Subsequently, the full data set, comprising 85 cilia from wild type MEFs and 91 cilia from *gpg6* mutant MEFs, was blinded, analysed and cilia were placed in the different classes; there was a statistically significant difference in the numbers in each class between wild type and mutant (Chi squared test, $p < 0.005$). For example, 86% of wild type cilia demonstrated “staining throughout cilia” whereas only 44% of *gpg6* mutant cilia demonstrated this phenotype. There was a concurrent increase in all other cilia types in *gpg6* mutant MEFs (Figure 25), suggesting abnormal cilia function. In particular, these data imply that the transport of Ift88 into and out of cilia is disrupted in *gpg6* mutant MEFs. It should be noted that this experimental approach cannot differentiate between anterograde or retrograde IFT defects or detect changes in the rates of IFT; it provides only a snap shot of a highly dynamic process. Despite these reservations, these data are indicative of a defect in IFT in *gpg6* mutants and warrant further investigation beyond the scope of this thesis. This would likely include adopting the methodology of Tran and colleagues (2008) to accurately measure IFT in *gpg6* mutant MEFs.

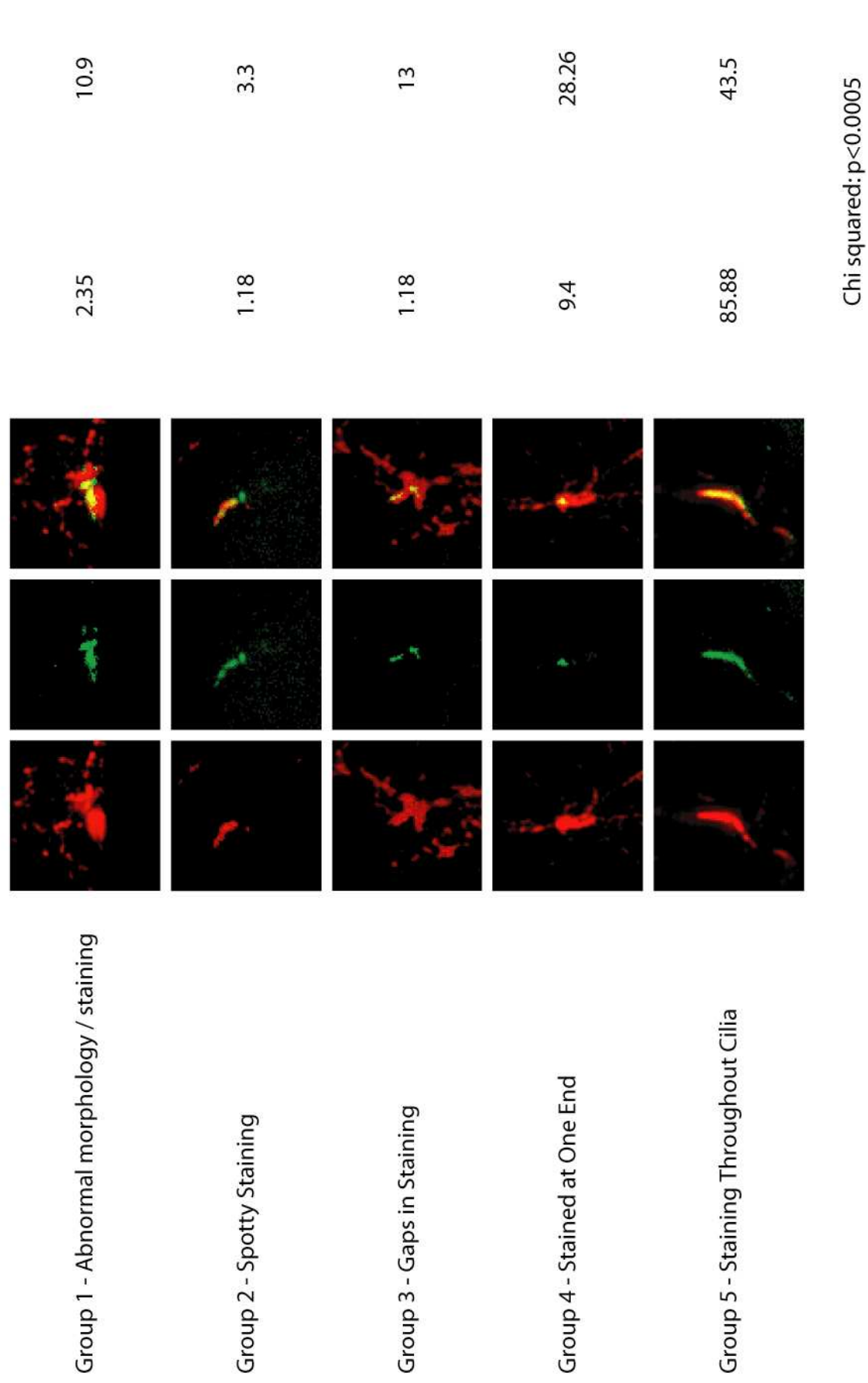


Figure 25. Ift88 antibody staining is abnormal in *gpg6* mutant MEFs

MEFs were derived from wild type and *gpg6* mutant 11.5dpc embryos and stained for acetylated tubulin (red) and Ift88 (green). Cilia were grouped blind into different groups: (i) abnormal morphology, (ii) spotty staining, (iii) gaps in staining, (iv) staining at one end and (v) staining throughout cilia (representative pictures are shown for each group). Most wild type cilia demonstrated “staining throughout cilia”, whereas *gpg6* mutant cilia demonstrated reduced numbers in this group and a concomitant increase in all other groups. The difference in cilia population was statistically significant.

5.3 Discussion

5.3.1 *Atmin* regulates expression of *DynIII* to control ciliogenesis

In previous chapters, it has been shown that *gpg6* mutants contain a point mutation in the zinc finger domain of *Atmin* and that this change is responsible for the *gpg6* phenotype (Chapter 4). Analysis of *gpg6* mutants revealed short node cilia; this was hypothesized to be a key feature of the *gpg6* phenotype (Chapter 3). Defects in ciliogenesis have previously been associated with abnormal L-R development and hh signalling (Huangfu et al., 2003; Nonaka et al., 1998). Consistent with *gpg6* being a cilia mutant, morphological and molecular defects in L-R development and hh signalling were detected in *gpg6* mutants (Chapter 3). In this chapter, the link between *Atmin* and ciliogenesis was investigated. It has been demonstrated that the zinc finger mutation in *Atmin* abolishes transcription of an *Atmin* target gene, strongly suggesting that levels of *Atmin* target genes are reduced in *gpg6* mutants and that these genes are normally required for ciliogenesis. Consistent with this, a putative target of *Atmin*, *DynIII*, has been found to be severely reduced in *gpg6* mutants.

Based on the evidence presented so far, a hypothesis can be proposed to explain the *gpg6* phenotype (Figure 26). *Atmin* activates (either directly or indirectly) transcription of *DynIII*, which is required for retrograde IFT and ciliogenesis. Therefore, *Atmin*, in addition to its role in DNA repair, regulates transcription of a component of the IFT machinery and is thus needed for ciliogenesis and by extension correct L-R patterning and hh signalling. A major prediction of this hypothesis is that retrograde IFT will be disrupted in *gpg6* mutants.

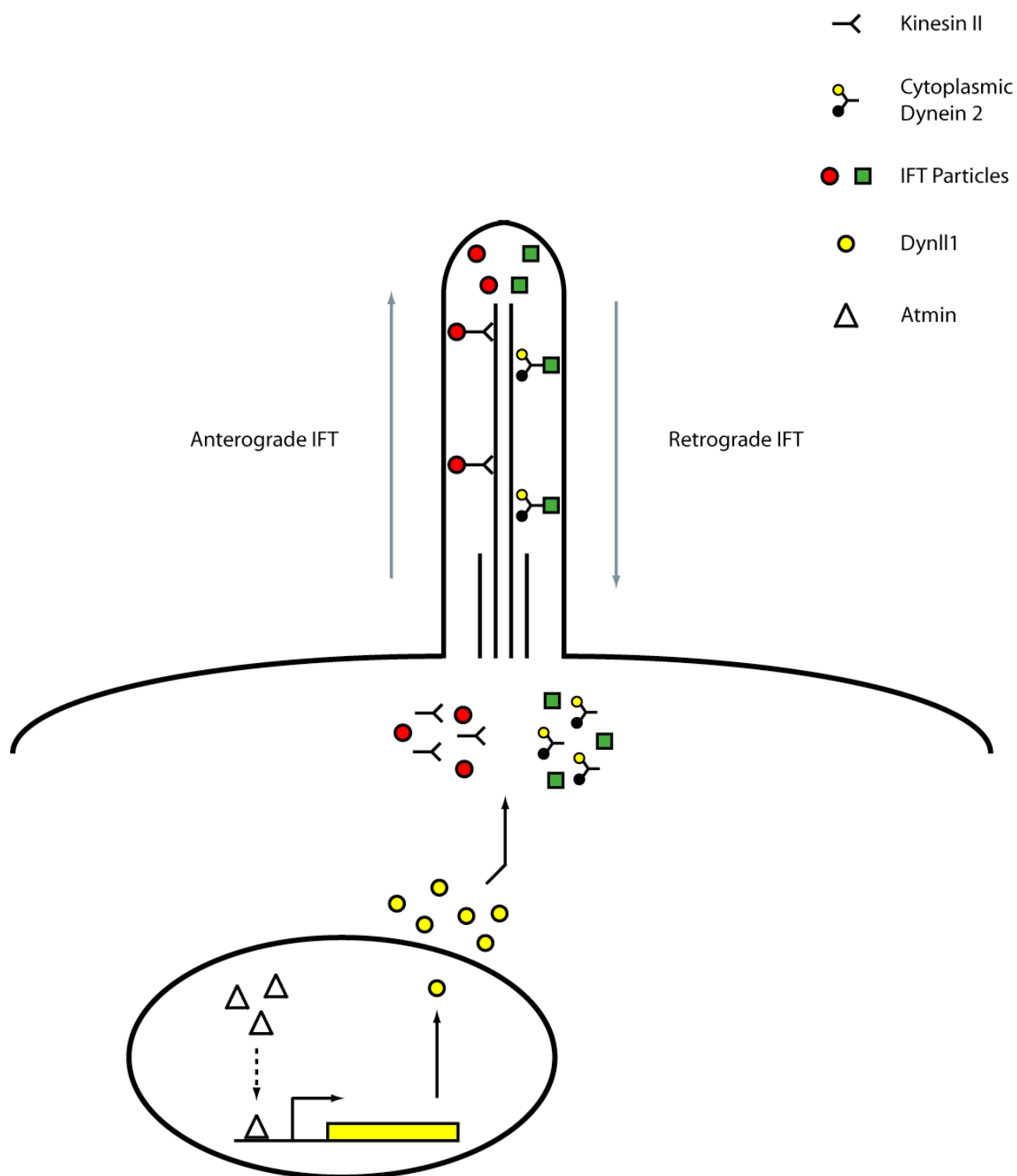


Figure 26. Hypothesis of the role of *Atmin* and *Dynll1* in ciliogenesis

Atmin activates (directly or indirectly) expression of *Dynll1*. *Dynll1* is transported to the basal body where it becomes incorporated into Cytoplasmic Dynein 2 and functions in retrograde IFT.

In *gpg6* mutants, *Atmin* fails to activate *Dynll1*, which leads to disruption of retrograde IFT and short cilia. This disrupts cilia-based L-R development and hh signalling, leading to the *gpg6* phenotype.

Measurement of retrograde IFT in *gpg6* mutant MEFs or *Dynll1* knockdown cells should be analysed in the future to test this prediction.

5.3.2 The *Chlamydomonas* homolog of *Dynll1* functions in retrograde IFT

To begin to understand how relevant the downregulation of *Dynll1* is to the *gpg6* phenotype and to provisionally assess the above hypothesis (Figure 25) it would be useful to explore previously published data on *Dynll1*. *Dynll1* has a *Chlamydomonas* homolog called LC8; LC8 was first identified as a low molecular weight component of axonemal Dynein in *Chlamydomonas* flagella (Pfister et al., 1982; Piperno and Luck, 1979). Subsequently, *Dynll1* was shown to be a member of the Dynein light chain family of proteins (King and Patel-King, 1995). *Dynll1* has since been extracted from vertebrates and shown (by co-immunoprecipitation and colocalisation) to be present in Cytoplasmic Dynein but not Kinesin II (King et al., 1996). This supports the prediction that *gpg6* mutants have defects in retrograde IFT, which is powered by Cytoplasmic Dynein 2, and not Kinesin II-dependent anterograde IFT. Consistent with this is the observation that the *Drosophila* homolog of *Dynll1* binds to Cytoplasmic Dynein components Ic74 and Dynlt1 in *Drosophila* (Makokha et al., 2002). In addition to being part of Cytoplasmic Dynein, LC8 is also present in the radial spoke complex and the outer and inner dynein arms of *Chlamydomonas* flagella (Yang et al., 2001), suggesting a role for LC8 in controlling the motility of flagella (Goodenough and Heuser, 1985; Warner and Satir, 1974; Witman et al., 1978).

A close paralogue of *Dynll1*, called *Dynll2*, has been identified (Naisbitt et al., 2000; Puthalakath et al., 2001). *Dynll1* and *Dynll2* are highly homologous with only a six amino

acid difference (Naisbitt et al., 2000). Expression of *Dynll2* is unaffected in *gpg6* mutants (Dr Paraskevi Goggliodou, personal communication), suggesting that Atmin specifically activates the *Dynll1* promoter region and that the regulatory elements of these close paralogues have diverged. Previously, *Dynll2* was found to localise to myosin V and to the actin cytoskeleton (Puthalakath et al., 2001), whereas *Dynll1* is associated with microtubules (Puthalakath et al., 1999); this argues against *Dynll1* and *Dynll2* functional redundancy in ciliogenesis since cilia are microtubule based structures (Porter, 1955). However, another study identified both *Dynll1* and *Dynll2* as components of Cytoplasmic Dynein isolated from rat brain, raising doubts about isoform specific functions of *Dynll1* and *Dynll2* (Wilson et al., 2001).

A key prediction of the hypothesis (Figure 26) is that *Dynll1* is required for retrograde IFT and when its expression is disrupted in *gpg6* mutants short cilia are manifested. A study of a *Chlamydomonas* *LC8* insertional mutant (Pazour et al., 1998) sheds important light on the credibility of this hypothesis. *LC8* mutants have short, immobile flagella and IFT particles accumulate at the flagellar tip and along the length of mutant flagella. Importantly, *LC8* mutants have a specific lack of retrograde IFT (Pazour et al., 1998). This provides strong support for the hypothesis that Atmin-dependent regulation of *Dynll1* is required for retrograde IFT and ciliogenesis (Figure 26). *LC8* mutant flagella also lack most radial spokes and have reduced numbers of outer and inner dynein arms. It was hypothesised that the role of *LC8* in retrograde IFT is important in the assembly of these structures and this is disrupted in mutants (Pazour et al., 1998). Alternatively, *LC8* may have multiple roles in *Chlamydomonas* in addition to its role in IFT, being further required for the stability and motility of flagella (Yang et al., 2001; Yang et al., 2009).

Although it has been elegantly demonstrated that *LC8* is required for retrograde IFT in the flagella of *Chlamydomonas* (Pazour et al., 1998), it remains unclear whether *DynIII* is required for ciliogenesis in vertebrates. It is therefore crucial to test whether *Atmin*-dependent activation of *DynIII* is indeed required for ciliogenesis. Only by testing this central prediction of the hypothesis (Figure 26) can progress be made in understanding the role of *Atmin* and *DynIII* in the mouse embryo.

Chapter 6

Atmin is required for Ciliogenesis

6.1 Introduction

6.1.1 Testing the hypothesis that *Atmin* and *Dynll1* are involved in ciliogenesis

It was hypothesised in Chapter 5 that *Atmin*-dependent downregulation of *Dynll1* in *gpg6* mutants causes abnormally short cilia. This hypothesis can be tested by disrupting expression of *Atmin* and *Dynll1* and then assessing the effect on ciliogenesis. Two experimental approaches exist to disrupt gene expression at the level of mRNA; blocking translation of mRNA using morpholinos (Heasman, 2002) and inducing degradation of mRNA by RNA interference (RNAi) (Rana, 2007). A recent large scale study has demonstrated the viability of using RNAi in ciliated cells to investigate ciliogenic gene function (Kim et al., 2010).

6.2 Results

The preliminary knockdown experiment (Table 15) was conducted in Leeds under the supervision of Gabrielle Wheway. I carried out Knockdown experiments 1-3 (Figure 28-29 and Table 17-18) and Dr Paraskevi Goggolidou validated the extent of knockdown by Real Time PCR (Figure 27 and Table 16). Knockdown experiment 4 was carried out by Gabrielle Wheway (Figure 31 and Table 20-21) and knockdown was confirmed by the Harwell Proteomics Facility (Figure 30 and Table 19).

6.2.1 48 hour knockdown of *Atmin* and *Dynll1*

Atmin and *Dynll1* were transiently knocked down for 48 hours in the ciliated kidney cell line IMCD3 and the percentage of cells containing a cilium and the length of cilia were measured to assess the effect of knock down. Due to the technical limitations of the microscope used in the subsequent data collection, cilia length was measured in the following way. The nuclei of IMCD3 cells are typically 10µm in length (Figure 31). Therefore, one nucleus was chosen at random and used as a standard reference of 10µm. This reference was used to build a 0-4µm ruler to measure cilia. Measurements across all images were taken using the same ruler. Around 100 randomly chosen cilia were measured in each knockdown and then grouped into lengths of <1µm, 1-2µm, 2-3µm, 3-4µm and >4µm.

6.2.1.1 Validation of knockdown

Predesigned siRNAs (Ambion) were used and validation was carried out by Real time PCR and Western Blotting. All knockdown experiments were conducted with a negative control that used a scrambled, non-homologous siRNA.

