

The Roles of the Homeobox Genes *ALX4* and *MSX2* in Skull Development



Lampros A. Mavrogiannis
Green College

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Thesis supervised by Professor Andrew O. M. Wilkie

Abstract

Heritable ossification defects of the skull vault often present as enlarged parietal foramina (PFM), bilateral oval openings of the posterior parietal bones. Isolated PFM may originate from wider defects in infancy and usually show an autosomal dominant mode of transmission, offering unexplored genetic insights into the molecular pathways of calvarial development. Haploinsufficiency of the homeobox gene *MSX2*, located at 5q34–q35, underlies a fraction of PFM families but the locus can be excluded in others, indicating heterogeneity. The proximal 11p deletion syndrome (P11pDS), characterised by multiple exostoses (due to haploinsufficiency of *EXT2*), occasional mental retardation, and PFM, pointed to a second locus at 11p11–p12.

The human orthologue of the mouse paired-like homeobox gene *Alx4* was identified adjacent to *EXT2*. *ALX4* was structurally characterised and heterozygous loss-of-function mutations were detected in association with skull vault defects in twenty-nine individuals from six families, including a new case of P11pDS. The calvarial phenotype of *ALX4* mutations was almost indistinguishable from the *MSX2*-caused defects and ranged from a midline gap to non-penetrance, nevertheless typified by classical PFM; abnormal morphology of the dural septa was also observed. The mutation spectrum and the subtle genotype-phenotype correlations suggested haploinsufficiency as the predominant pathophysiological mechanism. Interestingly, *Alx4*^{-/+} mice manifest polydactyly but no skull defects, illustrating species-specific dosage sensitivity. Two new *MSX2* mutations were also ascertained, one of which segregated with PFM and clavicular hypoplasia. The potential contribution of *ALX4* and *MSX2* to premature fusion of the cranial sutures – craniosynostosis – was investigated, but no unequivocally pathogenic variants were found.

To elucidate the functions of *Alx4* and *Msx2* in skull development, spatial expression analysis was performed in mouse embryos between embryonic days E12.5–E17. Transcripts of both *Alx4* and *Msx2* were seen in the early calvarial skeletogenic condensations and in later stages their expression displayed a more restricted pattern, overlapping minimally with the domains of mature bone. By assessing expression in embryonic heads of reciprocal knockout mice, activation of *Alx4* was found to be independent of functional *Msx2* and vice versa. Analysis of compound mutants demonstrated that the two loci exert roughly additive effects on the skull vault while protein interaction assays did not indicate any physiological interaction between *Alx4* and *Msx2*. Hence, *Alx4* and *Msx2* appear to regulate proliferation, differentiation, or survival of osteoblast precursors and pre-osteoblasts through parallel pathways.

Statement of originality

Unless declared otherwise below or in the acknowledgements section or in the main text, the work presented in this thesis was performed by the author himself at the Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford; and at the Department of Human Anatomy and Genetics, University of Oxford, Oxford, from October 1999 to December 2002 inclusive, and has not previously been submitted for a degree in this or any other University or institute of learning.

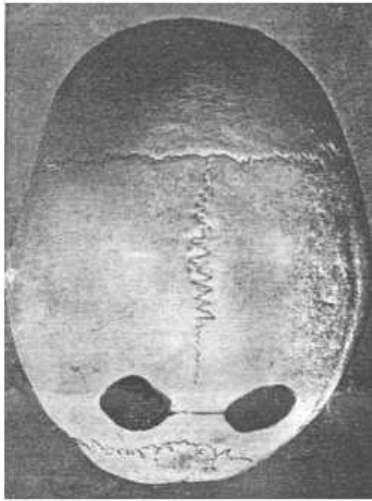
Significant contributions by other persons are as follows. Mating and skeletal staining of mice in chapter 6 (Figures 6.1, 6.2, 6.3, and 6.5) was conducted by Dr Ileana Antonopoulou and this material has appeared in her D. Phil. thesis (Antonopoulou 2002); however, the photography and the interpretations are of the author's. Figure 6.6 in the same chapter was also provided by Dr Antonopoulou and has, similarly, been included in her thesis. Part of the genotyping work involving markers around the *MSX2* gene, mentioned in chapter 3, was carried out by Prof. Andrew Wilkie and Mr Navaratnam Elanko.

To my father

Fishing comes the sea
and in its fragrance flash the fish
don't search in vain

Somewhere between Tuesday and Wednesday
your true day must have been mislaid

Odysseus Elytis



"EYES" IN THE BACK OF HIS HEAD

This is not, as it appears, an attempt on the part of nature to meet the proverbial small boy's demand for "eyes in the back of his head." The holes serve no such useful purpose, but show the inheritance of an entirely useless or even injurious character. (Fig. 11.)

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Acknowledgements

This thesis is a chapter in a collective story – largely a work in progress – of two genes, implicated in mammalian skull development in such ways that render them genetically visible. In many respects this contribution is a true child of its era. Starting from clinical and human genetics, one of the genes was identified through a hybrid approach, relying heavily on sequence analysis (pouring freely from the public human genome project) but also paying tribute to the tradition of positional cloning. Once the part of the exploration that I was somewhat equipped for was over, the project itself dragged me beyond descriptive genetics and into the waters of functional analysis — in fact, developmental functional analysis. The availability of mouse mutants offered no escape from certain questions, which I tried, hopefully not in a grossly amateurish way, to answer.

The bench work was performed at the Weatherall Institute of Molecular Medicine and the Department of Human Anatomy and Genetics from October 1999 to December 2002. It would have been impossible without a scholarship from the 'Alexander S. Onassis' Foundation (Greece) and a fees-only studentship from the Medical Research Council (UK); the Institute itself also provided support for two months that enabled me to bridge a funding gap.

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And a note. Although the text might convey the impression of a linear, uninterrupted, and coherent narrative, day-to-day laboratory research was slightly more entangling. As with any creative process, there was excitement and frustra-

tion, inspiration and fatigue — illuminative moments as well as deadlocks. Afterwards, writing in itself imposes selection: apparent mistakes are erased, retrospectively irrelevant lines of work are omitted, and negative or inconclusive data are concealed. Despite these unconscious or even deliberate compromises, I hope that the text is not unfaithful to the principles of the scientific method. Thesis writing along with a full-time job was more difficult than envisaged and my efforts to insulate it from other facets of life often failed dramatically. I am grateful to Dr Graham Taylor and Dr David Cockburn in the Yorkshire Regional Genetics Laboratory for their understanding and tolerance. Also, two of my new colleagues, Dr Rachel Robinson and Dr Kieran Bransfield, went through an advanced draft of the core of the thesis and I would like to thank them for their time. Obviously, I am fully responsible for all errors, omissions, and inconsistencies that remain. In other words, I learned through experience that the art is long yet life is short and time never convenient — *ο βίος βραχύς, η δε τέχνη μακρά, ο δε καιρός οξύς ...* Someone out there provided the inescapable but curiously vital ‘angelic and black, light’.