Experiments were carried out using 3 individual siRNAs for each gene that was knocked down. Preliminary experiments (under the supervision of Gabrielle Wheway, LIMM, Leeds) were carried out using each siRNA separately. Phenotypic analysis showed that each individual siRNA caused a reduction in cilia length compared to scrambled control. This was consistent using each siRNA, demonstrating that any off target effects are irrelevant to the ciliogenic phenotype (Table 15). For instance, in scrambled controlled cells only 1 cilium was less than 1µm, whereas in *Atmin* A, B and C knock down cells this increased to 24, 28 and 10 respectively; in *Dynl1l* A, B, C knock down cells this increased to 15, 7 and 36 respectively. Unfortunately, due to technical difficulties neither RNA nor protein was collected to measure levels of *Atmin* or *Dynl1l* following knockdown in this experiment. Despite this, the phenotypic analysis suggests that each siRNA was causing a similar phenotype and therefore the effect of knockdown was unlikely due to off target effects. Furthermore, it suggests that despite the limitations of the method chosen to assess cilia length, it nevertheless detects a change in the length of cilia and therefore a shift in cilia population in knockdown cells.

Kim and colleagues (2010) demonstrated that pooling siRNAs increased the effectiveness of knockdown. Therefore, individual siRNAs were pooled and used in 3 separate experiments

(1-3) to test whether knockdown resulted in defective ciliogenesis. *Atmin* and *Dynll1* mRNA levels following 48 hour knockdown were tested by Dr Paraskevi Goggolidou (MRC Harwell) using Real Time PCR (Figure 27 and Table 16). Both *Atmin* and *Dynll1* were knocked down in Experiment 2 but no statistically significant knock down was evident in Experiments 1 or 3 (Figure 27 and Table 16).

	<1μm	1-2μm	2-3μm	3-4μm	>4μm	p value
<i>Scrambled</i>	1	31	33	18	17	-
<i>Atmin A</i>	24	35	24	12	6	< 0.005
<i>Atmin B</i>	28	51	14	8	0	< 0.005
<i>Atmin C</i>	10	26	34	17	13	< 0.005
<i>Dynl1l A</i>	15	39	27	11	8	< 0.005
<i>Dynl1l B</i>	7	55	27	8	3	< 0.005
<i>Dynl1l C</i>	36	37	13	6	8	< 0.005

Table 15. Preliminary knockdown data using individual *Atmin* and *Dynl1l* siRNAs

All individual siRNAs demonstrated a statistically significant reduction in the length of cilia in knockdown cells compared to scrambled, suggesting that the effects seen with pooled siRNAs are not due to off target effects. p values are calculated across all 5 columns.

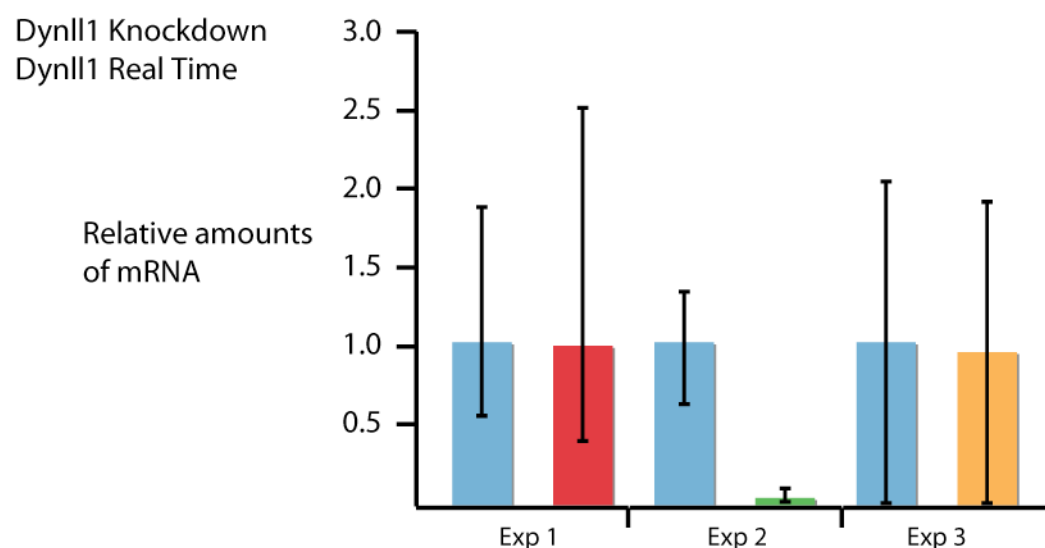
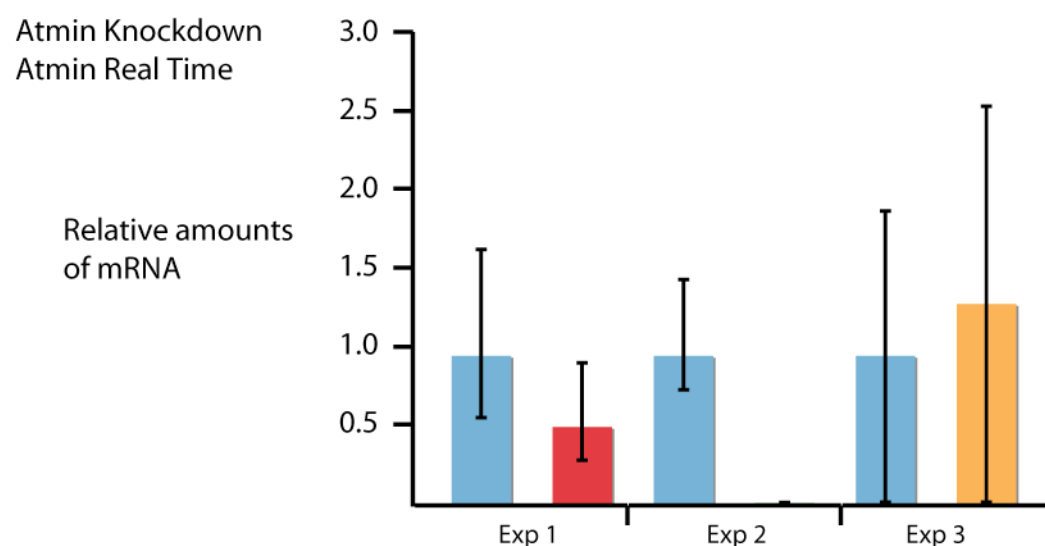


Figure 27. *Atmin* and *Dynl1* is downregulated in knockdown Experiment 2 but not Experiments 1 or 3

Atmin and *Dynl1* were knocked down in 3 different experiments (with scrambled siRNA as a negative control). cDNA from IMCD3 cells knocked down for *Atmin* and *Dynl1* was used in *Atmin* and *Dynl1* Real Time PCR analysis (all reactions were normalised to levels of β -actin). Both *Atmin* (a) and *Dynl1* (b) knockdown resulted in a statistically significant reduction in transcript levels only in Experiment 2.

	Knockdown	<i>Atmin</i> Real Time	<i>Dynl1l</i> Real Time
<i>Experiment 1</i>	Scrambled Av Δ CT	0.519	-0.135
	<i>Atmin</i> Av Δ CT	1.438	-
	<i>Dynl1l</i> Av Δ CT	-	-0.103
	$\Delta\Delta$ CT	0.919	0.032
	RQ	0.52	0.979
	Scrambled RQ Max	1.72	1.828
	Scrambled RQ Min	0.581	0.547
	<i>Atmin</i> RQ Max	0.952	-
	<i>Atmin</i> RQ Min	0.294	-
	<i>Dynl1l</i> RQ Max	-	2.438
	<i>Dynl1l</i> RQ Min	-	0.393
<i>Experiment 2</i>	Scrambled Av Δ CT	0.418	0.016
	<i>Atmin</i> Av Δ CT	7.527	-
	<i>Dynl1l</i> Av Δ CT	-	4.385
	$\Delta\Delta$ CT	7.109	4.519
	RQ	0.00724	0.044
	Scrambled RQ Max	1.514	1.31
	Scrambled RQ Min	0.76	0.619
	<i>Atmin</i> RQ Max	0.01	-
	<i>Atmin</i> RQ Min	0.006	-
	<i>Dynl1l</i> RQ Max	-	0.103
	<i>Dynl1l</i> RQ Min	-	0.019
<i>Experiment 3</i>	Scrambled Av Δ CT	7.15	5.852
	<i>Atmin</i> Av Δ CT	6.709	-
	<i>Dynl1l</i> Av Δ CT	-	5.94
	$\Delta\Delta$ CT	-0.441	0.088
	RQ	1.35	0.94
	Scrambled RQ Max	0.022	0.016
	Scrambled RQ Min	0.011	0.012
	<i>Atmin</i> RQ Max	0.016	-
	<i>Atmin</i> RQ Min	0.012	-
	<i>Dynl1l</i> RQ Max	-	0.02
	<i>Dynl1l</i> RQ Min	-	0.011

Table 16. Raw Real Time PCR presented in Figure 23

6.2.1.2 Effect of 48 hour knockdown on ciliogenesis

Based on the Real Time data demonstrating successful knockdown only in Experiment 2, it was hypothesised that if *Atmin* and *Dynl1l* were involved in ciliogenesis then Experiment 2 would show a cilia-related phenotype and Experiment 1 and 3 would be comparable to scrambled negative control. Similar to the preliminary experiments, following knockdown and staining of cells, all slides were viewed under a fluorescent microscope and images of cells from random positions around the slide were collected. Identical magnification was used for all images. The identity of the images was blinded and approximately 100 cells were analysed to determine the percentage of ciliated cells. Also, 100 cilia were measured to determine cilia length.

In all 3 experiments *Ift88*, a key IFT component, was used as a positive control (Dawe et al., 2007). Following *Ift88* knockdown there was a statistically significant shift in the cilia population towards shorter cilia compared to the scrambled control in all experiments (Figure 28 and Table 17). *Ift88* knockdown also resulted in a statistically significant reduction in the percentage of ciliated cells in experiment 1 and experiment 3 but not in experiment 2 (Figure 29 and Table 18). The pool of *Ift88* siRNAs that was used are a proven reagent (Dawe et al., 2007). Nevertheless, due to technical difficulties, validation of *Ift88* knockdown by Real Time PCR or Western blot was not possible. This raises doubts about whether the cilia phenotype that was detected can be attributable to a reduction in the level of *Ift88*.

Atmin and *Dynl1l* knockdown cells were analysed in a similar way to *Ift88* knockdown cells. *Atmin* knockdown resulted in a statistically significant shift towards shorter cilia in experiment 1 and experiment 3, but this shift was not detected in experiment 2 (Figure 28 and Table 17). There was a statistically significant reduction in the percentage of ciliated cells only in experiment 3 (Figure 29 and Table 18). *Dynl1l* knockdown did not result in a statistically significant reduction in cilia length in any experiment (Figure 29 and Table 17). There was however, a statistically significant reduction in the percentage of ciliated cells in experiment 1 and experiment 3 but not in experiment 2 (Figure 29 and Table 18).

It is readily apparent that there is an inconsistency with these data. Experiment 2 was the only experiment that showed, by Real Time PCR, a significant knockdown of both *Atmin* and *Dynl1l* mRNA levels (Figure 27 and Table 16). However, when cells from Experiment 2 were analysed no statistically significant changes in cilia length or the percentage of ciliated cells were detected (Figure 28-29, Table 17-18). Strikingly, the opposite was seen in Experiments 1 and 3: knockdown was not detected by Real Time PCR in these experiments (Figure 27 and Table 16), but a statistically significant reduction in the percentage of ciliated cells and cilia length was detected (Figure 28-29, Table 17-18).

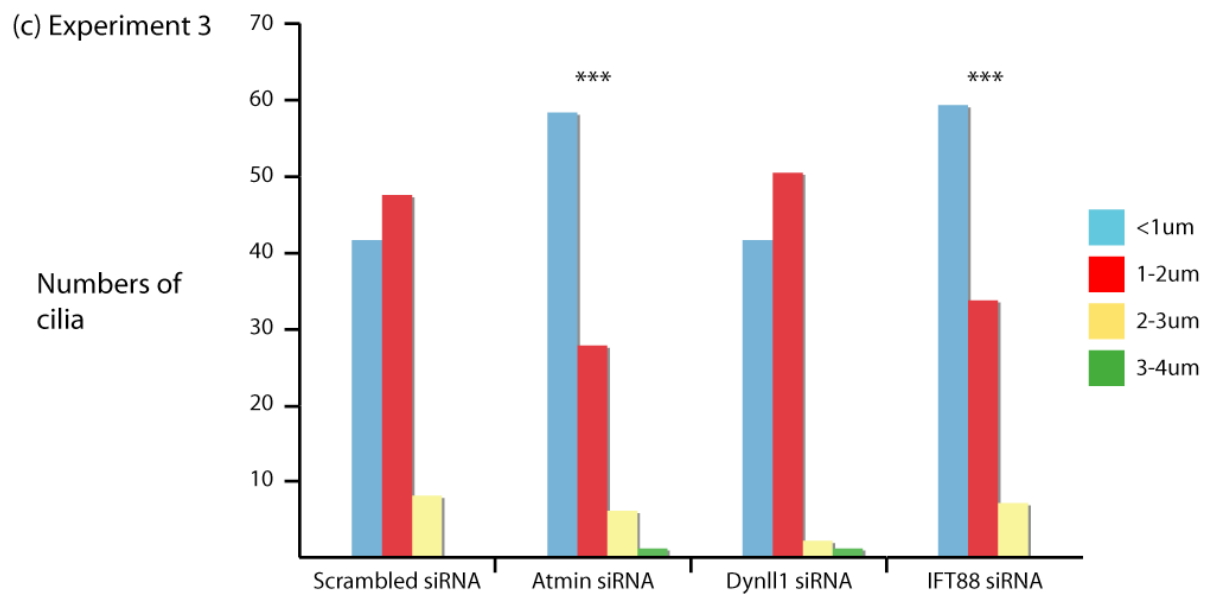
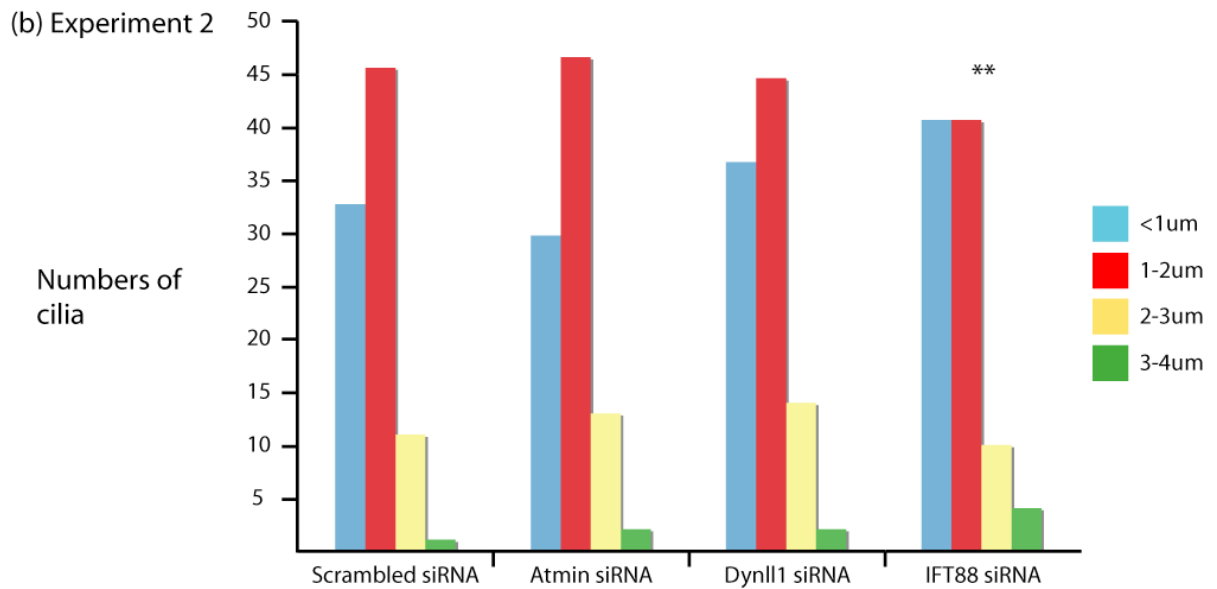
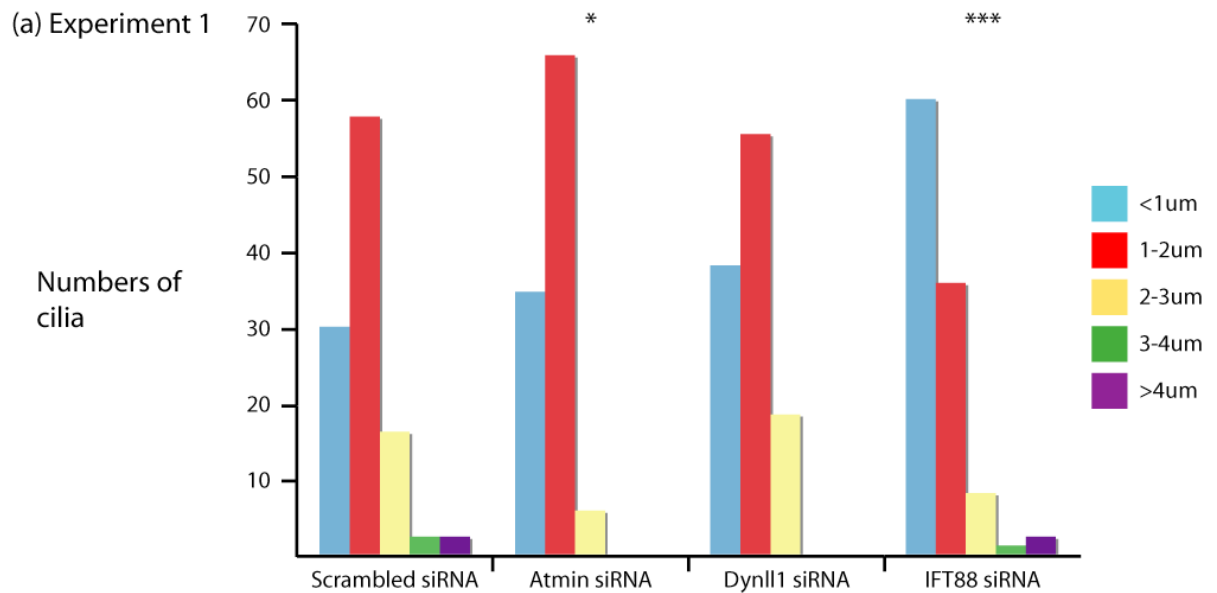


Figure 28. Cilia length in Knockdown Experiments 1-3

Atmin, *Dynl1l* and *Ift88* were knocked down in Experiments 1-3 (with scrambled siRNA as a negative control). (a-c) Cilia were then measured and grouped into different lengths (<1µm, 1-2µm, 2-3µm, 3-4µm, >4µm).

(a) Experiment 1. *Atmin* and *Ift88* knockdowns demonstrated a statistically significant shift towards shorter cilia.

(b) Experiment 2. Only *Ift88* knockdown demonstrated a statistically significant shift towards shorter cilia.

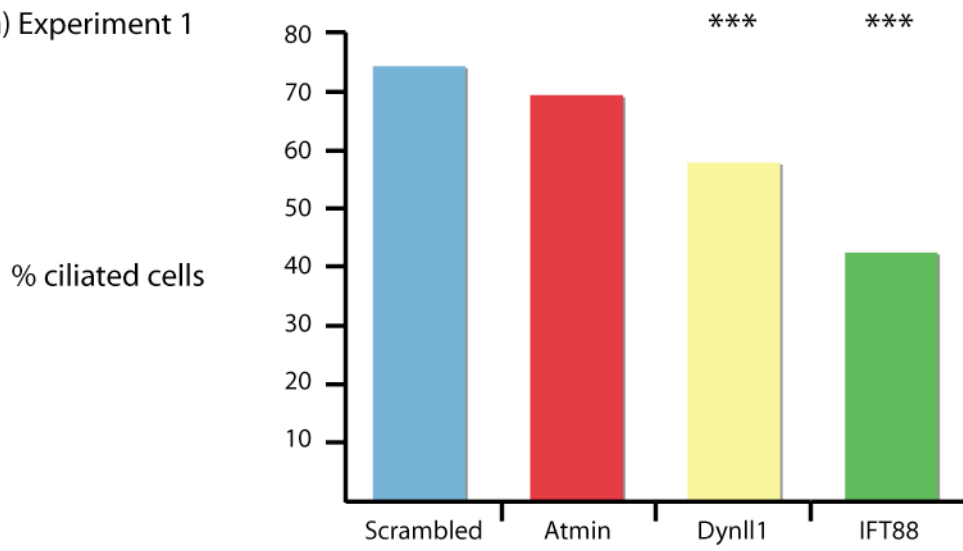
(c) Experiment 3. *Atmin* and *Ift88* knockdowns demonstrated a statistically significant shift towards shorter cilia.

* = $p < 0.05$

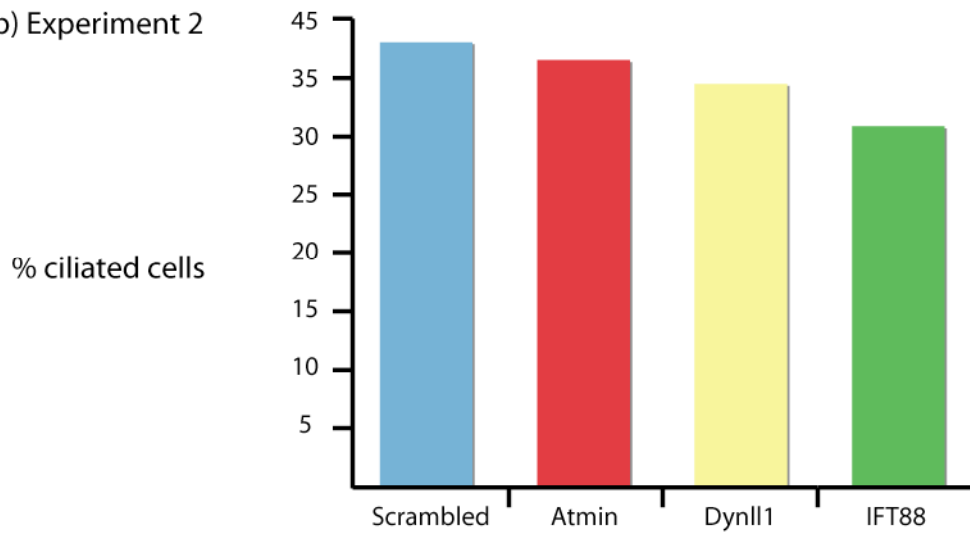
** = $p < 0.005$

*** = $p < 0.0005$

(a) Experiment 1



(b) Experiment 2



(c) Experiment 3

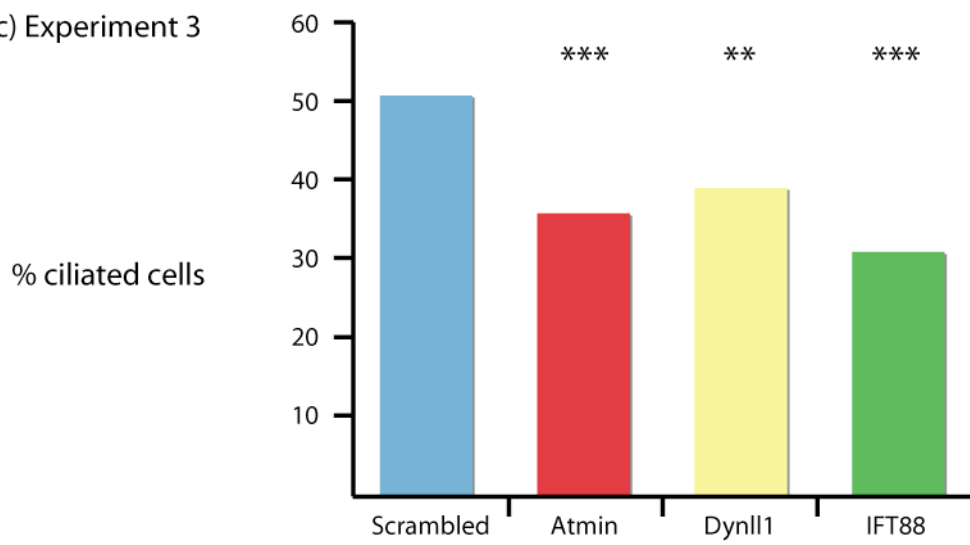


Figure 29. Percentage ciliated cells in Knockdown Experiments 1-3

Atmin, *Dynll1* and *Ift88* were knocked down in Experiments 1-3 (with scrambled siRNA as a negative control). Percentage of ciliated cells was measured in knockdown cells.

(a) Experiment 1. *Dynll1* and *Ift88* knockdowns demonstrated a statistically significant reduction in ciliated cells.

(b) Experiment 2. No knockdowns demonstrated a statistically significant reduction in ciliated cells.

(c) Experiment 3. *Atmin*, *Dynll1* and *Ift88* knockdowns demonstrated a statistically significant reduction in ciliated cells.

* = $p < 0.05$

** = $p < 0.005$

*** = $p < 0.0005$

siRNA	<1µm	1-2µm	2-3µm	3-4µm	>4µm	p value
<i>Scrambled</i>	26	50	14	2	2	-
<i>Atmin</i>	30	57	5	0	0	0.02
<i>Dynll1</i>	33	48	16	0	0	0.18
<i>IFT88</i>	52	31	7	1	2	0.0000016
<i>Scrambled</i>	33	46	11	1	0	-
<i>Atmin</i>	30	47	12	2	0	0.64
<i>Dynll1</i>	37	45	14	2	0	0.5
<i>IFT88</i>	41	41	10	4	0	0.009
<i>Scrambled</i>	42	48	8	0	0	-
<i>Atmin</i>	59	28	6	1	0	0.00039
<i>Dynll1</i>	42	51	2	1	0	0.096
<i>IFT88</i>	60	34	7	0	0	0.002

Table 17. Cilia length in knockdown Experiments 1-3. p values are calculated across all 5 columns.

siRNA	% ciliated cells	p value
<i>Scrambled</i>	73.7	-
<i>Atmin</i>	68.8	0.14
<i>Dynll1</i>	57.4	0.00006
<i>IFT88</i>	42.2	0.00000000000003
<i>Scrambled</i>	37.7	-
<i>Atmin</i>	36.2	0.3
<i>Dynll1</i>	34.2	0.1
<i>IFT88</i>	30.6	0.09
<i>Scrambled</i>	50.7	-
<i>Atmin</i>	35.8	0.00005
<i>Dynll1</i>	39.0	0.007
<i>IFT88</i>	30.9	0.00001

Table 18. Percentage of ciliated cells in knockdown Experiments 1-3

There is an obvious lack of correlation between the phenotype and the extent of knockdown. Preliminary data suggests that each individual siRNA causes a similar cilia phenotype, arguing that the effect of each siRNA is not due to off target effects. A possible reason for the lack of correlation could be that a longer knockdown period is needed to reduce the levels of transcript and protein to a level where ciliogenesis is consistently affected. Also, cells harvested for mRNA were grown in separate wells to those cells processed for phenotypic analysis. Although the same reagents were used, there may have been significant variation between wells.

There may be a more general problem with the methodology that was used to measure the knockdown phenotype. Due to the technical limitations of the microscope that was used a novel methodology was developed using a “ruler” and grouping cilia in ranges of length. It can be argued that this introduces greater inconsistency and that cilia defects might be missed. This method was tested in *Ift88* knockdown cells and a statistically significant shift in the cilia population towards shorter cilia was seen in all 3 experiments. However, in the absence of validation of *Ift88* knock down a strict correlation cannot be made between the extent of *Ift88* knockdown and the resulting phenotype.

To overcome these difficulties, a longer period of knockdown and a better method of data collection and analysis is needed to accurately describe the effect of *Atmin* and *Dynl1l* knockdown on ciliogenesis.

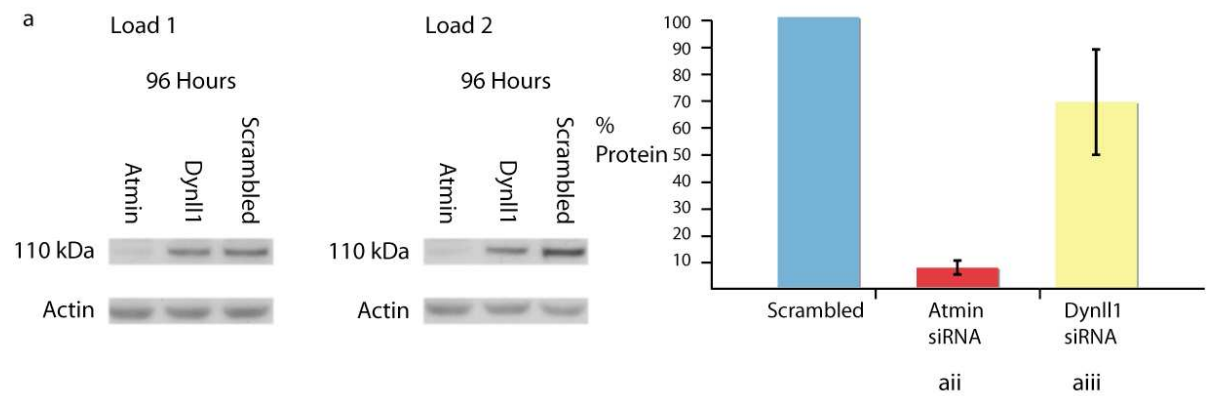
6.2.2 96 hour knockdown of *Atmin* and *Dynll1*

To overcome the technical limitations encountered in Experiment 1-3, Gabrielle Wheway (Colin Johnson's lab, LIMM, Leeds) carried out *Atmin* and *Dynll1* knockdown for 96 hours and collected and analysed data using a confocal microscope (Experiment 4). The use of confocal Z-stacks enabled accurate measurement of cilia length.

6.2.2.1 Validation of knockdown

Protein levels were analysed by Western Blotting to test whether knockdown had occurred in the 96 hour experiment (Figure 30 and Table 19). This work was carried out by the Proteomics facility (Helen Hilton and Rosario Romero) at MRC Harwell. Western blots were performed with duplicate protein samples (load 1 and load 2) using an *Atmin* antibody and a common *Dynll1*/*Dynll2* antibody. The very close homology between *Dynll1* and *Dynll2* excludes the possibility of a *Dynll1* specific antibody; therefore the following measurements of *Dynll* levels are likely an overestimate of the levels of *Dynll1*. In cells knocked down for *Atmin*, *Atmin* levels were severely reduced (to 7%; $p=0.009$, T-test) (Figure 30aii). In these cells *Dynll* levels were also downregulated (to 68.5%), however this was not statistically significant ($p=0.14$, T-test) (Figure 30bii). In cells knocked down for *Dynll1*, *Dynll* levels were reduced to 43% but this was not statistically significant ($p=0.074$, T-test) (Figure 30biii). These cells also demonstrated a reduction in *Atmin* levels (to 52%) but this failed to be statistically significant ($p=0.28$, T-test) (Figure 30aiii). Western blot analysis therefore demonstrated that *Atmin* levels were successfully knocked down in the 96 hour experiment.

Atmin Antibody



Dynll1 Antibody

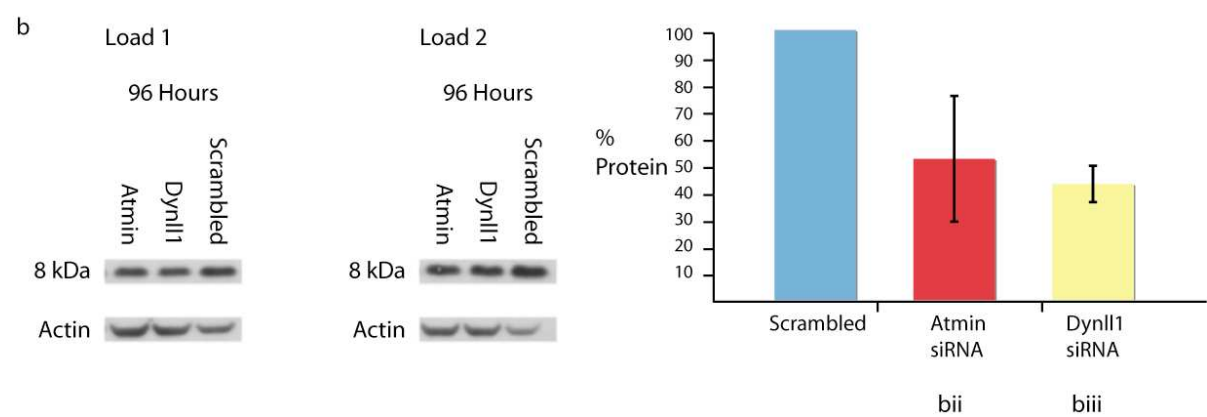


Figure 30. Atmin is knocked down in Experiment 4

Atmin and *Dynll1* were knocked down in Experiment 4 (with scrambled siRNA as a negative control). Protein from IMCD3 cells knocked down for *Atmin* and *Dynll1* was size fractionated, Western blotted and probed with Atmin and Dynll antibodies. (a) Levels of Atmin were measured in both *Atmin* and *Dynll1* knockdowns. (b) Similarly, Dynll levels were measured in both *Dynll1* and *Atmin* knockdowns. All measurements were normalised to β -actin.

(aii) Atmin protein levels were downregulated to 7% in cells knocked down for *Atmin* ($p=0.018$, T-test). (aiii) Atmin proteins levels were downregulated to 52% in cells knocked down for *Dynll1* ($p=0.28$, T-test).

(bii) Dynll protein levels were downregulated to 68.5% in cells knocked down for *Atmin* ($p=0.35$, T-test). (biii) Dynll protein levels were downregulated to 43% in cells knocked down for *Dynll1* ($p=0.074$, T-test).

<i>Load</i>	<i>siRNA</i>	<i>Antibody</i>	<i>Intensity of band</i>	<i>Actin band</i>	<i>Normalised Against Actin</i>	<i>% of Scrambled</i>
Load 1	Scrambled	Atmin	8139.9	16069.87	0.506	100%
	<i>Atmin</i>	Atmin	854.82	17134.54	0.049	9.68%
Load 2	Scrambled	Atmin	12526.29	10135.43	1.235	100%
	<i>Atmin</i>	Atmin	815.35	14042.82	0.058	4.7%
Load 1	Scrambled	Dynll	2505.57	2549	0.983	100%
	<i>Atmin</i>	Dynll	3174.69	4277.18	0.742	75.48%
Load 2	Scrambled	Dynll	3836.94	1150.99	3.33	100%
	<i>Atmin</i>	Dynll	1568.57	2400.4	0.653	19.6%
Load 1	Scrambled	Dynll	2505.57	2549	0.983	100%
	<i>Dynlll</i>	Dynll	1938.57	3962.35	0.489	49.74%
Load 2	Scrambled	Dynll	3836.94	1150.99	3.33	100%
	<i>Dynlll</i>	Dynll	2630.99	2172.7	1.21	36.33%
Load 1	Scrambled	Atmin	8139.92	16069.87	0.506	100%
	<i>Dynlll</i>	Atmin	6859.21	15398.4	0.445	87.94
Load 2	Scrambled	Atmin	12526.29	10135.43	1.235	100%
	<i>Dynlll</i>	Atmin	7280.38	12013.97	0.605	48.98%

Table 19. Raw Western Blot data presented in Figure 27

6.2.2.2 Effect of 96 hour knockdown on ciliogenesis

When the cilia length (100 cilia) and the percentage of ciliated cells (100 cells) were analysed in cells following knock down of *Atmin* and *DynIII*, a statistically significant affect was seen; there was an approximately 20% reduction in the number of ciliated cells and approximately 50% reduction in cilia length following both *Atmin* and *DynIII* knockdown (Figure 31, Table 20 and Table 21) (Gabrielle Wheway). This experiment was repeated and the same result was obtained (data not shown; Gabrielle Wheway).