Leeds,

December 2003

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List of Abbreviations

ACS	1-aminocyclopropane-1-carboxylate synthase
AER	Apical ectodermal ridge
ATP	Adenosine triphosphate
BAC	Bacterial Artificial Chromosome
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bisacrylamide	N, N'-methylene-bisacrylamide
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
C-	Carboxy-
CCD	Cleidocranial dysplasia
CT	Computed tomography
CTP	Cytidine triphosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine triphosphate
DHPLC	Denaturing high performance liquid chromatography
DMSO	Dimethylsulphoxide
dNTP	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol

dTTP	Deoxythymidine triphosphate
E	Embryonic day of mouse development
EBI	European Bioinformatics Institute
EDTA	Ethylenediamine tetraacetic acid
EST	Expressed sequence tag
FISH	Fluorescence in situ hybridisation
GDB	The Genome Database
GP	General practitioner
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HGMP-RC	Human Genome Mapping Project Resource Centre
HUGO	Human Genome Organisation
IPTG	Isopropyl- β -D-thiogalactoside
kb	Kilo base pairs
kDa	Kilo daltons
LB	Luria-Bertani medium
LOD	Logarithm of odds
Mb	Mega base pairs
Mes	2-(N-morpholino)-ethane sulphonic acid
M-MLV	Moloney murine leukemia virus
MRI	Magnetic resonance imaging
MW	Molecular weight
N-	Amino-
NBT	Nitroblue tetrazolium
NCBI	National Center for Biotechnology Information
OAR	Otp-aristaless-Rx
OMIM	Online Mendelian Inheritance in Man
OUBC	Oxford University Bioinformatics Centre

P	Postnatal day of mouse development
P11pDS	Proximal 11p deletion syndrome
PAC	Plasmid Artificial Chromosome
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBT	PBS with Tween-20
PCR	Polymerase chain reaction
PFM	Enlarged parietal foramina
PFMCCD	Parietal foramina with cleidocranial dysplasia
PFMCH	Enlarged parietal foramina with clavicular hypoplasia
rpm	Revolutions per min
RT-PCR	Reverse transcription-PCR
SCS	Saethre-Chotzen syndrome
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	Sodium chloride-sodium citrate buffer
SSCP	Single-strand conformation polymorphism
STS	Sequence tagged site
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBP	TATA-binding protein
TBS	Tris-buffered saline
TBST	TBS with Tween
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TFIID	Transcription Factor D for DNA polymerase II
TFIIF	Transcription Factor F for DNA polymerase II
TME	Tris-Mes-EDTA buffer

Tris	Tris(hydroxymethyl)aminomethane
UCSC	University of California at Santa Cruz
UTP	Uridine triphosphate
UTR	Untranslated region of mRNA

Chapter 1

General Introduction

1.1 Project Background

Genetic analysis of human heritable skeletal disorders is of dual interest — medical and biological. Individual disorders may be infrequent but collectively they comprise a large and diverse class of clinical entities, traditionally divided into malformations of specific elements – the dyschondroosteoses – and generalised defects of cartilage and bone formation – the osteochondrodysplasias –, with significant incidence that often pose diagnostic challenges. Mutational data confirm, supplement, or even override clinical and radiographic findings in diagnosis and nosological classification. From a biological perspective, they are viewed as patterning anomalies and perturbations of growth and differentiation that provide entry points into the molecular interactions controlling skeletal development. Their analysis, coupled with mouse genetics, has revealed a broad spectrum of important genes. Gene products include components of signal transduction networks, transcription factors, metabolic enzymes and extracellular matrix proteins — reviewed by Superti-Furga et al. (2001), Zelzer and Olsen (2003), and Kornak and Mundlos (2003). The power of the genetic approach, demonstrated in the discovery of pivotal regulators of bone and cartilage histogenesis like *Cbfa1* and *Sox9* amongst other molecules, resides in the relative absence of bias: prior assumptions or even knowl-

edge regarding functions is not necessary. On the other hand, it is constrained by pleiotropy and redundancy. Certain genes are used iteratively during development in various systems and global loss of function may cause early lethality, obscuring a requirement during later stages; and the presence of structurally and frequently functionally related members of multigene families in vertebrates may limit the number and severity of informative phenotypes.

The skull is, by far, the most elaborate part of the vertebrate skeleton and craniofacial malformations are common findings in numerous human disorders, often congenital, and mouse mutants — reviewed by Wilkie and Morriss-Kay (2001). Very broadly, these disorders can be considered as affecting: (1) patterning and growth of the branchial arches, as in the homonymous group of syndromes; (2) patterning and growth of the anterior midline structures, exemplified by holoprosencephaly and the various palatal/facial clefts; (3) neural tube closure — failure causing brain hypoplasia and exposure or protuberance, secondarily associated with absence or hypoplasia of the skull vault; (4) intrinsic patterning and growth of the skull vault, without neural involvement, as in the craniosynostotic conditions; (5) patterning and growth within and around the sensory capsules, usually linked to eye and ear defects; (6) maturation of bone and cartilage, reflecting arrest of differentiation or lack of extracellular matrix components; (7) bone remodelling, caused by dysregulated osteoclastic activity.

Over the last decade, a fruitful line of investigations has focused on the molecular basis of craniosynostosis, characterised by premature fusion of the cranial sutures. The initial identification of mutations in the fibroblast growth factor receptor genes, *FGFR1–3*, as well as in the transcription factors *TWIST1* and *MSX2* has evolved into a corpus of molecular, clinical and embryological data regarding development of the skull vault (Wilkie 1997; Muenke and Wilkie 2001). Study of somewhat contrasting anomalies, such as inherited defects of skull vault ossification, could complement and enrich this body of knowledge. Enlarged parietal



Figure 1.1: Radiographic appearance of typical enlarged parietal foramina. The paired, symmetric oval openings located on either side of the posterior sagittal suture are separated by a strip of bone traversed by a minor suture. Standard anteroposterior skull radiograph of the 12-year-old individual III-5 in family 1 (see section 3.2.4.1), courtesy of Dr Alberto Salamanca.

foramina (PFM), oval or round bilateral defects of the parietal bones (Figure 1.1), are encountered either in isolation or in a syndromic context. Isolated defects (OMIM 168500) usually exhibit an autosomal dominant mode of inheritance and represent a rare yet clinically well-documented familial trait, described in numerous reports in the literature, that attracted attention several decades ago — reviewed by O’Rahilly and Twohig (1952).

Four years ago, little was known about the molecular genetics of PFM. Wilkie et al. (2000) investigated the segregation of several candidate genes in families with isolated PFM. Amongst them was the homeobox gene *MSX2*, considered because a gain-of-function mutation associated with enhanced DNA binding had been causatively implicated in a craniosynostotic condition (Jabs et al. 1993). It was envisaged that loss-of-function mutations could correlate not with precocious fusion but with impaired growth of the flat bones. Indeed, a large deletion around *MSX2* was found to segregate with PFM and pathogenic intragenic changes were also identified (Wilkie et al. 2000): haploinsufficiency of *MSX2* can cause skull

defects, highlighting the gene as a pivotal regulator of skull vault development. However, the locus was definitively excluded by linkage analysis in certain families (Wilkie et al. 2000), indicating the existence of a second, at least, disease gene.

Hence, the initial goal of this project was to identify further gene(s) mutated in PFM. Genetic heterogeneity, when limited and dissectable, can present a valuable opportunity, exposing several interrelated genes in a biological system. Before resorting to genome-wide linkage analysis for the identification of a second gene, various clues may be considered — stemming from mouse mutations, existence and properties of paralogues, functional relationships in conserved pathways, and chromosomal aberrations. In this introductory section, a presentation of the disease model in its clinical and biological context — phenotypic and genetic aspects of skull vault defects in humans and mice in conjunction with fundamentals of underlying molecular pathways — and a discussion of the evidence for the second PFM locus, are preceded by an overview of the wider biological system, the mammalian skull — its structure, history, and development, focusing on the skull vault.

1.2 The Human and Mouse Skull

1.2.1 Introduction

The mammalian skull is of exceptional complexity: an assemblage of skeletal elements with intricate spatial relationships, developmental programmes exhibiting either intramembranous or endochondral ossification, dual tissue derivation from neural crest and mesoderm, and distinct evolutionary origins. Customarily, it is divided into the cranium proper, the braincase surrounding the brain, and the facial bones, protecting the nasal and oral cavities — an alternative classification, based on evolutionary grounds, is discussed in section 1.2.3.