Despite the consistent cilia phenotype in cells knocked down for *DynIII*, levels of Dynll1 were not reduced to a statistically significant level ($p=0.074$, T-test). Possible reasons for this include the antibody used reacts to both Dynll1 and Dynll2 and may therefore obscure the levels of Dynll1. Recent *DynIII* knockdown experiments carried out by Kim and colleagues (2011) also detected short cilia and a reduction in the number of ciliated cells in knocked down cells, supporting the hypothesis that the cilia phenotype detected here is real and due to reduced levels of Dynll1. Further work is needed to confirm *DynIII* knock down.

The 96-hour knockdown data clearly demonstrates that *Atmin*, the gene mutated in *gpg6*, is involved in ciliogenesis. These knockdown experiments, in combination with data published by Kim and colleagues (2011), provides support for the hypothesis that a failure of *Atmin* to activate transcription of *DynIII* is responsible for at least elements of the *gpg6* phenotype.

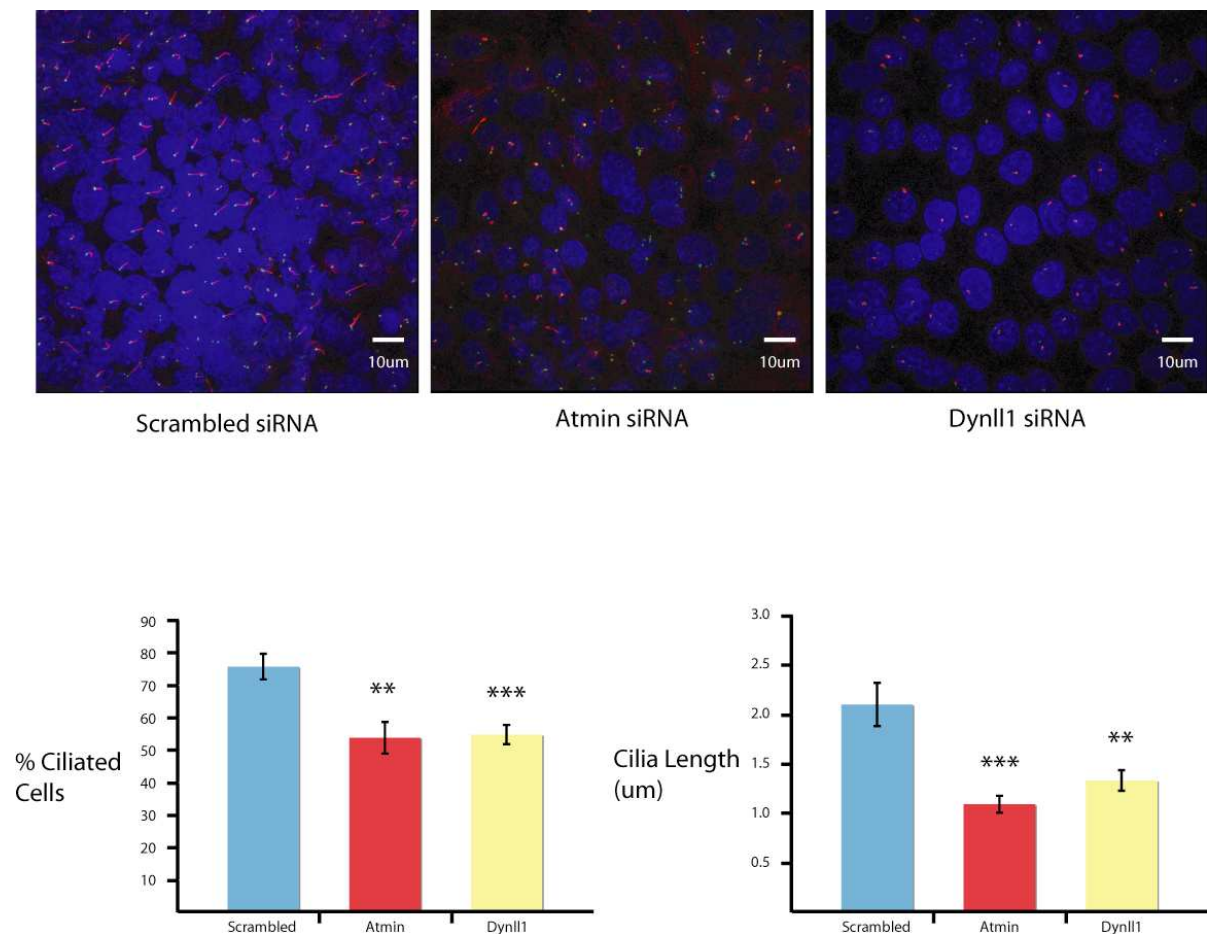


Figure 31. Knockdown Experiment 4 - mean cilia length and mean percentage ciliated cells were reduced following knock down of both *Atmin* and *Dynll1*

Atmin and *Dynll1* were knocked down in Experiment 4 (with scrambled siRNA as a negative control). (a) Cells were processed for immunofluorescence using DAPI (blue), acetylated tubulin (red) and γ -tubulin (green). (b) The mean percentage of ciliated cells was then measured in knockdown cells. Cells knocked down for *Atmin* and *Dynll1* demonstrated a statistically significant reduction in ciliated cells (55.7% and 56.8%, respectively, compared to scrambled control (78.8%)). (c) The mean cilia length was also measured. Cells knocked down for *Atmin* and *Dynll1* demonstrated a statistically significant reduction in cilia length (1.11 μ m and 1.36 μ m, respectively, compared to scrambled control (2.16 μ m)). ** = $p < 0.005$, *** = $p < 0.0005$. Experiment carried out by Gabrielle Wheway.

siRNA	% ciliated cells	p value
<i>Scrambled</i>	78.8	-
<i>Atmin</i>	55.7	0.005
<i>Dynl1l</i>	56.8	0.0005

Table 20. Mean percentage of ciliated cells in knockdown experiment 4

siRNA	Mean cilia length (μm)	p value
<i>Scrambled</i>	2.16	-
<i>Atmin</i>	1.11	0.00005
<i>Dynl1l</i>	1.36	0.0018

Table 21. Mean cilia length in knockdown experiment 4

6.3 Discussion

In this chapter RNAi experiments have been carried out to assess whether *Atmin* and *Dynl1l* are involved in ciliogenesis. These genes were knocked down in a ciliated kidney cell line, using three siRNAs against each gene. Knockdown was carried out for 48 and 96 hours. Preliminary data at 48 hours suggested that the siRNAs for each locus targeted the correct gene since each siRNA caused a consistent reduction in cilia length. Further experiments at 48 hours using pooled siRNAs encountered problems with variability between the extent of knockdown and the cilia phenotype. Possible reasons for this variability include: knocking down *Atmin* and *Dynl1l* for 48 hours is insufficient to reduce levels of expression to consistently effect ciliogenesis; problems with the sensitivity of the method of data collection used. Experiments carried out by Gabrielle Wheway addressed these problems. Knocking down *Atmin* and *Dynl1l* for 96 hours demonstrated a statistically significant reduction in cilia length and in the number of ciliated cells. However, only cells knocked down for *Atmin* demonstrated a statistically significant reduction in target protein levels. Therefore, these knockdown experiments demonstrate that *Atmin* is involved in ciliogenesis.

6.3.1 Comparing the knockdown experiments

Two different experimental methodologies were used to measure the effect of knocking down *Atmin* and *Dynl1l* expression on ciliogenesis. The difference between these approaches was in the length of the knockdown and also the methods of data collection and data analysis. Experiments 1-3 knocked down *Atmin* and *Dynl1l* for 48 hours and the resulting phenotype was measured by counting cells which had a cilium and also grouping cilia into

different classes of length. Significant problems with consistency were encountered during these experiments. Knockdown of mRNA levels were seen (using Real Time PCR) for both *Atmin* and *Dynll1* only in Experiment 2. However, when cells and cilia were measured in Experiment 2 no defect was detected. In contrast, knockdown was not confirmed by Real Time PCR in Experiment 1 and 3, but a cilia defect was detected. It is crucial in any knockdown experiment to correlate the extent of knockdown with any cellular phenotype. Possible reasons why this correlation is missing in Experiments 1-3 could be the period of knockdown is insufficient to consistently impact protein levels (e.g. a protein with a long half life still functions despite reduced mRNA levels). It could also be argued that the method of measurement may not accurately detect the consequence of knockdown on cilia. In addition, the RNA used to validate knock down was harvested from cells in a different well to those cells that were processed for immunofluorescence and phenotypic analysis.

There was a clear need to develop these knockdown experiments further. Subsequent experiments carried out by Gabrielle Wheway addressed the problems encountered at 48 hours by increasing the length of knockdown to 96 hours and changing the method of data collection (Experiment 4). The use of a confocal microscope made measurements of cilia more accurate (confocal Z-stacking allowed true measurement of cilia length). Using this improved methodology it was clear that when *Atmin* was knocked down cilia length and the percentage of ciliated cells were significantly reduced. Importantly, these defects in ciliogenesis correlated with a clear reduction in *Atmin* levels as assessed by Western Blot analysis. However, a statistically significant reduction in *Dynll1* levels, despite a clear cilia phenotype, was not detected in *Dynll1* knocked down cells. The lack of a specific *Dynll1* antibody may be responsible for this; levels of *Dynll2*, which are also detected by the antibody, may obscure the levels of *Dynll1*.

6.3.2 The difficulty of understanding population versus individual defects in knockdown experiments

The presence of two defects in *Atnin* and *Dynl1l* knockdown cells (reduced cilia length and absence of cilia) raises a difficulty of interpretation that applies to all knockdown experiments. In siRNA experiments, knockdown is validated using mRNA or protein harvested from a *population* of knockdown cells whereas *individual* cells and *individual* cilia are measured. The extent of knockdown in an individual cell may not be the same as the average knockdown, identified by Real Time PCR or Western Blot, in a population of cells. This causes difficulty when the extent of knockdown results in different phenotypes. For example, it is reasonable to propose that absence of cilia (a severe defect) correlates with greater knockdown in a particular cell whereas short cilia (a less severe defect) correlates with a less severe knockdown in a different cell. It is technically very demanding to differentiate between these possibilities and quantitatively measure a cilia phenotype in a particular cell and also accurately measure the level of knockdown in that cell. Normally, one experimental approach (immunofluorescence) excludes the possibility of the other (Real Time PCR or Western Blotting). This difficulty reduces the interpretive power of knockdown experiments in that cellular phenotypes can be said to be affected by knockdown but not by how much in a particular cell. A qualitative approach to overcome this difficulty could be to stain the cells that are measured for cilia phenotypes with antibodies against the gene that is knocked down. A more demanding, quantitative approach would be to use Laser Capture Technology to isolate individual cells (Emmert-Buck et al., 1996) following cilia measurement. RNA from individual cells could then be extracted and used to assess the extent of knockdown by Real Time PCR. Alternatively, the development of cell lines stably

expressing an inducible siRNA would reduce the likelihood of variation in the extent of knockdown.

Despite these difficulties, siRNA mediated knockdown has been shown to be a powerful technique to test whether genes are involved in particular cellular processes and has successfully been used to systematically study ciliogenesis (Kim et al., 2010). The results described here clearly demonstrate a cilia phenotype following *Atmin* and *Dynl1l* knockdown. Nevertheless, these data need to be developed further. For example, the knockdown experiments did not determine *how* ciliogenesis is affected. A role for *Dynl1l* in retrograde IFT seems most likely, since it has been shown that the *Chlamydomonas* homolog of *Dynl1l* is required for retrograde IFT (Pazour et al., 1998), but this needs to be tested directly in vertebrate cells. Live cell imaging of IFT (Tran et al., 2008) using knockdown cells will determine whether IFT is affected. It is also crucial to analyse the function of *Dynl1l in vivo* by developing a *Dynl1l* null mouse. This will allow extensive analysis of *Dynl1l* in the context of a whole organism. It can be argued from analysis of *gpg6* mutants (Chapter 3), the identification of *Dynl1l* as a putative target of *Atmin* (Chapter 4 and 5) and the role of both *Atmin* and *Dynl1l* in ciliogenesis (this Chapter), that *Dynl1l* mutants will resemble *gpg6* mutants and demonstrate cilia-based defects in L-R development and hh signalling.

6.3.3 Knockdown data supports the proposed hypothesis

The knockdown experiments that have been described in this chapter directly tested the hypothesis that *Atmin* and *Dynl1l* are required for ciliogenesis (Figure 26). The presence of

consistent cilia defects in 96 hour knockdown cells confirms that downregulation of *Atmin* results in impaired ciliogenesis. Recently, Kim and colleagues (2011) demonstrated that knocking down *Dynl1l* resulted in short cilia and a reduction in the percentage of ciliated cells, similar phenotypes to those described here. Further work is needed to confirm *Dynl1l* knock down in Experiment 4. Despite this, the data generated during this chapter, in combination with the study of Kim and colleagues (2011), does support a link between the cilia defects seen in *gpg6* mutants and the proposed *Atmin*-mediated activation of *Dynl1l*. It remains to be seen whether *Atmin* activates expression of other cilia genes and if these are important in the *gpg6* phenotype. However, the similarity between the cilia phenotypes in *Atmin* and *Dynl1l* knockdown cells argues, at least in IMCD3 cells, that activation of *Dynl1l* expression is a major effector downstream of *Atmin* in ciliogenesis. The hypothesis (Figure 26) is supported by these knockdown experiments.

Chapter 7

Discussion

7.1 Summary

7.1.1 Chapter 3

The aim of this thesis was to characterise the embryonic lethal ENU mutant *gpg6*. As detailed in Chapter 3, gross phenotypic analysis of 13.5dpc *gpg6* mutant embryos suggested that the complex *gpg6* phenotype could be separated into defects associated with L-R patterning (pulmonary isomerism and outflow tract defects) and hh signalling (exencephaly, pulmonary hypoplasia, eye and craniofacial defects). Molecular phenotyping supported the hypothesis that *gpg6* mutants have defects in L-R development and hh signalling. Analysis of *Nodal* expression in *gpg6* mutants revealed an anterior truncation in the left lateral plate expression domain of *Nodal*, arguably due to inefficient nodal flow and inefficient asymmetric calcium signalling at the node. This suggested a novel requirement for the *gpg6* mutated gene in the correct activation of *Nodal* expression in the left lateral plate. Hh signalling in *gpg6* mutants was investigated using genetic interaction studies with *Smo* mutants; *+/gpg6;Smo/Smo* and *gpg6/gpg6;Smo/Smo* embryos demonstrated a mild rescue of the *Smo* phenotype. Furthermore, *Ptch1* expression was increased in *gpg6* mutants and there was a mild increase in the levels of full length Gli3, suggesting that *gpg6* is a mild negative regulator of the hh pathway.

The defects seen in *gpg6* mutants are milder than defects associated with loss of components of both pathways. For example, *gpg6* mutants undergo embryonic turning and demonstrate normal cardiac situs, whereas L-R mutants that abolish leftward nodal flow (*Kif3a* and *Kif3b*) or the perception of flow (*Pkd2*) demonstrate abnormal turning and randomised heart

looping (Marszalek et al., 1999; Nonaka et al., 1998; Pennekamp et al., 2002; Supp et al., 1999; Supp et al., 1997; Takeda et al., 1999). This argues that a form of nodal flow still occurs in *gpg6* mutants and that it is perceived. The L-R mutant *inv* more closely resembles *gpg6* mutants in that they undergo embryonic turning; nevertheless, *inv* mutants demonstrate *situs inversus*, a more severe L-R defect than seen in *gpg6* mutants (Okada et al., 1999). Okada and colleagues (1999) showed that nodal flow in *inv* mutants cannot develop beyond the uncoordinated stage. Given the milder nature of the *gpg6* L-R phenotype, this argues that *gpg6* mutants generate smooth but not laminar leftward nodal flow, a stage beyond the uncoordinated flow detected in *inv* mutants.

gpg6 mutants demonstrate mild hh signalling associated defects. Indeed, *gpg6* mutants die around 13.5dpc, whereas *Shh/Ihh* double mutants and *Ptch1* and *Smo* single mutants demonstrate severe early embryonic defects and die around 9.5dpc (Goodrich et al., 1997; Zhang et al., 2001). Mutations affecting positive regulators of the hh pathway (e.g. *Shh* and *Smo*) result in dorsalisation of the neural tube (Chiang et al., 1996; Zhang et al., 2001) while loss of negative regulators of the pathway (e.g. *Ptch1*, *Rab23*, *Pka* and *Sufu*) results in ventralisation of the neural tube (Cooper et al., 2005; Eggenschwiler et al., 2001; Goodrich et al., 1997; Huang et al., 2002; Svard et al., 2006). The absence of neural tube D-V patterning defects in *gpg6* mutants further highlights the mild nature of the hh associated *gpg6* phenotype.

A common structure linking hh signalling and L-R development is the cilium (Huangfu et al., 2003; Nonaka et al., 1998). Analysis of the embryonic node in *gpg6* mutants revealed

abnormally short node cilia, suggesting a retrograde IFT defect. Therefore, analysis of *gpg6* mutant embryos strongly suggests that *gpg6* is a cilia mutant.

7.1.2 Chapter 4

Chapter 4 provided compelling evidence that *gpg6* is an *Atmin* mutant. Mapping and sequencing of *gpg6* mutants revealed a mutation in exon 3 of *Atmin*. This base pair change resulted in an amino acid change affecting a critical residue in the third zinc finger of *Atmin*. A second allele, affecting the fourth zinc finger of *Atmin*, was identified. This failed to complement the *gpg6* mutation, demonstrating the mutation in *Atmin* underlies the *gpg6* phenotype.

Atmin has previously been associated with DNA repair (Kanu and Behrens, 2007; McNees et al., 2005). It is argued to recruit Rad51 to DNA repair foci to prevent DNA damage induced apoptosis (McNees et al., 2005). It has also been proposed to interact, at DNA repair foci, with active Atm, a key cell cycle checkpoint and DNA repair protein. This interaction activates and stabilises p53, initiates cell cycle arrest and activates DNA repair (Kanu and Behrens, 2007; Kanu et al., 2010). The role of *Atmin* in DNA repair was recently demonstrated in adult mice with the observation of increased DNA damage and neural cell loss in a brain-specific conditional *Atmin* mutant (Kanu et al., 2010).

The identification of the *Atmin* mutation as the cause of the *gpg6* embryonic lethal phenotype has uncovered a novel role for *Atmin* during embryonic development. This is

confirmed by the recent report of two mutant alleles of *Atmin* demonstrating a similar phenotype to *gpg6* mutants (Jurado et al., 2010; Kanu et al., 2010).

7.1.3 Chapter 5

In Chapter 5 the hypothesis that *Atmin*, through its zinc finger domain, regulates expression of cilia related genes was tested. The *gpg6* mutation was shown to abolish transcriptional activity of *Atmin* in an *in vitro* assay. To identify potential transcriptional targets of *Atmin* during embryonic development, expression analysis was carried out. Following identification of a list of putative *Atmin* target genes by microarray based analysis of *Atmin*^{-/-} MEFs, Real time PCR analysis confirmed *Dynl1l* is significantly downregulated in *gpg6* mutant embryos. This led to the hypothesis that *Atmin* activates transcription of *Dynl1l*, which is required to build normal cilia, thereby allowing correct L-R patterning and hh signalling.

7.1.4 Chapter 6

In Chapter 6 the hypothesis that *Atmin* and *Dynl1l* are required for ciliogenesis was tested using siRNA to knockdown *Atmin* and *Dynl1l* in ciliated kidney cells. It was found that knocking down *Atmin* and *Dynl1l* resulted in fewer ciliated cells and that those cilia were abnormally short. However, only levels of *Atmin* were shown to be reduced in *Atmin* knock down cells. It has recently been shown that knocking down *Dynl1l* impairs ciliogenesis (Kim et al, 2011). Therefore, the hypothesis that *Atmin* and *Dynl1l* are required for ciliogenesis is supported.

7.2 Atmin has roles in DNA Repair and Ciliogenesis

The main finding of this thesis is a previously unappreciated transcriptional function of *Atmin* in ciliogenesis, L-R development and hh signalling. The identification of *Dynl1l* as a target of Atmin has extended understanding of the transcriptional role of *Atmin* in ciliogenesis. For example, the effect of knocking down *Dynl1l* is similar to the cilia defects seen in cells knocked down for *Atmin* (Chapter 6 and Kim et al (2011)), which supports *Dynl1l* as being a relevant mediator of the cilia function of *Atmin*.

Previous work identified Atmin as being required to form DNA damage foci and to stabilise Atm, thereby initiating cell cycle arrest and DNA repair (Kanu and Behrens, 2007; McNees et al., 2005). Atmin has also been shown to be required for phosphorylation and stabilisation of p53 through its role in stabilising active Atm (Kanu and Behrens, 2007). The identification of *Dynl1l* as a putative target of Atmin may also be relevant to the DNA repair function of *Atmin*. It has been shown that Dynl1l forms a complex with p53 interacting protein (53bp1) and p53. Importantly, Dynl1l is required to transport p53 into the nucleus to initiate cell cycle arrest following DNA damage (Lo et al., 2005). In light of the work presented here, it can be proposed that, in addition to activating and stabilising p53, a previously unappreciated DNA repair function of *Atmin* is to activate expression of *Dynl1l* to facilitate transport of p53 into the nucleus following DNA damage. Atm phosphorylates p53 and active p53 initiates transcription of *p21*, which functions to inhibit Cdk2-CyclinE and block the transition from G1 to S phase of the cell cycle (Bakkenist and Kastan, 2004; Shiloh, 2003). Overexpression of *Atmin* in U2OS cells leads to an increase in cells within G1 phase of the cell cycle following methylation DNA damage, which is consistent with a role

for *Atmin* in initiating the G1 checkpoint (McNees et al., 2005). It remains to be seen whether activation of *Dynll1* expression by *Atmin* plays a role in controlling the G1-S phase transition following DNA damage. The DNA repair role of *Atmin* or *Dynll1* is likely masked in *gpg6* mutants by the greater requirement for the cilia function of *Atmin*, via *Dynll1*, during embryonic development.

7.3 Defining the relationship between *Atmin* and *Dynll1* and their role in ciliogenesis

This thesis has demonstrated roles for *Atmin* and *Dynll1* in ciliogenesis but there still remains a need to clarify the functional relationship between *Atmin* and *Dynll1*. Although the microarray, Real Time PCR and knockdown data argues that *Atmin* activates transcription of *Dynll1*, it is not clear whether this is direct or indirect. Gel shift assays using the enhancer/promoter regions of *Dynll1* could be used to test whether *Atmin* itself physically binds to the *Dynll1* locus. If *Atmin* does interact directly with *Dynll1* then DNaseI protection assays of the *Dynll1* enhancer/promoter could be used to discover the consensus binding sequence of *Atmin*. This would allow *in silico* database searches for additional targets of *Atmin*. Alternatively, chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq) could be used to identify genome wide targets of *Atmin*.

Real Time PCR data that was presented in Chapter 5 demonstrated that *Dynll2* expression is unaffected in *gpg6* mutants, suggesting that *Atmin* targets only *Dynll1*. Furthermore, it suggests that in the context of ciliogenesis *Dynll1* and *Dynll2* do not have redundant roles; ciliogenesis is still affected in *gpg6* mutants despite normal expression of *Dynll2*. Data published on the role of *Dynll1* and *Dynll2* in apoptosis (see below) suggests that *Dynll1*

associates with microtubules and Dynll2 associates with actin (Puthalakath et al., 1999; Puthalakath et al., 2001). Therefore, it can be speculated that Dynll1 associates specifically with cilia. However, this is undermined by the observation that in rat brain Dynll1 and Dynll2 are both present in Cytoplasmic Dynein (Wilson et al., 2001). These data still leave open the possibility that Dynll1 and Dynll2 both associate with Cytoplasmic Dynein 1, which is involved in intracellular transport while only Dynll1 associates with the retrograde IFT motor Cytoplasmic Dynein 2 (Pfister et al., 2006).

There is a strong suggestion, given the similarity of the cilia phenotypes following knockdown of *Atmin* and *Dynll1*, that the effect of *Atmin* on ciliogenesis is mediated solely by *Dynll1*. However, it is perhaps naïve to predict that *Atmin* will have only one transcriptional target relevant to ciliogenesis. To fully understand ciliogenesis in *gpg6* mutants, other targets of *Atmin* need to be found and tested for their involvement in ciliogenesis. The approach taken in Chapter 5 of validating putative *Atmin* targets identified by microarray analysis was successful in uncovering the downregulation of *Dynll1* in *gpg6* mutants. However, expression analysis in *Atmin*^{-/-} MEFs is limited in that these cells are an *in vitro* system and as such may not reflect the *in vivo* state. Therefore, to identify a more comprehensive and relevant list of potential *Atmin* targets further expression analysis comparing wild type and *gpg6* mutant embryos should be carried out. Further knockdown experiments will then have to be conducted to validate whether these candidate genes are required for ciliogenesis.

So far, the role of *Dynll1* in ciliogenesis has been studied solely *in vitro*. There is an obvious need for *in vivo* analysis of *Dynll1* in the mouse. Mutant alleles of *Dynll1* can be generated

by homologous recombination or insertional mutagenesis. Alternatively, mutant alleles may already exist in ENU-induced mutant archives or within the international mutagenesis programs such as KOMP and EUCOMM (Collins et al., 2007). It will be informative to analyse cilia in these mutants. If the retrograde IFT role of LC8, the *Chlamydomonas* homolog of *Dynl1l* (Pazour et al., 1998), is conserved in mammals then it can be predicted that node cilia will be short in *Dynl1l* mutants. Furthermore, if *Dynl1l* is the sole mediator of the ciliogenic role of *Atmin* then *Dynl1l* mutants should resemble *gpg6* mutant embryos. The severity of the *Dynl1l* mutant phenotype when compared to *gpg6* mutants will indicate the relative importance of *Dynl1l* in the *gpg6* phenotype. For example, a less severe phenotype would indicate that *Atmin* targets other genes and that these genes also influence ciliogenesis, whereas a more severe phenotype would indicate the mutant allele of *Dynl1l* has a stronger effect than its downregulation in *gpg6* mutants. Epistatic analysis, conducted by crossing *gpg6* with *Dynl1l* mutants, will help clarify whether *Dynl1l* is genetically downstream of *Atmin*: if double mutants resemble *Dynl1l* mutants then the function of *Atmin* in ciliogenesis is dependent on *Dynl1l*.

7.4 The Potential Role of Dynl1l in the Mouse Embryo

Dynl1l is required for ciliogenesis *in vitro* (Chapter 6 and Kim et al (2011)). However, these knockdown experiments did not address *how* ciliogenesis is affected. In addition, the precise role of *Dynl1l* in the mouse embryo remains unclear. A review of the published data on *Dynl1l* reveals at least four possible hypotheses to explain its function during embryonic development:

Hypothesis 1. *Dynll1* is required for retrograde IFT

As outlined in Chapter 5, *Dynll1* could be required for retrograde IFT. Several lines of evidence support this hypothesis. Briefly, *LC8*, the *Chlamydomonas* homolog of *Dynll1*, was found specifically associated with the retrograde IFT motor Cytoplasmic Dynein but not the anterograde motor Kinesin-II (King and Patel-King, 1995). Importantly, mutants of *LC8* demonstrated short flagella and a lack of retrograde IFT (Pazour et al., 1998). If the retrograde IFT function of *LC8* in *Chlamydomonas* is conserved in mouse, it would be reasonable to predict, *a priori*, that *Dynll1* mutants would demonstrate short cilia. This is consistent with the observation that *gpg6* mutants demonstrate both severe downregulation of *Dynll1* and short node cilia. Furthermore, mutants of other components of the vertebrate retrograde IFT motor Cytoplasmic Dynein 2 (Huangfu and Anderson, 2005) demonstrate short node cilia. It is unlikely that *Dynll1* functions in anterograde IFT since mutants of *Kif3a* and *Kif3b*, components of Kinesin II, lack node cilia (Marszalek et al., 1999; Nonaka et al., 1998; Takeda et al., 1999). Collectively, these data strongly support the hypothesis that *Dynll1* is required for retrograde IFT and ciliogenesis.

A direct test of the requirement of *Dynll1* in retrograde IFT would be to measure the rate of IFT in live cells using fluorescent-based video microscopy. Tran and colleagues (2008) used this technology to demonstrate a lack of retrograde IFT in cells knocked down for *Thm1*. *Dynll1* mutant MEFs or *Dynll1* stably knocked down cells could be used in similar analysis. If changes in the rate of retrograde IFT were detected it would strongly argue that *Dynll1* has a role in the motor function of Cytoplasmic Dynein. Since *Dynll1* is present outside of the motor domain of Dynein (Vale, 2003), *Dynll1* may function to stabilise the Dynein complex

or may be required to bind cargo. This can be tested by coimmunoprecipitation of the Dynein complex in *gpg6* mutant or *Dynll1*^{-/-} cells.

If a change in retrograde IFT is not detected, it is likely that Dynll1 is dispensable for the motor function of Dynein. It is tempting to speculate that Dynll1 may be required to bind specific cargos that are transported within cilia. In *gpg6* mutant or *Dynll1*^{-/-} cells, retrograde IFT would function normally but in the absence of Dynll1 components of cilia would fail to bind to Cytoplasmic Dynein 2 and would not be transported within cilia. This could disrupt ciliogenesis and the signalling that is dependent on cilia (e.g. hh and L-R signalling), leading to the *gpg6* phenotype. Co-immunoprecipitation and immunofluorescence could be used to identify potential binding partners of Dynll1 within cilia.

Hypothesis 2. *Dynll1* is required to monitor centrosome damage

It has been demonstrated that Dynll1 forms a complex with p53 (via 53bp1) to transport p53 into the nucleus, thereby initiating cell cycle arrest following DNA damage (Lo et al., 2005). Interestingly, p53 also accumulates at damaged centrosomes (Mikule et al., 2007). Mikule and colleagues (2007) found that siRNA-mediated knockdown of centrosomal proteins resulted in centrosome damage and cilia defects. Centrosome damage initiates G1 cell cycle arrest and centrosome repair in a p53-dependent manner. It was also shown that p38 (known to phosphorylate and activate p53) is required to induce G1 arrest following centrosome damage. Consistent with these data, p38 and active p53 were found to be concentrated at centrosomes. This led to a hypothesis: following centrosome damage, p53 is recruited to centrosomes where it is activated by p38. Active p53 then activates expression of *p21*, which

inhibits CyclinA-Cdk2 to induce G1 arrest. This ultimately leads to centrosome repair (Mikule et al., 2007).

This raises an alternative, IFT independent, hypothesis to explain the ciliogenic defects in *gpg6* mutants. Since Dynll1 transports p53 into the nucleus (Lo et al., 2005) and it has been shown to transport other proteins to centrosomes (Petit et al., 2003), it can be hypothesised that Dynll1 normally recruits p53 to damaged centrosomes. Therefore, in *gpg6* mutants, downregulation of *Dynll1* disrupts the transport of p53 to centrosomes, blocking both G1 arrest and centrosome repair. Damaged centrosomes will escape repair and form abnormal cilia, resulting in the *gpg6*-related defects in hh signalling and L-R development. A major prediction of this hypothesis is the presence of centrosome damage in *gpg6* mutants. It should be noted that a putative target of Atmin that was identified by microarray analysis was *Centrin 4*, a centrosome associated protein (Gavet et al., 2003) (Chapter 5). It can be speculated that in *gpg6* mutants, downregulation of *Centrin 4* causes centrosomal damage. Parallel downregulation of *Dynll1* results in failure of p53-mediated repair of this damage and causes subsequent defects in ciliogenesis.

The failure to detect, by Real Time PCR, a statistically significant downregulation of *Centrin 4* in *gpg6* mutant embryos argues against this hypothesis. However, it may reflect the efficiency of the Real Time PCR reaction, the age of the embryos that were used, or the low levels of *Centrin 4* expression in the embryo. Further Real Time PCR and knockdown analysis is needed to investigate the involvement of *Centrin 4*.

The knockdown experiments do not differentiate between a retrograde IFT (hypothesis 1) or centrosomal role (hypothesis 2) of *Dynll1* in ciliogenesis, they only show that ciliogenesis is affected. Further work is needed to differentiate between these possibilities. For example, centrosome damage in *gpg6* mutants could be assessed by Western blot analysis of the levels of activated p53 and p21. Immunofluorescence labelling of Dynll1 could determine whether Dynll1 colocalises with centrosomes, which would be consistent with hypothesis 2. Further knockdown of *Dynll1* and live cell analysis of the dynamics of p53 could reveal whether Dynll1 is involved in the transport of p53 to centrosomes.

Hypothesis 3. *Dynll1* is required to transport cilia components intracellularly

The cilium is a dynamic structure (Cole et al., 1998; Iomini et al., 2001; Qin et al., 2004) requiring ciliary components to be continually sent to cilia from inside the cell (Follit et al., 2006). Dynll1 is found in Cytoplasmic Dynein 1, which is involved in intracellular transport (Pfister et al., 2006). Moreover, in *Drosophila*, the homolog of mouse *Dynll1* is required to transport swallow along microtubules to the anterior region of developing oocytes (Schnorrer et al., 2000; Wang et al., 2004). In the light of this, it is reasonable to speculate that Dynll1 has an intracellular transport function within the cell to send ciliary components to cilia.

This raises the possibility that the cilia defects seen in *gpg6* mutants are due not to retrograde IFT defects but result from a lack of the correct ciliary components being transported to the base of cilia; this would disrupt the normal turnover of these components, resulting in defective ciliogenesis. Although it could be argued that a total absence of intracellular

transport to cilia would result in a lack of cilia, it is equally possible that inefficient transport would result in more subtle defects and thus the short cilia that are seen in *gpg6* mutants.

Hypothesis 4. *Dynll1* is required to prevent apoptosis

Data has been published demonstrating that Dynll1 and Dynll2 interact with BimL and Bmf, components of the apoptotic machinery (Puthalakath et al., 1999; Puthalakath et al., 2001). BimL and Dynll1 bind to microtubules but during induction of apoptosis they are released from microtubules (Puthalakath et al., 1999). A mutant form of BimL that fails to bind to Dynll1 causes an increase in apoptosis (Puthalakath et al., 1999). It was therefore proposed that Dynll1 normally sequesters BimL to microtubules, blocking inhibition of the anti-apoptotic factor Bcl2 and promoting cell survival. When apoptosis is induced the Dynll1/BimL complex is released from microtubules to inhibit Bcl2, thereby promoting apoptosis (Puthalakath et al., 1999). The interaction between Dynll1 and BimL has been proposed to be abolished by p21-activated kinase 1 (Pak1)-mediated phosphorylation of Dynll1 serine residue 88, causing destabilisation of Dynll1 dimers (Song et al., 2008; Vadlamudi et al., 2004).

It has also been shown that Dynll2 binds specifically to Bmf and Bcl2. A mutant form of Bmf that fails to bind Dynll2 causes an increase in apoptosis. However, unlike the microtubule-based interaction between Dynll1 and BimL, Dynll2 and Bmf are associated with myosin V and actin. Therefore, Dynll2 sequesters Bmf to the actin cytoskeleton to prevent apoptosis (Puthalakath et al., 2001), while Dynll1 sequesters BimL to the microtubule cytoskeleton to prevent apoptosis (Puthalakath et al., 1999). This is consistent

with isoform specific roles for *Dynll1* and *Dynll2*; however this argument is undermined by the observation that both *Dynll1* and *Dynll2* are found in Cytoplasmic Dynein derived from rat brain (Wilson et al., 2001).