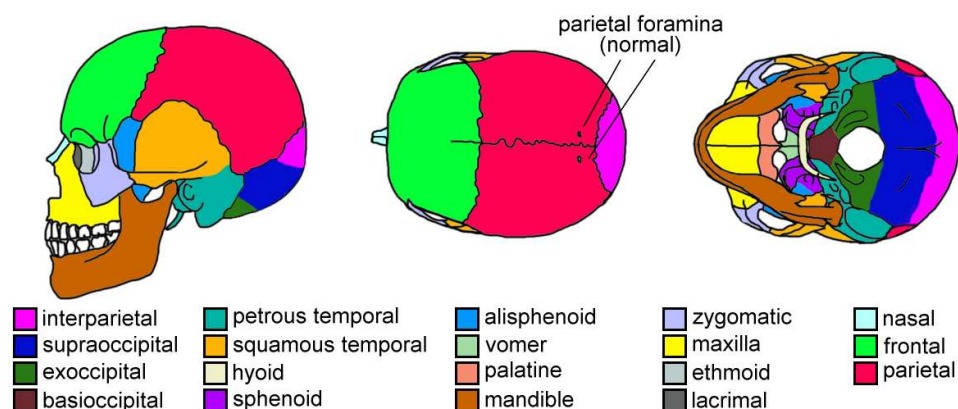


Figure 1.2: Generalised scheme of the adult human skull — lateral, superior (dorsal), and inferior (ventral) views. Apart from the auditory ossicles – incus, malleus, stapes – and the inferior nasal conchae all distinct osseous elements are depicted. The composite occipital and temporal bones are subdivided to demonstrate their constituent parts; in the latter, the tympanic ring is not shown. From the composite sphenoid only the alisphenoid is singled out. For comparison with the murine skull the same colour code is employed in Figure 1.3. Drawings adapted from Martini and Timmons (1997).

1.2.2 Anatomical Aspects

Figure 1.2 illustrates the structural organisation of the adult human skull (Williams and Warwick 1980; Martini and Timmons 1997). The composite occipital bone – a fusion of the interparietal, supraoccipital, paired exoccipital, and basioccipital segments – surrounds the foramen magnum. Anteriorly, it meets the compound sphenoid bone (its body is formed by the basisphenoid and presphenoid parts; laterally, the orbitosphenoid and alisphenoid segments participate in the lesser and greater wings respectively; and ventrally, the pterygoid processes project downwards). The temporal bone consists of the petrous part, the squamous part and the embedded tympanic ring. With the addition of the ethmoid bone in the nasal region, these elements form the skull base and contribute to much of the lateral and posterior surfaces. Numerous bones participate in the construction of the face. Superficially, the mandible, the maxilla, the nasals, the zygomatics, and the lacrimals

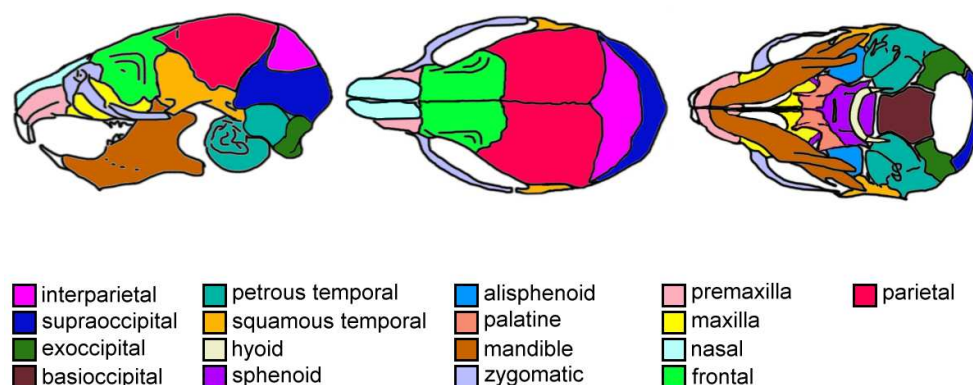


Figure 1.3: Generalised scheme of the adult mouse skull — lateral, superior (dorsal), and inferior (ventral) views. With the exception of the auditory ossicles – incus, malleus, stapes –, the lacrimals, the ethmoid (including the turbinates), and the vomer all distinct osseous elements are depicted. The composite occipital and temporal bones are subdivided to demonstrate their constituent parts; in the latter, the tympanic ring is not shown. From the composite sphenoid only the alisphenoid is singled out. For comparison with the human skull the same colour code is employed in Figure 1.2. Drawings based on material by Forsthoefel (1962) and Depew et al. (2002).

define its contour. More deeply, the ethmoid, the lower nasal conchae, the palatines, and the vomer assist in shaping the nasal and oral cavities. Below the mandible the hyoid bone is suspended. Superiorly, the cranial cavity is completed by the large, paired frontal and parietal bones.

Although of different proportions, with more distinct elements, and demonstrating rodent-specific adaptations, the mouse skull, outlined in Figure 1.3, conforms to the same basic pattern (Kaufman and Bard 1999; Depew et al. 2002).

1.2.2.1 The Calvaria

This term describes the upper part of the braincase, consisting of several flat bones that encase the cerebral hemispheres. In this work it is used in the narrow sense, to refer to the ancestral series of roofing dermal bones (see section 1.2.3.1): the paired frontal and parietal bones as well as the single interparietal bone. When viewed

from above (Figures 1.2 and 1.3), the frontal bones form a cap in the forehead region and articulate posteriorly with the parietal bones. Each parietal bone is irregularly quadrilateral, slightly elevated near its centre, the parietal eminence. The interparietal bone, which is separate in the mouse skull, extends the cover to the occipital region. On section, the calvarial bones display compact outer and inner tables with intervening cancellous osseous tissue – the *diplöe* – and are lined by a fibrous periosteal membrane which is continuous with the *dura mater*.

Sutures, technically fibrous synarthroses, mark the boundaries of the calvarial bones. Only traces of the metopic suture between the frontal bones can be noticed in the adult human skull; and in the mouse, its posterior part obliterates equally early. By contrast, the coronal, sagittal, and lambdoid sutures remain patent into adulthood and traverse the calvarial surface. At the bevelled coronal suture the anterior borders of the parietal bones overlap with the posterior edges of the frontals. The serrated sagittal suture separates the parietal bones, running from the frontal to the interparietal region. And at the lambdoid suture, the parietal bones interlock with the interparietal bone. Occasionally, few small intrasutural (Wormian) bones are encountered in normal skulls.

Two minute bilateral perforations, usually not exceeding 1 mm in diameter, located 2–5 cm in front of the meeting point of the sagittal and lambdoid sutures and in close proximity to the former, interrupt the surface of the parietal bone in a significant proportion of human skulls (Figure 1.2). These parietal foramina transmit minor anastomotic vessels and are an established, normal anatomical variant (O’Rahilly and Twohig 1952; Currarino 1976; Penteado and Santo Neto 1985).

1.2.3 Evolutionary Aspects

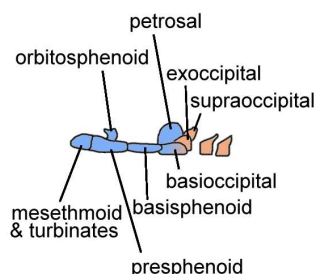
Components with different evolutionary histories have been brought together to construct the mammalian skull. The most apparent parts are: (1) the cartilaginous

neurocranium, which first arose in the agnathans as a protective case for the brain and the sense organs; (2) the cartilaginous viscerocranium, whose evolutionary predecessor is the tissue reinforcing the pharyngeal slits in acraniates; (3) and the dermatocranium, derived from the osseous armour that covered certain agnathans (Kardong 1998). A refined scheme (Morriss-Kay 2001) would add a fourth component: (4) the occipital arch region, which can be traced to the sclerotomes of a number of occipital somites that have been annexed into the skull.

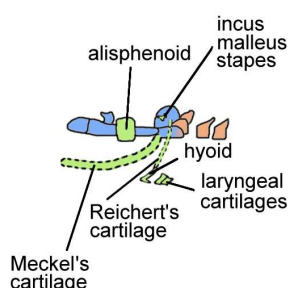
The cartilaginous neurocranium, incorporating the sensory capsules, and the occipital sclerotomes establish a supportive keel for the brain and the sense organs, forming the floor and part of the side walls of the cranial cavity (Figure 1.4). The mesethmoid with the turbinates, the presphenoid, the basisphenoid, and the basioccipital define a platform which is extended by the occipital sclerotomes, concealed, at least, in the exoccipitals and the supraoccipital. The nasal capsule has been integrated in the most anterior element – the mesethmoid with the turbinates – while the orbitosphenoid is the successor of the optic capsule. The otic capsule has expanded as the petrous part of the temporal bone.