In light of these data it is reasonable to predict that in *gpg6* mutants downregulation of *Dynll1* will result in increased apoptosis. It should be noted that *Atmin*^{-/-} cells or cells knocked down for *Atmin* demonstrate increased apoptosis (Kanu and Behrens, 2007; Kanu et al., 2010; McNees et al., 2005). This was explained by the role *Atmin* has in recruiting Rad51 to DNA repair foci to prevent apoptosis (McNees et al., 2005). The identification of *Dynll1* as a target of *Atmin* raises an alternative explanation that downregulation of *Dynll1* is responsible for this increased apoptosis. However, it is unclear how this could lead to the cilia-based *gpg6* phenotype. If *Bcl2*, *BimL* and *Bmf*, the apoptotic components that bind to *Dynll1* and *Dynll2*, are directly involved in ciliogenesis it would be expected that mutants of *Bcl2*, *BimL* and *Bmf* would demonstrate a *gpg6*-like cilia phenotype. Yet, *Bcl2*, *BimL* and *Bmf* mutants do not manifest a ciliogenic phenotype (Bouillet et al., 1999; Labi et al., 2008; Nakayama et al., 1994). It is possible that an increase in apoptosis of a subset of ciliated cells that normally express *Dynll1* (e.g. in the embryonic node or rostral neural tube) could disrupt L-R development and hh signalling.

7.5 Distinguishing between the potential roles of *Dynll1* in *gpg6* mutants

A number of approaches could be used to distinguish between the various hypotheses. For example, determining the sub-cellular localisation of *Dynll1* by co-immunoprecipitation and immunofluorescence could distinguish between the retrograde IFT, centrosomal repair and

intracellular transport hypotheses: If *Dynll1* is present in cilia then a retrograde IFT function is more likely, whereas if it localises specifically to the basal body then a centrosomal repair function is likely. Conversely, if *Dynll1* is excluded from cilia then it is more likely to have a role in intracellular transport or apoptosis. Measuring apoptosis in *gpg6* mutants, by TUNEL analysis, could further differentiate the intracellular transport and apoptosis hypotheses. Of course, it is possible that *Dynll1* has multiple functions in the vertebrate cell and that downregulation of *Dynll1* in *gpg6* mutants may affect all of these functions. This raises the question of which role of *Dynll1* is most functionally relevant to the phenotype detected in *gpg6* mutants.

A key finding in *gpg6* mutants was the observation of short node cilia. This provided strong evidence that *gpg6* is a cilia mutant. This is consistent with the L-R and hh signalling defects found in *gpg6* mutants. Therefore, to fully understand the *gpg6* phenotype requires explanation of the cilia defect. The apoptosis hypothesis (hypothesis 4) is unlikely to provide an adequate explanation because mutants of *Bcl2* and *BimL*, which are known to interact with *Dynll1*, do not have a *gpg6*-like phenotype (Bouillet et al., 1999; Labi et al., 2008; Nakayama et al., 1994). The intracellular hypothesis (hypothesis 3) is also unlikely because any defect in intracellular transport will disrupt cellular processes more globally and while cilia will be affected as a secondary consequence, so will many other organelles. In the absence of strong evidence demonstrating centrosome damage in *gpg6* mutants, hypothesis 2 is speculative and requires further investigation before it can adequately explain the *gpg6* phenotype. The previously published finding that LC8, the *Chlamydomonas* homolog of *Dynll1*, has a role in retrograde IFT (Pazour et al., 1998) is consistent with the observation of short cilia in *gpg6* mutants and in cells knocked down for *Dynll1*. Therefore, the retrograde

IFT function of *Dynll1* (hypothesis 1) is likely to be the most functionally relevant to the *gpg6* phenotype and should be the focus of future work.

7.6 Conclusion

This thesis has provided evidence that *gpg6* is a cilia mutant demonstrating defects in L-R and hh signalling. A mutation in the zinc finger domain of *Atmin* has been identified in *gpg6* mutants and shown to be responsible for the *gpg6* phenotype. This mutation results in the failure to activate expression of *Atmin* target genes. Putative targets of *Atmin* were sought and *Dynll1* was shown to be severely downregulated in *gpg6* mutant embryos. To test the hypothesis that *Atmin* and *Dynll1* are required for ciliogenesis, *Atmin* and *Dynll1* were knocked down *in vitro* and it was discovered that ciliogenesis was disrupted. This provides support for the hypothesis that *Atmin* activates expression of *Dynll1* to control ciliogenesis.

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Appendices

Appendix A

gpg6 was crossed to *Smo* mutants to assess genetic interaction and epistasis with the hh pathway (Chapter 3)

Smo-gpg6 embryos

$+/+ ; +/+$

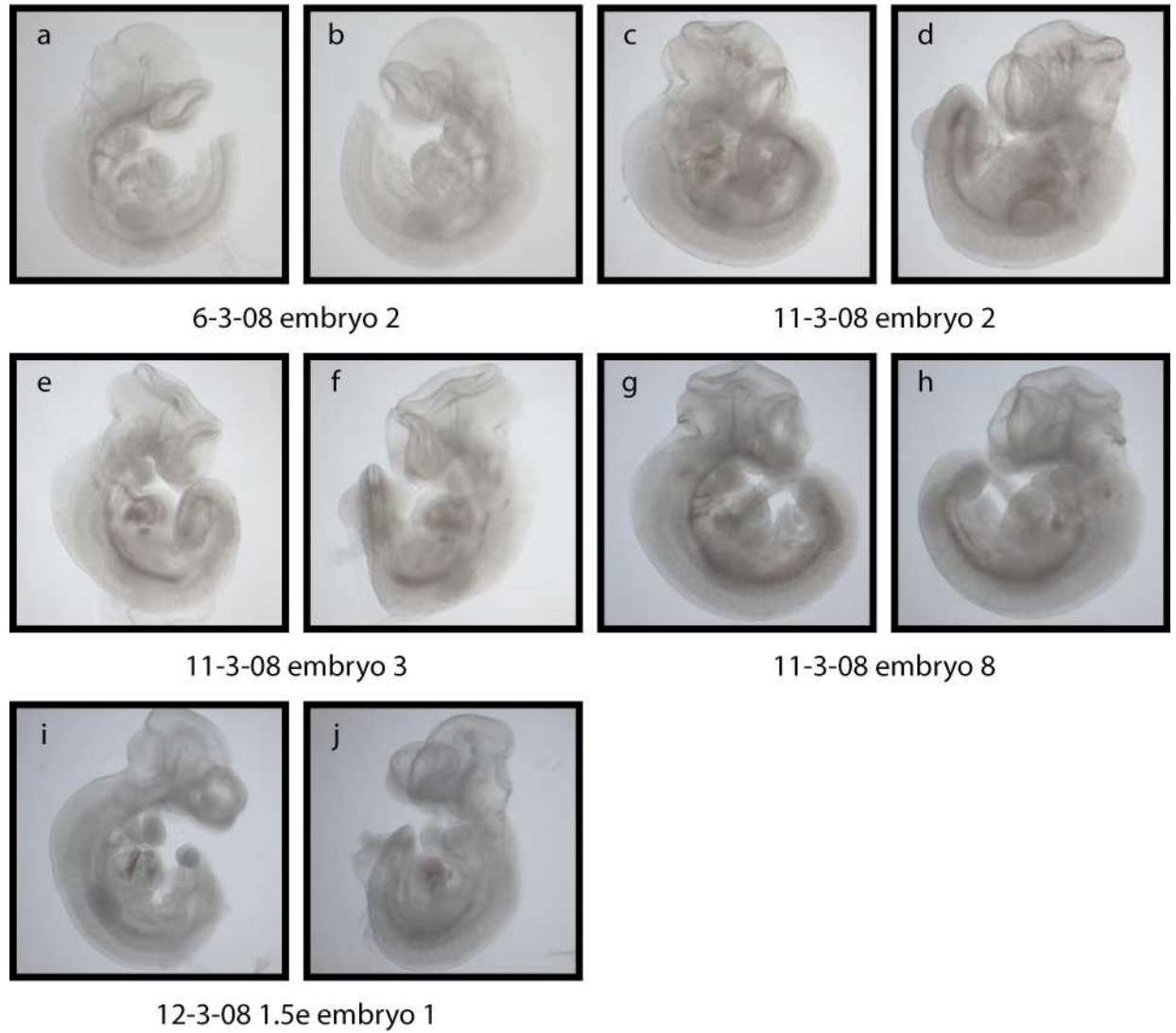


Figure 1. $+/+ ; +/+$ embryos.

$+/+ ; smo/+$

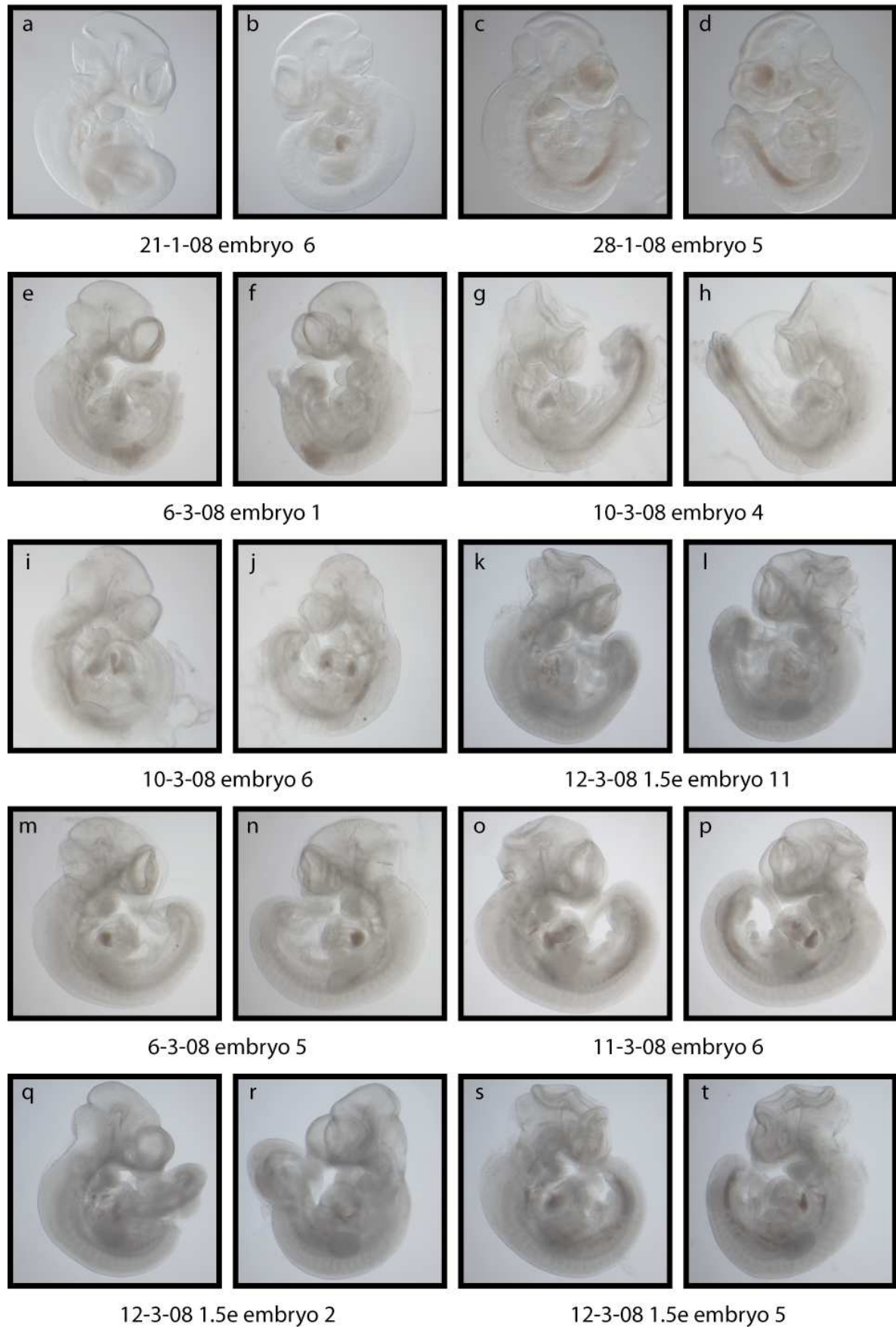


Figure 2. $+/+ ; smo/+$.

gpg/+ ; +/+

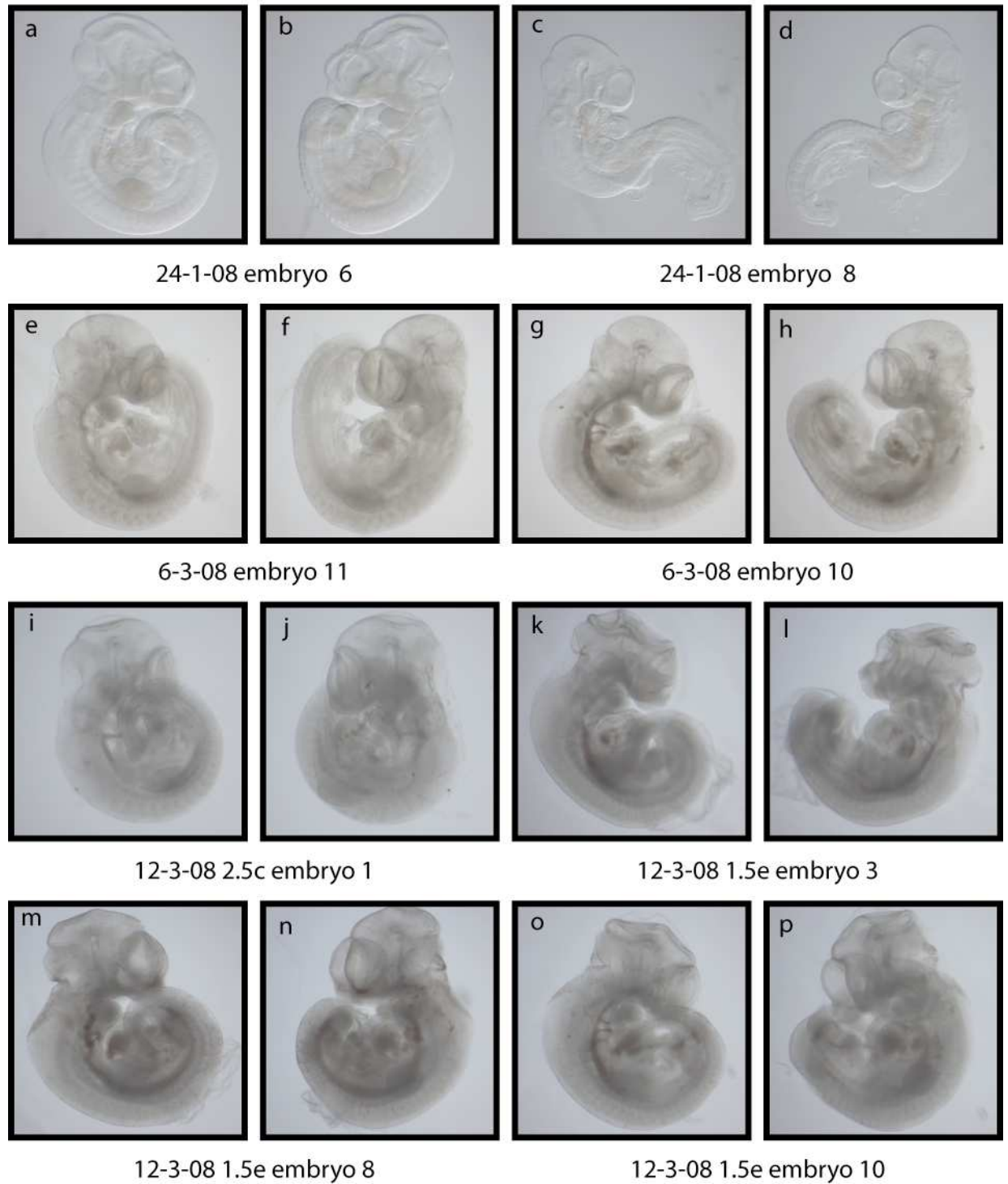


Figure 3. gpg/+ ; +/+ embryos.