The cartilaginous viscerocranium has a minor contribution, providing few primary structures and certain derivatives (Figure 1.4). In the first branchial arch, Meckel's cartilage appears during embryonic development yet degenerates almost completely, giving rise to the malleus at its posterio-dorsal end. The incus and the alisphenoid cartilage (corresponding only partially to the alisphenoid bone in the adult) are remnants of a second element, the palatoquadrate of early gnathostomes. The second arch cartilage – Reichert's cartilage – forms part of the hyoid, the styloid process of the temporal bone as well as the stapes. The cartilaginous bar of the third arch contributes to the hyoid while certain laryngeal cartilages are derived from the fourth and sixth arches.

Cartilaginous neurocranium & occipital sclerotomes



Cartilaginous viscerocranium



Dermal bones

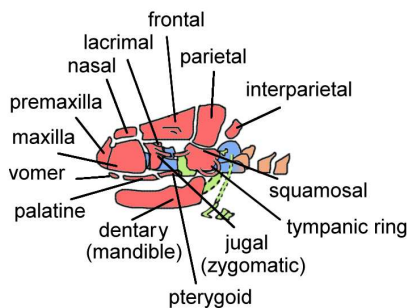


Figure 1.4: Phylogenetic deconstruction of the mammalian skull. Four components with distinct origins contribute to the overall structure. The cartilaginous neurocranium (*blue*) consists of a supportive platform from the ethmoid to the basal region with the associated sensory capsules — olfactory, optic, and otic. The very posterior part that forms the occipital arch is segmented, derived from the sclerotomes of the occipital somites (*beige*). Derivatives of the branchial arch skeletal rods make up the cartilaginous viscerocranium (*green*) — transitory structures are outlined. The alisphenoid cartilage corresponds only partially to the alisphenoid bone which may have a dermal contribution. The dermal bones (*red*) cover the majority of other elements and define the contour of the skull. Schemes adapted from Kardong (1998).

1.2.3.1 The Dermal Bones

The dermatocranium dominates the mammalian skull, providing numerous membranous bones that overlie, ensheathe, or even replace other skeletal elements (Figure 1.4). Phylogenetically, several series of bony plates arose in the integument of early fishes but in mammalian skulls certain elements have sunk inwards, complexed with other structures, fused, overgrown, diminished, or disappeared. The facial subset consists of the nasals, the premaxilla and the maxilla that shape the snout. Only the jugals (zygomatics) and the lacrimals remain from the original orbital series that housed the eyeball. Equivalently, the original temporal series is reduced to the squamosals. The palatal subset consists of the vomer, the palatines and the pterygoids while the dentary (mandible) is the sole component of the mammalian mandibular series. However, the vault series that roofs the brain is representative of the archetypical organisation, although some fusion may have taken place in the anterior part. It comprises the paired frontals, the paired parietals and the single interparietal bone.

1.2.4 Embryological Aspects

Cephalisation of the embryo starts with expansion of the neural plate at the anterior end. Subsequently, the primitive head forms around the neural tube, the anterior tip of the notochord, and the pharynx. Cell populations from all three conventional germ layers and from the cranial neural crest participate in its construction. The space between the epidermis and the neuroepithelium as well as the core of the branchial arches is occupied by proliferating undifferentiated mesenchyme of neural crest and mesodermal origin; these are the sites where skeletogenic condensations develop.

1.2.4.1 Head and Neck Shaping

Morphogenesis of the head and neck begins just after closure of the anterior neuro-pore with the appearance of five swellings around the stomodeum (Larsen 1997; Carlson 1999; Kaufman and Bard 1999). Around embryonic day (E) 9 in mice and at the beginning of the fourth week in humans the first pair of branchial arches has developed and each arch splits into a maxillary and a mandibular process. On an unpaired bulge over the forebrain, the frontonasal process, the developing nasal pits define a medial and lateral nasal process on each side. Fusion of the mandibular processes in the midline along with fusion of the maxillary processes with the medial nasal processes produces the rudiment of the face.

1.2.4.2 Histogenesis of Cartilage and Bone

A mesenchymal condensation is the earliest histologically discernible event during skeletogenesis (Patten 1964; Hamilton and Mossman 1972). Cells aggregate, undergo a shape change from spindle-like to round, proliferate in a controlled fashion, and alter locally the composition of the extracellular matrix. It is likely that inductive interactions with an epithelial sheet, either epidermal or neuroepithelial, during cell migration precede condensation which in itself is a multi-phasic process comprising initiation, establishment of boundaries, focal proliferation, adhesion, and overall growth in size. When a critical mass is reached, overt differentiation to osseous or cartilaginous tissue is instigated — cellular and molecular aspects of these early phases have been reviewed by Hall and Miyake (2000).

Whereas expansion of chondrification centres is mostly interstitial, as the dividing chondrocytes are pushed apart by their own matrix, growth during intramembranous ossification is appositional. Pre-osteoblasts, committed but not terminally differentiated cells that have developed from mesenchymal precursors, line the edges of dense fibrous strands of osteoid — unmineralised matrix consisting of bone-specific glycosaminoglycans and proteoglycans, collagen fibers, and

specialised glycoproteins. The mature, matrix-secreting cell which contributes to further expansion of the osteoid is designated as the osteoblast. Subsequent impregnation with calcium, in the form of hydroxyapatite, gives rise to the primitive osseous trabecula. Coalescence of trabeculae produces primary cancellous bone — an irregular latticework with ample unossified space. Once trapped within the calcified matrix, osteoblasts are referred to as osteocytes. Synchronised waves of matrix deposition coupled with extensive re-modelling through osteoclastic action can form compact bone. In endochondral ossification, an external osseous collar forms and, concurrently, a periosteal bud invades the cartilaginous anlage; osteoid is laid down along the strands of deteriorating cartilage at the growth plate.

1.2.4.3 Appearance and Growth of Cartilaginous and Osseous Elements — an Overview

The series of schemes in Figure 1.5 summarise the development of the human skull during the early fetal period and illustrate its status at birth (Patten 1964; Hamilton and Mossman 1972; England 1990; Larsen 1997; Carlson 1999). At the sixth week, the bauplans of the cartilaginous neurocranium and viscerocranium have emerged. Blocks of paired cartilages, the parachordal cartilages, have appeared and fused around the anterior end of the notochord, whose extremity reaches the level of the future hypophyseal fossa. Posteriorly, metameric cartilages have developed from the occipital sclerotomes. Anteriorly, a pair of small hypophyseal cartilages is followed by the elongated trabeculae cranii. Laterally to these mid-line structures, centres associated with the primordia of the sense organs have appeared — the nasal capsule, the ala orbitalis (future orbitosphenoid), and the otic capsule — joined by the ala temporalis (future alisphenoid cartilage), possibly derived from the first branchial arch. Ventrally, cartilaginous elements have formed in the branchial arches: the prominent rod of Meckel's cartilage spans the mandibular process. Ossification centres are evident in the eighth week and correspond to