gpg/+ ; smo/+

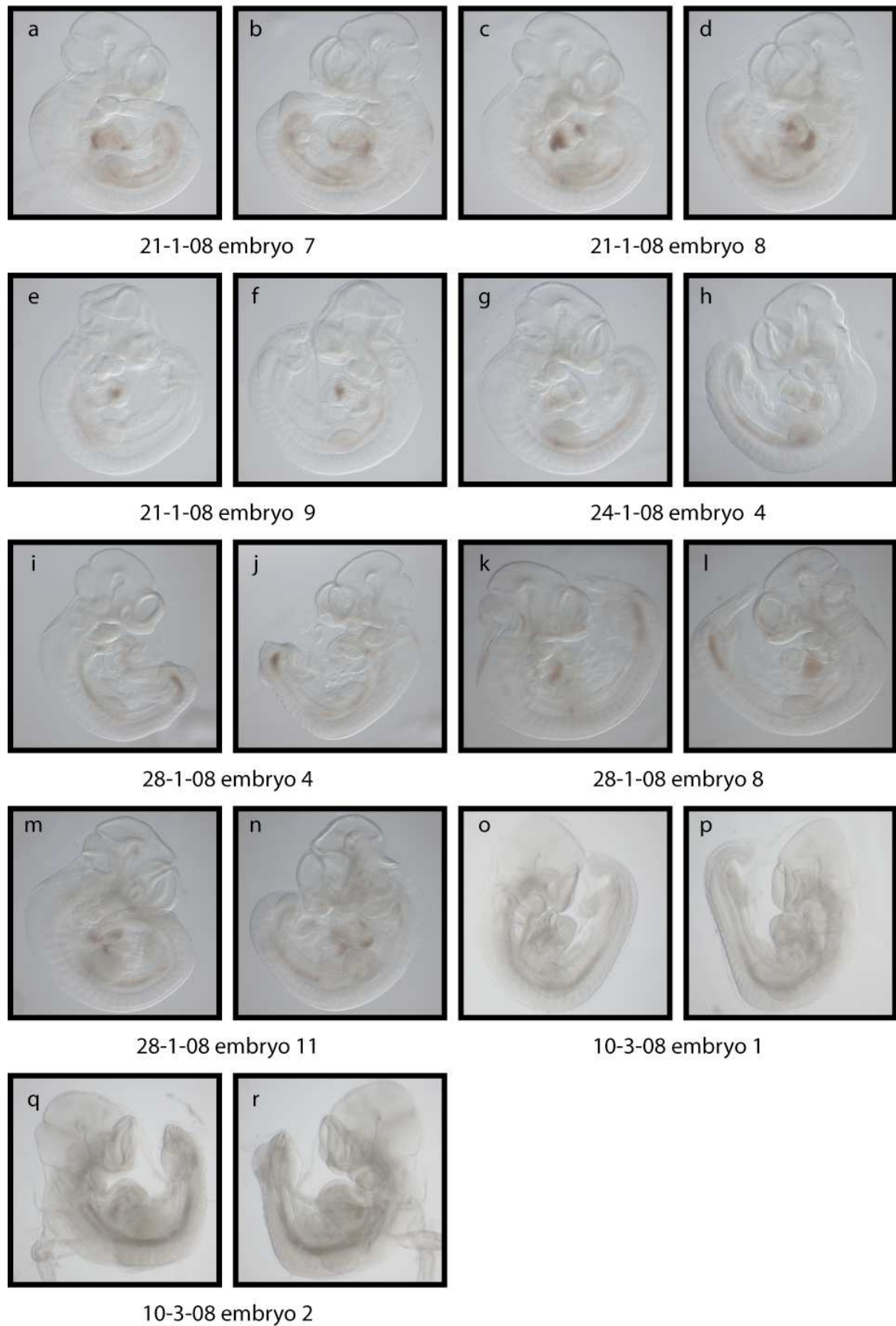


Figure 4. gpg/+;smo/+

$+/+ ; \text{smo-smo}$

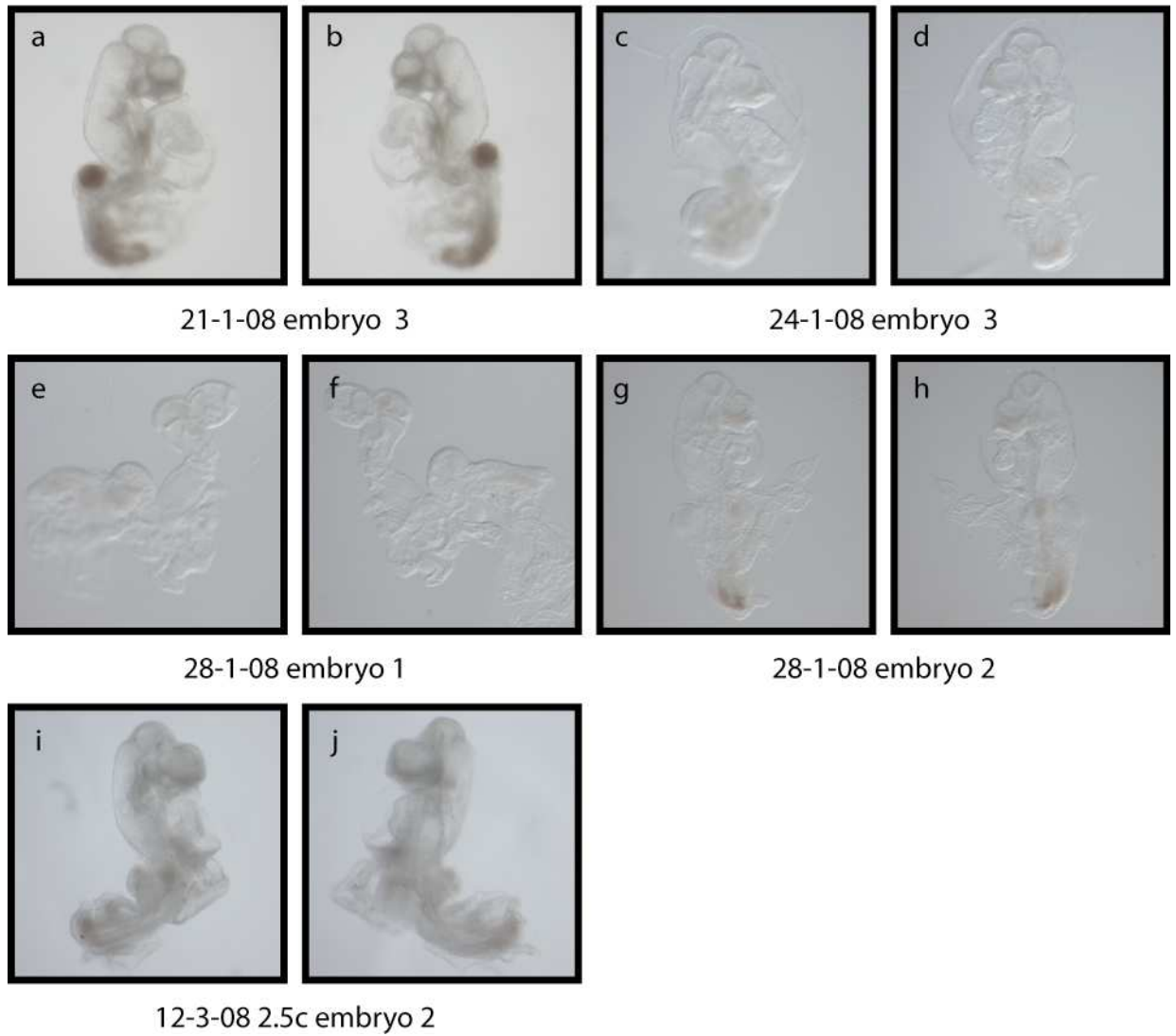


Figure 5. $+/+ ; \text{smo/smo}$

gpg/gpg ; +/+

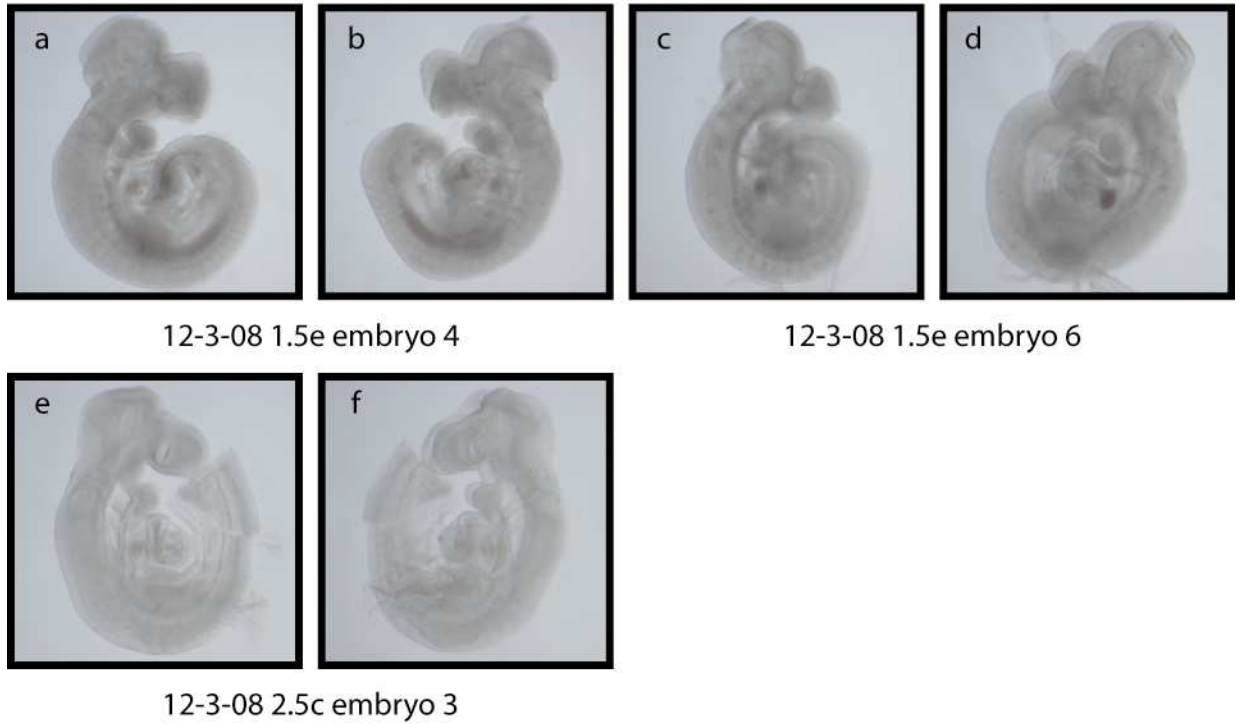


Figure 6. gpg/gpg;+/+

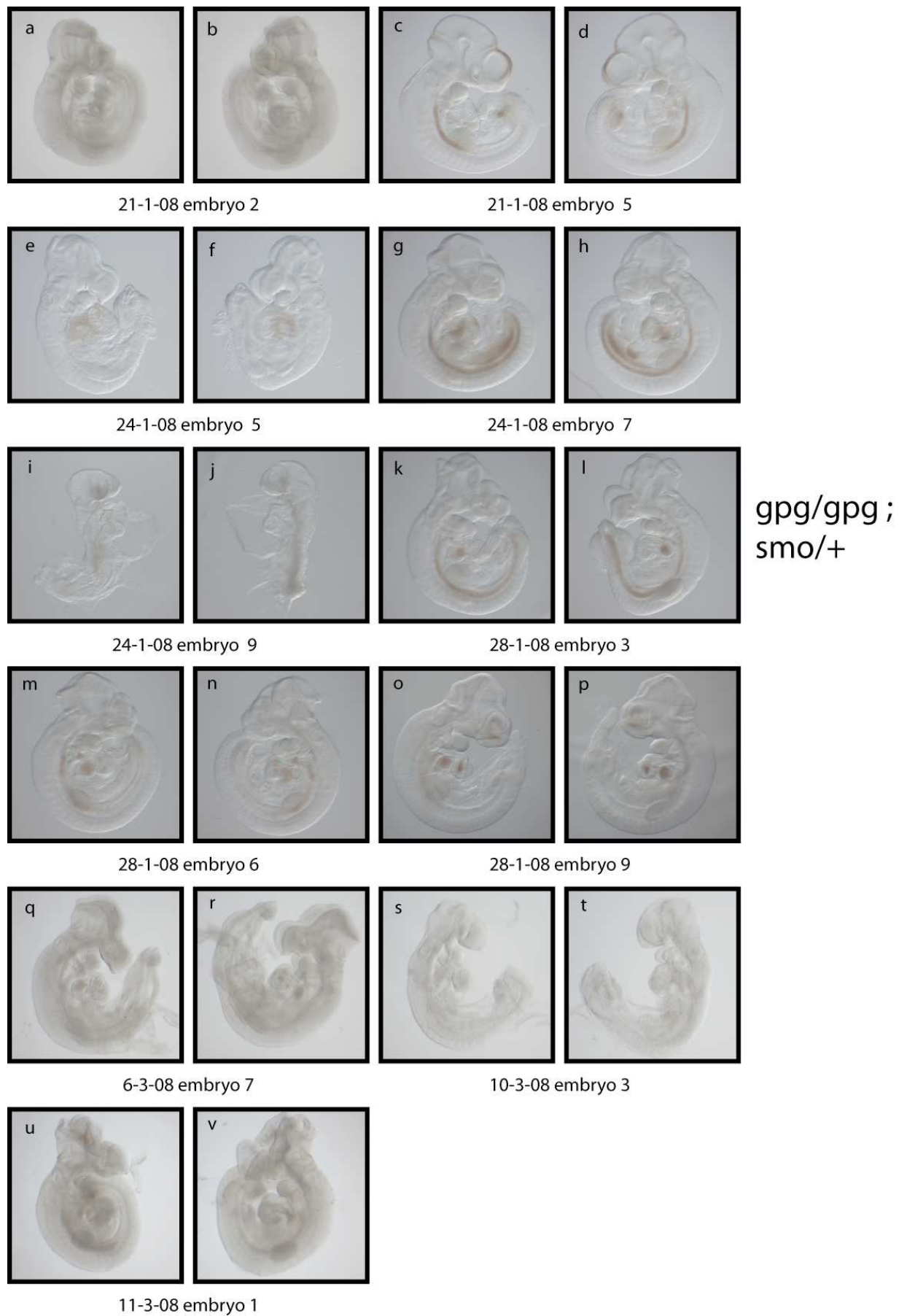
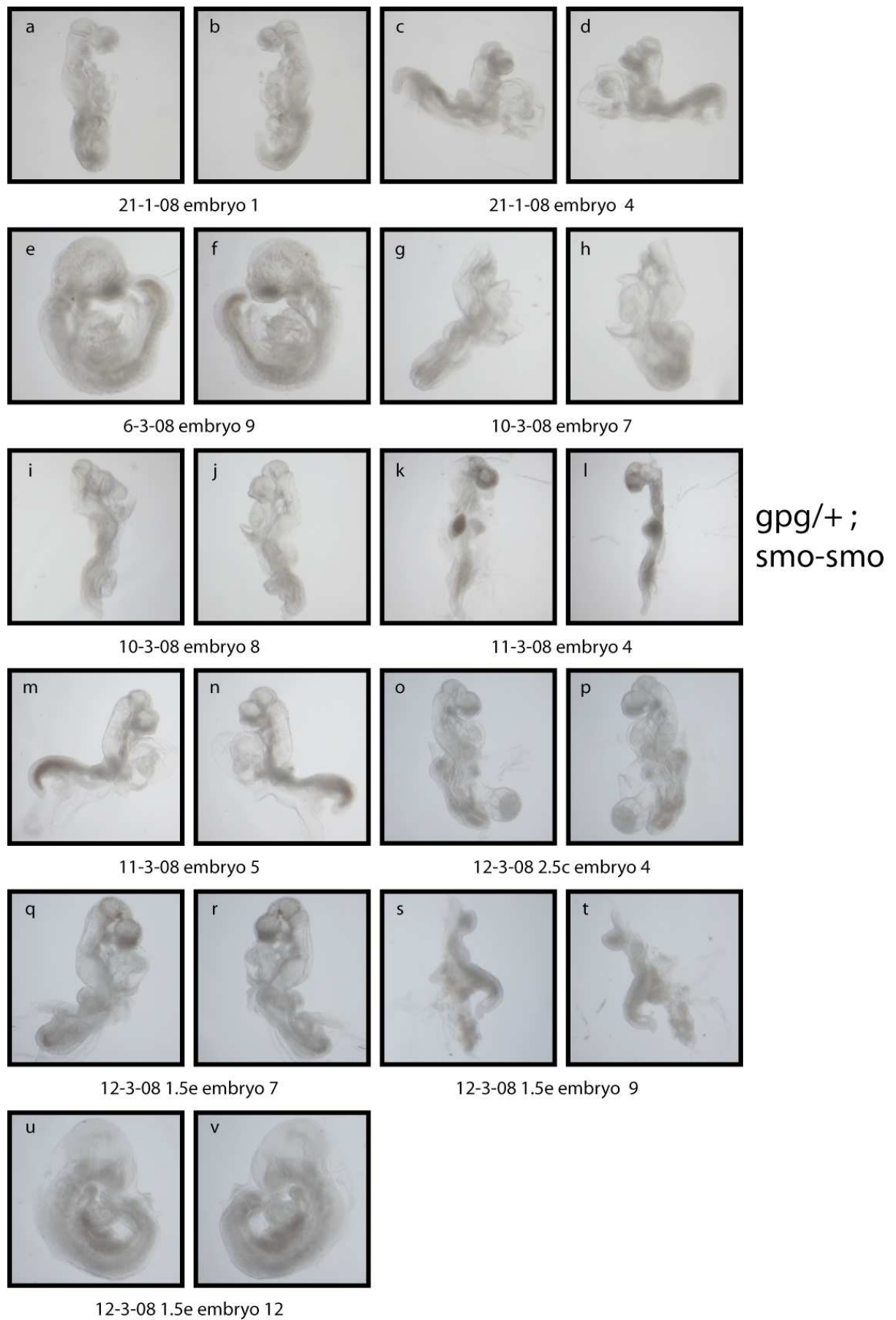


Figure 7. gpg/gpg;smo/+



gpg/gpg ; smo/smo

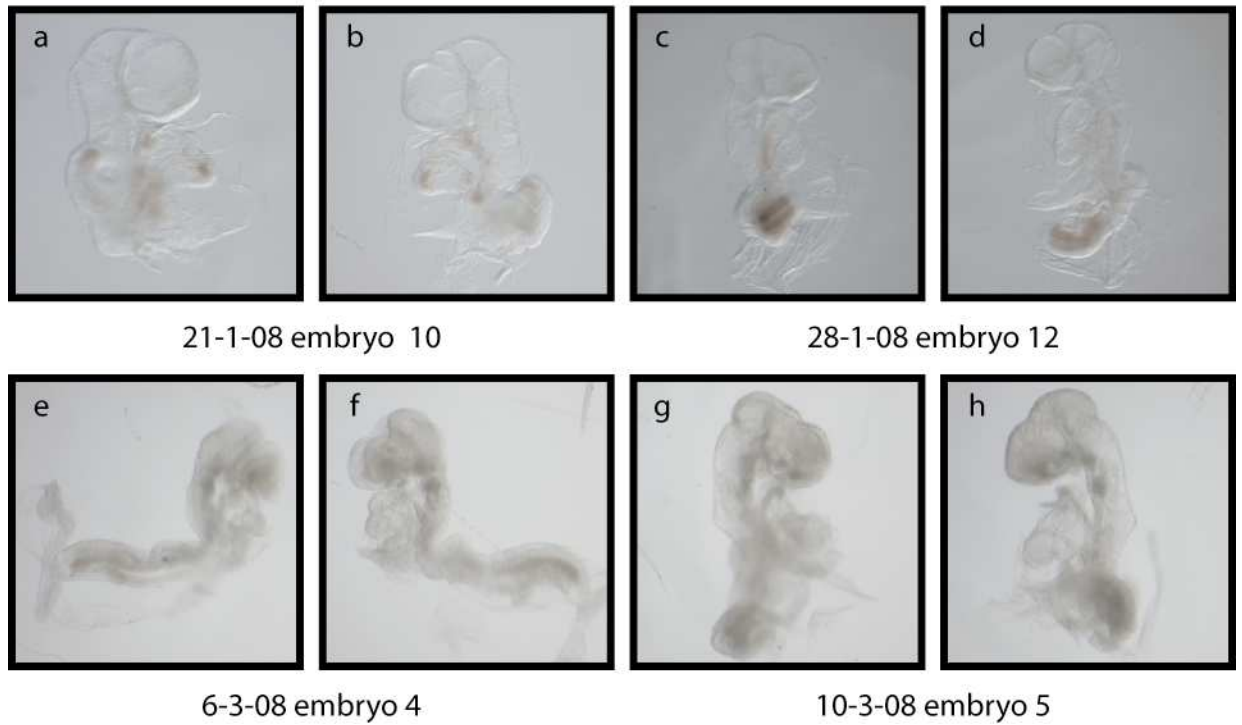
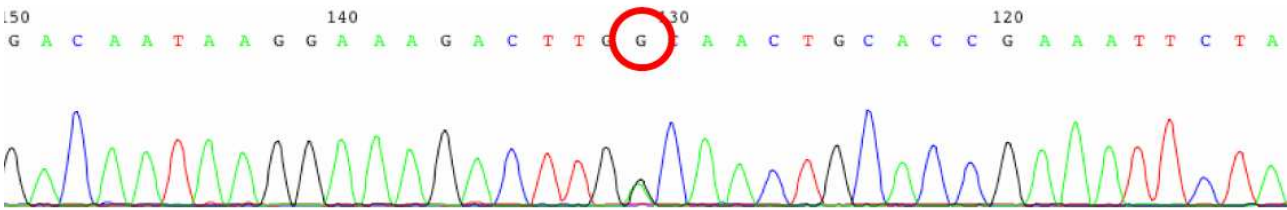


Figure 9. gpg/gpg;smo/smo

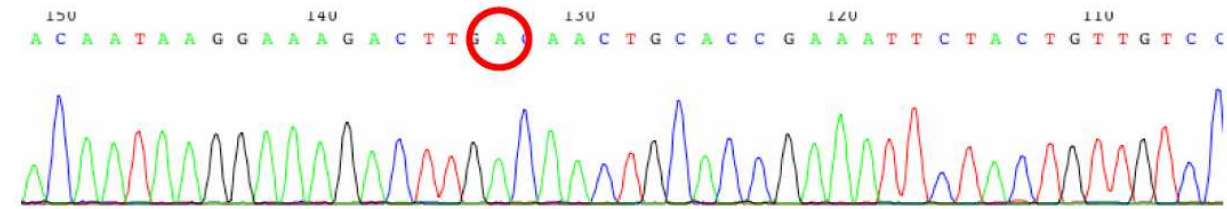
Appendix B

Atmin exon 2 archive mutant

a

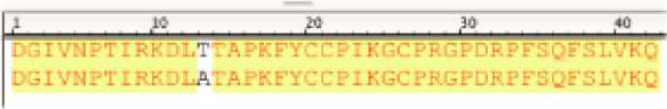


archive exon 2 40 D1



archive exon 2 balb

archive exon 2 balb
archive exon 2 40 D1



b

		130	
mouse bco	-VNPTIRKDLT	TAPKFYCC	
rat bco	-VNPTIRKDLT	TAPKFYCC	
human bco	-VNPTIRKDLT	TAPKFYCC	
chimp bco	-VNPTIRKDLT	TAPKFYCC	
macaca bco	-VNPTIRKDLT	TAPKFYCC	
rabbit bco	-VNPTIRKDLT	TAPKFYCC	
cat bco	-VNPTIRKDLT	TAPKFYCC	
cow bco	-VNPTIRKDLT	TAPKFYCC	
dog bco	-VNPTIRKDLT	TAPKFYCC	
bush baby bco	-VNPTIRKDLT	TAPKFYCC	
opposum bco	-VNPTIRKDLT	TAPKFYCC	
platypus bco	-VNPTIRKDLT	TAPKFYCC	
chicken bco	-FNAPIRKGL	TPQKFYCC	
xenopus trop bco	-LNPTIRKDL	KTASQKLYCC	
zebrafish bco	-VNPTIRKDL	KTASQKLYCC	
stickleback fish bco	-VNPTIRKDL	KTASQKLYCC	
medaka fish bco	-VNPTIRKDL	KTASQKLYCC	
sea urchin bco	-VQSKTNQ	PRVHTQIKNC	
drosophila bco	-HGEVIPP	SVIGPIVAEHC	
anopheles mosquito bco	-VQSKTNQ	PRVHTQIKNC	
aedes mosquito bco	-VQSKTNQ	PRVHTQIKNC	
c elegans bco	-LISPDPA	PSSSSSSSASCS	
arabidopsis bco	-KQRSNK	LVVVR--KKVYVC	
barrel clover bco	-KQRNKLE	VIR--KKVYVC	
common rice bco	-KQRSNG	KEPRK--RVYVC	
yeast bco	-SESSESL	NSLTSTRSSSS	
consensus	VNPTIRKDL	K YCCF	

Figure 1. A second allele of *Atmin* was sought in the Harwell ENU DNA archive (Chapter 4). Archive exon 2 mutation in *Atmin*. (a) A to G change results in a Threonine (T) to Alanine (A) amino acid change. (b) However, Threonine is unique in mouse; Lysine (K) is present at this position in other species and is conserved to medaka fish.

Appendix C

Below is a list of primers used throughout the project.

Primers

Mapping Primers

D8mit167 LEFT	ATGGAAGAAGAGAACTGACTCCC	56
D8mit167 RIGHT	TTTTTCAATTCATTTATTTGCTTTG	
D8mit272 LEFT	TGTTTGTCTCTGTCTCATCTAGCC	56
D8mit272 RIGHT	CCAAGCATATGTGTATGCACG	
D8mit321 LEFT	ACACTGAACCATACTGCCTGG	56
D8mit321 RIGHT	CTGAGCAGTTTTTTTCAGCACC	
D8mit139 L	TCCCCATTCCCATCTGTCT	54
D8mit139 R	GGAGAACTCCCAATCCAACA	
D8mit49 L	TCTGTGCATGGCTGTGTATG	56
D8mit49 R	TGGTGTGCTGCTGATGCT	
Rs13480001 LEFT	CCCACAGCCTTTATTTGCAT	54
Rs13480001 RIGHT	GCGGAACAATTAAACCCAAA	
Rs13480012 LEFT	CCTATGGCTCCAGGAAACAG	58
Rs13480012 RIGHT	CACACATTCTCCCAGCACAG	
Rs4227432 LEFT	CGATAAGCTGTCTGCAGGTG	54
Rs4227432 RIGHT	AAGGCACGTGTTTTTCTGCT	

RT-PCR

GSP6 novel 74073 LEFT	TGGTAGATTAGAACTAGGATGTGAAA53	
GSP6 novel 74073 RIGHT	CAGTTGTGGTCTGCACAGCTA	
GSP6 novel 74068 LEFT	TTGGGAGCGACGTGTCTG	53
GSP6 novel 74068 RIGHT	GCGGATGCTAAGCTGTTGTC	
PKD112 RTPCR 2 LEFT	CTTTGCAACCACCTCACCTT	53

PKD112 RTPCR 2 RIGHT	AAGCCCGTGGAGGTTTTAGT	
Bcmo1 RTPCR 2 LEFT	TCTATGCAACCACGGAGACC	53
Bcmo1 RTPCR 2 RIGHT	ACCTCCTGAGGGTAGGCACT	
1700030J22Rik RTPCR 2 LEFT	ATGTCTGCTGCCTTCTTGGT	53
1700030J22Rik RTPCR 2 RIGHT	GTTGTGGGGAGAGCTTTTCA	
Cdyl2 RT PCR LEFT	AAGAAGGGGTCTTCGGGTAA	53
Cdyl2 RT PCR RIGHT	GGCCGATCAGGTAGGAATAG	
2310061C15Rik RT PCR LEFT	GTGCTTCTTGTAGGGGCTGA	53
2310061C15Rik RT PCR RIGHT	GTGGCACTCAAGTTCAGCAG	
2610510J17Rik RT PCR LEFT	TGTCGACCTTTTTGATATGGA	53
2610510J17Rik RT PCR RIGHT	GCTTCCTGCAGAGACGTTGT	
Atmin RT PCR LEFT	TGACAACTGCACCGAAATTCT	53
Atmin RT PCR RIGHT	GACGCTCTGCTTTTTTGGTGT	
1700030J22Rik RT PCR LEFT	GAAGAGACGGCACCAACTTT	55
1700030J22Rik RT PCR RIGHT	GAGAGGCCGCTCCAGTTC	
Gesh RT PCR LEFT	CTGCAAGTGTCGAGGAGCTT	53
Gesh RT PCR RIGHT	TTTTCGGCAAGAGCTTCATT	
pkd 112 RT PCR LEFT	TGGCTCCTCTCACCTACAT	53
pkd 112 RT PCR RIGHT	TGTAGAGGTGGTGGTGTGTC	
4933407C03Rik RT PCR LEFT	CGCCTCTGCAGGATGATT	53
4933407C03Rik RT PCR RIGHT	CGTTTCGGATCTCTTTCAGG	
4632417N05Rik RT PCR LEFT	TAGGTGAAGGGAGCATGTGG	53
4632417N05Rik RT PCR RIGHT	CCCGGAGGATGTTCTCTGT	
Hsd17b2 RT PCR LEFT	TCCTCTGCACCTTGTTCTC	54
Hsd17b2 RT PCR RIGHT	GGTTAGAGCTGCCTTTGTGG	
Mphosph6 RT PCR LEFT	GAAGAGGAGGAGCGGAAGAT	53
Mphosph6 RT PCR RIGHT	TTTAGAGGGAAACGGTGCTTT	

Dynlrb2 Exon Scanning

dynlrb2 alt exon 1 LEFT	AGAAACAGCCTAACCGTTGC	59
dynlrb2 alt exon 1 RIGHT	CCTTTCCAGCTGTCATCCAG	
dynlrb2 alt exon 4 LEFT	GGTACAGCCATTTGTCTACGC	59
dynlrb2 alt exon 4 RIGHT	CCGATACTGTAGTTTTTATTTGTTTGA	
dynlrb2 rt per 1 LEFT	CATCGGAACGATGGTGGT	59
dynlrb2 rt per 1 RIGHT	TCACATGGGTTCTGAATGACA	
dynlrb2 rt per 2 LEFT	TGGAGGAAACCCTCAAGAGA	59
dynlrb2 rt per 2 RIGHT	CCATGATTTTCATGTTTCTTCG	
dynlrb2 rt per 3 LEFT	ACCCTGGACAACCTCCACAAC	59
dynlrb2 rt per 3 RIGHT	TCACATGGGTTCTGAATGACA	
dynlrb2 rt per 4 LEFT	GGGGAAGCGTTGTTGACAT	59
dynlrb2 rt per 4 RIGHT	CTTCTGCATTGACCACCATC	
dynlrb2 rt per 5 LEFT	ACCCTGGACAACCTCCACAAC	59
dynlrb2 rt per 5 RIGHT	CACAATTCAAAGGGGACTTAGG	
dynlrb2 rt per 6 LEFT	GGGGAAGCGTTGTTGACAT	59
dynlrb2 rt per 6 RIGHT	CAATTCAAAGGGGACTTAGGG	

Atmin Exon Scanning

Bco exon 1a LEFT	acatgggacttcaacctcca	65
Bco exon 1a RIGHT	GGACAGCTCGCTCACAGTC	
Bco exon 1.1 LEFT	acatgggacttcaacctcca	65
Bco exon 1.1 RIGHT	gctcctcttggaaacctgag	
Bco exon 2 LEFT	acagttctgagccaggtgt	65
Bco exon 2 RIGHT	ccaatgcccttaacctgtc	
Bco exon 3 LEFT	tgcagtacctggtgatcctg	65
Bco exon 3 RIGHT	cccttgtaaagccacaggt	
Bco exon 4.1 LEFT	tccaagggtacccatttcag	65

Bco exon 4.1 RIGHT	CAGAGAGCAGGCCTCTGAGT	
Bco exon 4.21 LEFT	CTGTTACCAAAGCCCAAGGT	65
Bco exon 4.21 RIGHT	TGGGTGTGGACACTAACAGG	
Bco exon 4.31 LEFT	TCCTGTTGTTGAGCCAATGA	65
Bco exon 4.31 RIGHT	ACTCTGAGGTGGCAGACCAT	
Bco exon 4.41 LEFT	GCTTCTCCATGAGTACCGACT	65
Bco exon 4.41 RIGHT	TCAATATCAAAATCAATCCCAGAA	
Bco exon 4.51 LEFT	ATGGTCTGCCACCTCAGAGT	65
Bco exon 4.51 RIGHT	TTTCTGTGCTGCTCAACTGG	
Bco exon 4.6 LEFT	AGAGCGGGGTCAGGCTTC	65
Bco exon 4.6 RIGHT	AGCTGCCCCACTGACTACAT	
Bco exon 4.7 LEFT	CAGTTCAGCTCCGTGGAAAC	65
Bco exon 4.7 RIGHT	ACATGACAAGGCAAGGTGGT	
Bco exon 4.8 LEFT	CAAGCTTTGTGGCTGCTTTA	65
Bco exon 4.8 RIGHT	TGGAGTCATAGGAACCCCAAT	
Bco exon 4.9 LEFT	AATCTGTGGGATTGCCATGT	65
Bco exon 4.9 RIGHT	GCTGGTAGCATCTGGGTAGC	
Bco exon 4.101 LEFT	TAGCCTCGTAGGAACCTCCA	65
Bco exon 4.101 RIGHT	CAATTGCTCTCCAGATTTTCC	
Bco exon 4.11 LEFT	GCCAGTATGCTGCACTTGTT	63
Bco exon 4.11 RIGHT	CAGGACAGCAGCAATACCAA	
Bco exon 4.12 LEFT	TCTAGCCTGGATGGAAAAGC	63
Bco exon 4.12 RIGHT	TTTGTGGGGACAGTTCTTGC	
Bco exon 4.131 LEFT	TGTTTTGGACTCGTCCACTG	65
Bco exon 4.131 RIGHT	tgaaagggcagcaaggtatc	

Atmin Pyrosequencing

Gasping6_April08_For_B	Biotin_TCCGCAGCACTTTATGAA	58
Gasping6_April08_Rev	AGCTACTCAACCGCATCTC	
Gasping6_April08_SeqR	GGAAGGTCTTGCCAC	
<i>Atmin</i> ²¹¹ _June08_For_B	Biotin_TCTCACATCTACCGAACTGG	60
<i>Atmin</i> ²¹¹ _Rev	GGTCCCATTACAGCTACTCA	
<i>Atmin</i> ²¹¹ _SeqR	GTGCTCTGCAGGGAT	

Point Mutagenesis

BCO mut primer 1	AGACACGAGGAGGATaGTGGCAAGA	63
BCO primer 1	ACAGTACCGGCTGCAGAACT	
BCO mut primer 2	GTGGAGGCCGCCCCGAGTC	63
BCO primer 2	GGAAGGTCTTGCCACtATCCTCCTCGTGTCT	

Myc tag primers

BCO-myc LEFT PRIMER (Sall)	gcgtcgacCCATGGCGGCCACGGAGG	68
BCO-myc RIGHT PRIMER (NotI)	gcgcggccgcTGCCTGGCTGCACCTTCAGA	

Primers to Sequence Atmin Plasmids

Fusion seq 1 LEFT	CAGCCGGCCGAGACC	65
Fusion seq 1 RIGHT	GCGCTACAGGAGTCTTCGAG	
Fusion seq 2 LEFT	CAGAGCACAGAGACCCACCT	65
Fusion seq 2 RIGHT	CACGATGACACAGAGGAATCA	
Fusion seq 3 LEFT	GTCAGAGGACCAGCATTTCC	65
Fusion seq 3 RIGHT	TCGCTCAGCAGATCTATGGTT	
Fusion seq 4 LEFT	ATGGTCTGCCACCTCAGAGT	65
Fusion seq 4 RIGHT	GGCTCGAAGCCAGAACTCAT	
Fusion seq 5 LEFT	CCTCTGACACAGAGACCCAAA	65

Fusion seq 5 RIGHT	GCTCTGCGCATGTCTGG	
Point seq 1 LEFT	ACTTGACAACTGCACCGAAA	63
Point seq 1 RIGHT	GCAGGTAGCTTTCCATTTTCC	

Archive Scan Primers

Asciz 1AF	TGCTAATGAGCCCGGAGG	66
Asciz 1AR	TCTCGCCGTGCTCGGTC	
Asciz 1AF(2)	TGCTAATGAGCCCGGAG	66
Asciz 1AR(2)	GCAGGGCACGGCGCAAA	
Asciz 2AF	GGCTACATAGAGAGACCCT	66
Asciz 2AR	GATCATGGTACTAGCAAAGTAATTAAC	
Asciz 2AF(2)	CCTTTAGTTCCAGGCAAATCT	66
Asciz 2AR(2)	TCGCAGCGTTTGATCAT	
Asciz 3AF	ATGCTCTCTCTTGTCTTCAT	66
Asciz 3AR	AGGAGGTCCCATTACAGCTA	
Asciz 3AF(2)	TACTGGCAAATGCAGTACC	66
Asciz 3AR(2)	TTTGTGGCTGGCTTGTC	

Real Time PCR (ABI Taqman Assays)

Dynll1	Mm00850282
Dynlt1	Mm00839582
Cetn4	Mm00782119

Smo genotyping

Wild type	Smo-5	GGC CTG CGC TGC TCA ACA TGG	64
	Smo-Exon1	GCA ACT GCA GCA GCC TCC GG	
Mutant	Smo-5	GGC CTG CGC TGC TCA ACA TGG	67
	Smo-NEO	GGC GCT ACC GGT GGA TG	