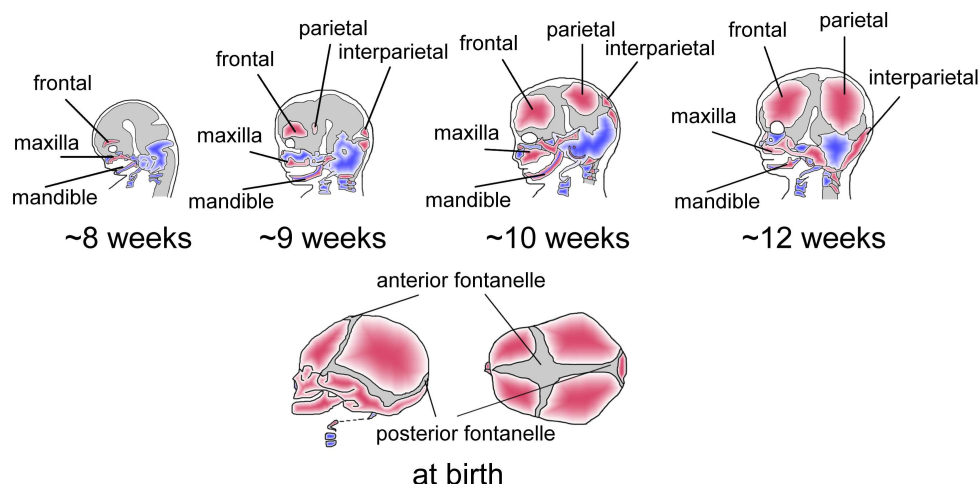


Figure 1.5: Schematic account of early stages in human skull development and appearance of the skull at birth — emergence and growth of cartilaginous (*blue*) and osseous (*red*) elements. At ~ 8 weeks, the cartilages of the neurocranium have fused in a continuous platform and the cartilaginous elements of all branchial arches are apparent; intramembranous ossification commences in the mandible, the maxilla, and the frontal region. At ~ 9 weeks, ossification centres for the nasal, the parietal, the squamous temporal, and the interparietal membranous bones develop while localised endochondral ossification is seen in the occipital cartilages. At ~ 10 weeks, bony spicules have spread over the frontal and parietal regions, the zygomatic arch has shaped, and the tympanic ring appears; endochondral ossification has also advanced. At ~ 12 weeks, the frontal and parietal plates are already large and most of the adult structures are recognisable; part of the skull base is still cartilaginous. In the skull of the neonate wide sutures separate the growing calvarial bones and two larger gaps are palpable, the anterior and posterior fontanelles. The schemes, based on primary material in Patten (1964), Hamilton and Mossman (1972), and England (1990), are indicative and not drawn to scale.

dermal bones: the inferior part of the frontal bone and the mandible. Within the next two weeks, intramembranous ossification is in progress at other sites, including the parietal bones, and endochondral ossification has commenced in the cranial base; by twelve weeks most of the adult structures are recognisable. The neonatal skull differs from the adult in its shape and proportions. The plasticity required to accommodate the enlarging brain is reflected in two adaptations: the synchron-

droses of the skull base have not fused and the bony plates of the skull vault are separated by wide strips of fibrous tissue.

Gross skull development in the mouse, depicted schematically in Figure 1.6, follows a comparable course of events (Roberts 1990; Kaufman 1992; Kaufman and Bard 1999). The foundations of the cartilaginous neurocranium and viscerocranium have been laid down by E12–12.5 and intramembranous ossification commences in the inferior frontal bone and the mandible around E13.5–E14. Ossification advances rapidly and by E18.5 the adult configuration is reached. At birth, relatively narrow sutural spaces demarcate the calvarial bones and cartilaginous structures persist, chiefly in the occipito-temporal region.

1.2.4.4 Embryology of the Calvaria — a Descriptive Account

The membranous sheath of the brain splits into an outer skeletogenic layer, the ectomeninx, which differentiates into the dermal bones and the dura mater, and an inner layer, the endomeninx, which gives rise to the arachnoid and pia mater (Hamilton and Mossman 1972). Within the condensation of the ectomeninx, osteoblastic differentiation is triggered (Patten 1964; Hamilton and Mossman 1972; Shapiro and Robinson 1980; England 1990; Roberts 1990; Kaufman 1992; Kaufman and Bard 1999). Thin trabeculae are seen initially in the supra-orbital part of the frontal bone (Figures 1.5 and 1.6), over the orbitosphenoid cartilage, around the eight week in humans and E13.5–14 in mice; ossification rapidly spreads laterally and dorsally, presenting as radiating bony spicules. One, or maybe two, centres develop in the inferior parietal region, over the alisphenoid/temporal cartilages, soon afterwards (Figures 1.5 and 1.6) and expand dorsolaterally in a similar fashion. Ossification in the interparietal region is apparent slightly later. As the osteogenic fronts approximate each other, sutures are induced along the lines of apposition; at their points of confluence, where more than two bones meet, wider gaps are formed.

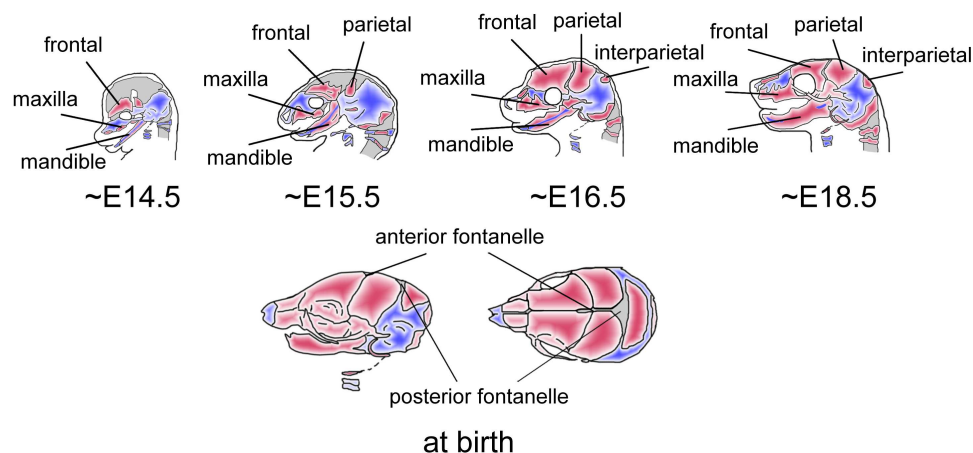


Figure 1.6: Schematic account of early stages in mouse skull development and appearance of the skull at birth — emergence and growth of cartilaginous (*blue*) and osseous (*red*) elements. At $\sim$ E14.5, the cartilaginous neurocranium and viscerocranium are well-developed; intramembranous ossification is under way in the mandible, the maxilla, and the frontal region; the parietal region lags slightly behind. At $\sim$ E15.5, intramembranous ossification has advanced in the calvaria and the jaws, while centres of endochondral ossification have appeared in the occipital cartilages. At $\sim$ E16.5, the expanded frontal and parietal bones are joined by the growing nasal, interparietal, and squamous temporal elements; the tympanic ring appears as well; endochondral ossification in the skull base has also progressed. At $\sim$ E18.5, most of the mature structures are recognisable. At birth, the frontal and parietal plates have approximated along the coronal and sagittal sutures which meet at a very narrow anterior fontanelle; on the contrary, a wide posterior fontanelle is present in front of the interparietal bone; several cartilages in the nasal, sphenoid, temporal, and occipital regions persist. The schemes, based on primary material in Roberts (1990) and Kaufman (1992), are indicative and not drawn to scale.

However, ossification in the parietal plates does not advance at a uniform pace, at least in the human skull. In the supero-posterior region, along the sagittal suture and in front of the junction with the lambdoid suture, slowing results in a notch which is usually obliterated by the fifth fetal month (Currarino 1976). Remnants of this parietal notch present as normal anatomical variants in postnatal skulls — minute bilateral parietal foramina (see section 1.2.2.1), a tiny sagittal fontanelle,

or a parietal fissure. Abnormally delayed or inadequate ossification can produce pronounced local defects: a pair of enlarged parietal foramina, a single medial foramen, or an oblong midline gap (Currarino 1976). These developmental anomalies can be striking at birth and early postnatal life when compared with the normal calvarial pattern (Figure 1.5). The sutural spaces are relatively wide in the neonate but only two medial fontanelles are constantly present — the anterior, at the intersection of the coronal and sagittal sutures, and the posterior, at the intersection of the sagittal and lambdoid sutures. The posterior fontanelle disappears within the first two months, whereas the anterior closes by the end of the second year (Hall et al. 1989).

1.2.4.5 Tissue Origins of the Skull

The vertebrate skull owes its existence to the evolution of the neural crest. Only cranial neural crest can form skeletal tissue and cell detachment and migration is under way by E8.5 in mice and the third week in humans. Moving ventrally, streams from the posterior forebrain and midbrain populate the frontonasal process while streams from the midbrain and the first three hindbrain rhombomeres enter the maxillary and mandibular processes. Clearly, skeletal elements that develop in the frontonasal region and the branchial arches are of neural crest origin and when the skull base is considered the boundary between mesodermal and neural-crest derived mesenchyme is drawn at the future hypophyseal fossa — discriminating between parachordal, somitic or cephalic, and prechordal mesenchyme respectively (Le Douarin and Kalcheim 1999).

However, the derivation of the calvarial bones has been controversial. Interpretations based on traditional grafting experiments considered the entire skull vault as either of neural crest (Couly et al. 1993) or mesodermal origin (Noden 1988), although there was consensus regarding the neural crest origin of the dura mater. Recent studies, employing a *Wnt1*-activated reporter system to mark and

follow neural crest cell populations, indicate that the frontal bones, the dura mater, and the mid-sutural mesenchyme of the sagittal suture are of neural crest origin whereas the parietal bones are mesodermal (Jiang et al. 2002).

1.3 Genetic Determinants of Calvarial Defects

1.3.1 Introduction

In principle, disturbances during cell specification, migration, condensation, growth, and differentiation may result in incomplete calvarial ossification. Genetic and environmental factors, including teratogenic drugs, can cause defects of variable presentation and severity, with or without extracranial skeletal or non-skeletal anomalies. Several heritable human conditions feature skull vault defects as an obligatory or accessory clinical and/or radiographic finding. Apart from the case of isolated, non-syndromic PFM, partially attributable to *MSX2* mutations, two other disorders of interest have been molecularly elucidated so far: mutations of *RUNX2* cause cleidocranial dysplasia (CCD); and mutations in *TWIST1* underlie Saethre-Chotzen syndrome (SCS). However, other clinical entities exist and available clinical and/or genetic data may point to new disease loci. Additionally, skull vault defects are encountered in the phenotypic spectrum of numerous mouse mutants, including the ones for *Msx2*, *Runx2*, and *Twist1*, and known mouse genes could flag human counterparts as candidates. In this section a short list of relevant human genetic disorders is presented, the genetics of known loci are discussed in brief, and the disease model of the study is introduced in more detail. A list of selected mouse mutants complements this approach.

1.3.2 Human Genetic Disorders

1.3.2.1 Inherited Disorders Associated with Skull Vault Defects: a List

Table 1.1 is a non-exhaustive compilation of clinical entities where skull vault defects are a prominent or frequent finding. Metabolic causes and conditions related to primary neural defects, like the majority of meningoencephaloceles, hydrocephaly, cranial cysts, etc., have been omitted. Even after this filtration, short-listed entries demonstrate significant phenotypic and genetic diversity; the calvarial defects themselves range from suture widening to absence of all roofing bones at birth.

In the dominantly inherited Adams-Oliver syndrome, vascular disruption underlies the scalp and skull defects as well as the limb anomalies. The autosomal recessive Al-Awadi/Raas-Rothschild syndrome features severe phocomelia with skull vault defects. Aplasia cutis congenita is clinically and probably genetically heterogeneous (see section 1.3.2.4). Thin, poorly differentiated, and almost transparent skin can cover a skull defect. CCD is the prototype disorder associated with clavicular hypoplasia or aplasia; in addition, a wide midline gap spans the skull vault. The craniomicromelic syndrome is an extremely rare, apparently autosomal recessive condition characterised by global delay of skeletogenesis, skull defects, and visceral abnormalities. The entry 'delayed membranous cranial ossification' comprises two familial incidences of a remarkable disorder: complete absence of the frontal, parietal, squamous temporal, and interparietal dermal bones in infancy without other major skeletal abnormalities. In one kindred the defect was found to segregate with a balanced translocation, $t(2;3)(p15;q12)$. The combination of multiple calvarial defects with adjoining sclerotic bone, producing the impression of doughnut-shaped lesions, is probably the result of abnormal bone remodelling. Frontonasal dysplasia is characterised by facial dysmorphism and a defect in the frontal region. In the recessive entity Meckel syndrome type 1, a parieto-occipital encephalocele is associated with renal and hepatic degeneration

and, also, polydactyly. Abnormally wide sutures and fontanelles are seen in the neonatal progeroid syndrome. PFM stand out as a characteristic bilateral defect and occur both in isolation and in certain syndromes — as in conjunction with defective ossification of the clavicles (PFMCCD), in the proximal 11p deletion syndrome (P11pDS), and, variably, in SCS. The manifestations in the rare autosomal recessive pseudoaminopterin syndrome mimic the effects of exposure to the teratogens aminopterin and methotrexate and include skull defects. Finally, calvarial defects have been observed in the Schinzel-Giedion syndrome while suture widening has been noticed in combination with micrognathia, clavicular hypoplasia, and defects of the digits in the Yunis-Varon syndrome.

1.3.2.2 Established Disease Genes: *RUNX2* in Cleidocranial Dysplasia and *TWIST1* in Saethre-Chotzen Syndrome

The hallmark features of CCD (OMIM 119600), showing autosomal dominant inheritance with almost complete penetrance, are: (1) hypoplasia/aplasia of the clavicles, affecting mostly the lateral ends; (2) defective ossification of the skull vault, presenting as a central gap from the frontal to the occipital region; (3) dental anomalies, including delayed eruption as well as supernumerary teeth; (4) and short stature due to global bone and cartilage dysplasia. Certain malformations in specific axial or appendicular elements and characteristic facial dysmorphism complete the clinical picture which shows some variability (Mundlos 1999). Haploinsufficiency of *RUNX2*, located on chromosome band 6p21 and encoding a transcription factor of the Runt family, causes CCD: not only missense, nonsense, frameshift, and splice site mutations, but also large deletions removing the entire gene segregate with CCD (Mundlos et al. 1997; Lee et al. 1997; Quack et al. 1999; Zhou et al. 1999; Otto et al. 2002) — the mutational spectrum was reviewed by Otto et al. (2002).

Table 1.1: Human Inherited Diseases Associated with Skull Vault Defects

OMIM Number	Disorder	Craniofacial Features	Other Major Anomalies	Gene	References
100300	Adams-Oliver syndrome	Scalp and skull vault defects	Limb truncations, cardiovascular anomalies	—	Whitley and Gorlin (1991)
276820	Al-Awadi/Raas-Rothschild syndrome	Skull vault defects	Phocomelia, pelvic hypoplasia/aplasia	—	Olney et al. (2001)
107600	Aplasia cutis congenita	Skull vault defects	Scalp defects	—	Evers et al. (1995)
119600	Cleidocranial dysplasia (CCD)	Midline skull vault defect	Clavicular hypoplasia/aplasia, dental anomalies, abnormalities of the axial and appendicular skeleton	<i>RUNX2</i>	Mundlos (1999)
602558	Craniomicromelic syndrome	Skull vault defects	Generalised growth retardation, digital anomalies	—	Baralle and Firth (1999)
155980	Delayed membranous cranial ossification	Delayed calvarial ossification, mild dysmorphic features	—	—	Gonzalez-del Angel et al. (1992) Cargile et al. (2000)
126550	Doughnut lesions of skull	Multiple skull vault defects surrounded by sclerotic bone	—	—	Baumgartner et al. (2001)
136760	Frontonasal dysplasia	Cranium bifidum, facial asymmetry	Limb abnormalities, mental retardation	—	Nevin et al. (1999)
249000	Meckel syndrome, type 1	Encephalocele	Polydactyly, renal and hepatic cysts	—	Paavola et al. (1997)
264090	Neonatal progeroid syndrome	Wide sutures and fontanelles	Progeroid appearance, severe growth delay, hair defects, abnormal lipid metabolism	—	Pivnick et al. (2000)
168500	Enlarged parietal foramina/Cranium bifidum (PFM)	Enlarged parietal foramina, cranium bifidum	—	<i>MSX2</i>	Wilkie et al. (2000)
168550	Enlarged parietal foramina with cleidocranial dysplasia (PFMCCD)	Enlarged parietal foramina	Clavicular hypoplasia	—	Golabi et al. (1984)
601224	Proximal 11p deletion syndrome (P11pDS)	Enlarged parietal foramina	Multiple exostoses, mental retardation	—	Bartsch et al. (1996)

Table 1.1 — Continued

OMIM Number	Disorder	Craniofacial Features	Other Major Anomalies	Gene	References
600325	Pseudoaminopterin syndrome	Skull vault defects	Occasionally other skeletal anomalies, body wall defects	—	Barnicoat et al. (1994)
101400	Saethre-Chotzen syndrome (SCS)	Craniosynostosis, persistent fontanelles, enlarged parietal foramina	Soft tissue syndactyly	<i>TWIST1</i>	Howard et al. (1997) El Ghouzzi et al. (1997)
269150	Schizel-Giedion midface retraction syndrome	Wide sutures and persistent fontanelles, Wormian bones, sclerotic skull base, midface retraction	Hydronephrosis, hypertrichosis, brain anomalies, limb defects	—	Labrune et al. (1994)
216340	Yunis-Varon syndrome	Wide sutures, micrognathia	Clavicular hypoplasia/aplasia, digital defects	—	Hughes and Partington (1983)

Sources: The list was compiled mostly from secondary sources: structured queries against OMIM, material in a reference text (Gorlin et al. 2001), and data in the review by Wilkie and Morriss-Kay (2001).

Notes: Both the OMIM number and the most common or appropriate designation are listed, followed by a description of the major craniofacial features and a brief note of the abnormalities in other systems. There are three established major loci, *RUNX2*, *TWIST1*, and *MSX2*, associated with cleidocranial dysplasia, Saethre-Chotzen syndrome, and non-syndromic enlarged parietal foramina respectively. Disorders of calcium and phosphate metabolism as well the majority of clinical entities with pronounced neural involvement – as in encephaloceles or hydrocephaly – are excluded. In addition, extremely rare syndromes represented by a single case report are omitted.

SCS falls within the acrocephalosyndactyly syndromes. It is inherited in an autosomal dominant fashion but with incomplete penetrance and shows pronounced phenotypic variability. Common features include craniosynostosis, low frontal hairline, ptosis of the eyelids, facial asymmetry, a pointed nose, prominent ear crus, and mild cutaneous syndactyly between the second and third digits (Rear-don and Winter 1994). Paradoxically, suture obliteration, usually of the coronal suture, can co-exist with skull ossification defects. Several reports of PFM in SCS suggest that the association is not coincidental (Friedman et al. 1977; Thompson

et al. 1984; Young and Swift 1985; Wilkie et al. 1995b). Haploinsufficiency of the *TWIST1* gene that maps at chromosome band 7p21 and encodes a basic helix-loop-helix transcription factor underlies SCS. Nonsense, missense and frameshift mutations, as well as recurrent small intragenic duplications without any apparent genotype-phenotype correlation point to a loss-of-function mechanism (El Ghouzzi et al. 1997; Howard et al. 1997). This is corroborated by the characterisation of deletion events around the *TWIST1* locus (Johnson et al. 1998); Muenke and Wilkie (2001) tabulated the mutations. Oppositely, trisomies encompassing the 7p21 segment, including *TWIST1*, are consistently associated with large fontanelles and wide sutures (Stankiewicz et al. 2001; Megarbane et al. 2001).

1.3.2.3 Non-Syndromic Enlarged Parietal Foramina and the *MSX2* Gene

Also known as foramina parietalia permagna, fenestrae parietales symmetricae, and giant parietal foramina, PFM are typically oval or round, symmetric defects of the parietal bones alongside the posterior part of the sagittal suture (Figure 1.1 and Figures 3.6, 3.7, 3.8, and 3.9). Although located in the same region as the normal, minute foramina, they tend to be unmistakable because of their much greater size as soft spots upon palpation and/or as striking lucencies on plain radiographic examination — exceeding 5 mm in diameter (Stallworthy 1932; O’Rahilly and Twohig 1952; Lodge 1975; Currarino 1976; Pang and Lin 1982; Little et al. 1990). A fibrous membrane, continuous with the dura mater, covers the openings and the overlying skin is usually grossly normal; no large vessels traverse the gaps. Furthermore, no prominent cerebral anomalies underlie the defects and intelligence is unaffected (modern imaging has revealed subtle anatomical variations of the brain (Reddy et al. 2000): atypical patterning of the network of cranial venous sinuses and peculiar infolding of the cortex in the occipital lobe). However, recurrent episodes of fierce headaches, vomiting, nausea, and intense local pain are sometimes indis-

putably associated with the defects, especially on application of even mild pressure to the unprotected cerebral cortex (Pang and Lin 1982).

Being a rather benign anatomical abnormality, PFM usually come to medical attention incidentally — during prenatal scanning or routine postnatal paediatric assessment and after head injuries. It seems that few hundred reports have accumulated since the original description in the nineteenth century, scattered in the anatomical, paediatric, neurological, radiological, and genetic literature (Gorlin et al. 2001). The first report in English is credited to Turner (1866), although his case was an atypical one. An example in a living person (Greig 1892) was followed by several case reports. Goldsmith (1922) noted familial occurrence in a large kindred but failed to recognise the mode of inheritance. Around 1920–1930 it had been registered as a rare, erratic hereditary disorder of calvarial ossification (Greig 1927; Pepper and Pendergrass 1936). On review of the literature Pang and Lin (1982) concluded that PFM are usually inherited as an autosomal dominant Mendelian trait, complicated by incomplete penetrance and variable expressivity. PFM have been observed in non-Caucasians (Travers and Wormley 1938; Kinoshita and Sunami 1980; Little et al. 1990; Kortesis et al. 2003) and there is evidence to suggest a prevalence figure in the range of 1/15,000 – 1/50,000 (Moore 1949; Lodge 1975).

Developmentally, the circumscribed openings reflect impaired ossification at the lateral margins of the fetal parietal notch and tend to close slowly with age, although there is significant variability even within families. Meticulous recordings of the natural history of the disorder have showed that PFM are in continuum with a more severe midline defect, confluent with the anterior and posterior fontanelles, known as cranium bifidum occultum — literally, split or cleft skull. In several patients, progress from cranium bifidum in infancy to PFM during childhood has been documented (Pendergrass and Pepper 1939; Terrafranca and Zellis 1953; Murphy and Gooding 1970; Fein and Brinker 1972; Pang and Lin 1982; Little et al. 1990). The oblong defect closes anteriorly and in the posterior parietal region two narrow

strips of bone grow towards each other in the midline; they eventually meet, dividing the large opening into left and right giant foramina. A minor suture, perpendicular to the sagittal, often develops on this bridge and small islands of ossification may give rise to Wormian bones. The openings tend to narrow further with age through circumferential bone growth. Sometimes ossification stops and the defects persist in elderly individuals (Figures 3.10 and 3.11).

Because of its characteristic appearance and absence of accompanying neural or skin lesions, differential diagnosis of PFM can be straightforward. Usually, they can be distinguished from genuine meningoencephaloceles, ventricular cysts, arachnoid cysts, ectopic glial tissue, secondary tumours, scalp defects, craniofacial/craniofenestria, osteoporosis, local inflammation, injury, infections, etc. (Lodge 1975; Currarino 1976; Pang and Lin 1982); and, importantly, from syndromic associations — as in P11pDS, SCS, PFMCCD, and aplasia cutis congenita with parietal foramina. Surgical intervention is sometimes undertaken, although its clinical indications and benefit remain uncertain. Cranioplasty employing autologous grafts or mesh plates has been performed successfully during early childhood to alleviate fear of injury and resolve neurological symptoms (Kortesis et al. 2003).

The homeobox gene *MSX2* maps to chromosome band 5q34–q35 and was the first gene to be implicated in the pathogenesis of craniosynostosis, uncovered through a mutation in the homeodomain (P148H) that increases DNA binding affinity without altering specificity in a single family, although this gain of function appears rather subtle (Warman et al. 1993; Jabs et al. 1993; Semenza et al. 1995; Ma et al. 1996). The dominant positive nature of this unique variant and the antithesis between suture obliteration and calvarial defects prompted Wilkie et al. (2000) to analyse the gene in a panel of families with non-syndromic PFM. Heterozygous loss-of-function mutations – a large deletion, a missense substitution, and a small in-frame intragenic deletion – were identified and further nonsense and missense changes were reported independently by Wuyts et al. (2000b) soon afterwards.

Nevertheless, not all PFM families have *MSX2* mutations (Wilkie et al. 2000; Wuyts et al. 2000b) and exclusion of linkage to 5q in two kindreds demonstrated unambiguously locus heterogeneity (Wilkie et al. 2000).

1.3.2.4 Clinical Entities with Syndromic Enlarged Parietal Foramina

PFM have been scored in combination with craniosynostosis, craniofacial dysmorphism, microcephaly, reduced calvarial thickness, facial clefting (O’Rahilly and Twohig 1952; Gorlin et al. 2001), and could be a minor, variable feature in the FG syndrome (OMIM 305450) (Chrzanowska et al. 1998; Rauch et al. 1998; Gorlin et al. 2001) and the Rubinstein-Taybi syndrome (OMIM 180849) (Gorlin et al. 2001). Moreover, there is a single report of co-occurrence with eye defects, genital anomalies, and mental retardation (Warkany and Weaver 1940). However, excluding SCS and even CCD proper, three adequately documented syndromic associations (Table 1.1) of dominantly inherited PFM remain. P11pDS is characterised by PFM, exostoses of the long bones, and mental retardation, caused by deletions of proximal 11p (Bartsch et al. 1996). The extremely rare entity ‘parietal foramina with cleidocranial dysplasia’ (PFMCCD) is represented by two families (Eckstein and Hoare 1963; Hall 1982; Golabi et al. 1984), where affected members presented with hypoplastic clavicles and PFM – not a midline gap – in absence of globally delayed skeletal development. In aplasia cutis congenita, scalp defects, notably local alopecia, may coexist with underlying bone defects in the form of PFM (Preis et al. 1995); Wuyts et al. (2000b) identified an *MSX2* mutation in a single family (Preis et al. 1995).

1.3.3 Mouse Mutants

1.3.3.1 Single Mutants Manifesting Skull Vault Defects: a List

A plethora of mouse mutants both from gene targeting experiments and from classical stock exhibit skull vault defects within their phenotypic spectrum (Table 1.2).

On the one hand, this list is non-exhaustive; and on the other, it is inflated by many mutations that interfere primarily with neural tube closure. However, as available delineations of head phenotypes are not always clear-cut and reliable, compared to human conditions, many of these mutants have been included. Interestingly, transcription factors and nuclear proteins in general, including the products of *Msx2*, *Runx2*, and *Twist1* genes, dominate this compilation.

1.3.3.2 Mutants of the *Msx2*, *Runx2*, and *Twist1* Genes

Msx2^{-/+} mice appear normal but homozygous null animals exhibit multiple abnormalities of the skeleton, skin, and brain, although they can reach adulthood (Satokata et al. 2000). A skull vault opening, resembling an enlarged anterior fontanelle, is seen at birth and impaired endochondral ossification results in reduced overall size. Ectodermal derivatives, including hair, teeth, nails and mammary glands, are also affected — for a detailed description see section 6.1.

Runx2^{-/-} mice die perinatally and are devoid of mature osteoblasts; the endochondral skeleton remains unossified and the condensations prefiguring the dermal bones do not form. Heterozygous null animals are viable and model, to a great extent, human CCD (Komori et al. 1997; Otto et al. 1997): a midline calvarial gap occurs in combination with hypoplastic clavicles, although the medial end – not the lateral – is missing, in absence of dental anomalies.

Table 1.2: Mutations Causing Skull Vault Defects in Mice

Mutation	Gene Name	Protein Properties	Skull Phenotype	Other Major Anomalies	Human Disease	References
<i>Alx4</i> ^{-/-}	Aristaless 4	Homeoprotein, transcription factor	Midline defect involving the frontal and parietal bones	Polydactyly, abdominal body wall defects	—	Qu et al. (1997b)
<i>Apaf</i> ^{-/-}	Apoptotic protease activating factor 1	Activator of the caspase cascade	Exencephaly due to failure of neural tube closure	Brain abnormalities, infertility	—	Yoshida et al. (1998) Honarpour et al. (2000)
<i>Apob</i> ^{-/+}	Apolipoprotein B	Component of plasma lipoproteins	Exencephaly due to failure of neural tube closure	Low levels of plasma lipoproteins, infertility	Familial hypobetalipoproteinemia	Huang et al. (1995)
<i>Bmp1</i> ^{-/-}	Bone morphogenetic protein 1	Procollagen protease	Reduced size of frontal, parietal, and interparietal bones	Abdominal body wall defects	—	Suzuki et al. (1996)
<i>Cart1</i> ^{-/-}	Cartilage homeoprotein 1	Homeoprotein, transcription factor	Acrania due to failure of neural tube closure	Meroanencephaly	—	Zhao et al. (1996)
<i>Cited2</i> ^{-/-}	Cbp/p300-interacting transactivator	Nuclear protein	Acrania/exencephaly due to failure of neural tube closure	Heart defects	—	Bamforth et al. (2001) Barbera et al. (2002)
<i>Ct</i>	Cranioschisis		Acrania/exencephaly	—	—	Brown and Harne (1973)
<i>Crebbp</i> ^{-/+}	CREB binding protein	Transcriptional co-activator	Delayed ossification of frontal bones, enlarged anterior fontanelle	Abnormalities of the axial skeleton, defective haemopoiesis	Rubinstein-Taybi syndrome (OMIM 180849)	Tanaka et al. (1997) Yao et al. (1998)
<i>Dlx5</i> ^{-/-}	Distal-less homeobox 5	Homeoprotein, transcription factor	Defective ossification of the parietal and interparietal bones, exencephaly, abnormal nasal and otic capsules, defects in the branchial arches	—	Within the minimal critical region of split-hand/split-foot malformation (OMIM 183600)	Acampora et al. (1999) Depew et al. (1999)
<i>Eya1</i> ^{-/-}	Eyes absent 1 homologue	Transcription factor	Reduced skull ossification	Absent/malformed inner ear and kidney	Branchiootorenal dysplasia syndrome (OMIM 113650), branchiootic syndrome (OMIM 602588)	Xu et al. (1999)

Table 1.2 — *Continued*

Mutation	Gene Name	Protein Properties	Skull Phenotype	Other Major Anomalies	Human Disease	References
<i>Fgf18</i> ^{-/-}	Fibroblast growth factor 18	Secreted growth factor	Wide fontanelles, delayed suture obliteration	Abnormalities of the axial and appendicular skeleton	—	Liu et al. (2002) Ohbayashi et al. (2002)
<i>Foxc1</i> ^{-/-}	Forkhead box C1	Winged helix transcription factor	Acrania/exencephaly	Abnormalities of the axial skeleton, eye defects, cardiovascular anomalies, urogenital anomalies	Rieger anomaly (OMIM 601090), Axenfeld anomaly (601090), iris hypoplasia (601090), iridogoniodygenesis type 1 (OMIM 601631)	Kume et al. (1998)
<i>Gja1</i> ^{-/-}	Gap junction membrane channel protein α1	Connexin 43	Reduced size of frontal and parietal bones, generalised ossification delay	Heart defects, urogenital abnormalities	Oculodentodigital dysplasia (OMIM 164200)	Reaume et al. (1995) Lecanda et al. (2000)
<i>Gli2</i> ^{-/-}	GLI-Kruppel family member 2	Zinc finger protein	Midline defect involving the frontal and parietal bones, truncation of the jaws, absence of incisors, cleft palate	Deficient ossification in the axial and appendicular skeleton	—	Mo et al. (1997)
<i>Gli3</i> ^{-/-}	GLI-Kruppel family member 3	Zinc finger protein	Exencephaly due to failure of neural tube closure, enlarged maxilla	Abnormalities of the axial and appendicular skeleton (including polydactyly) brain anomalies, eye defects, hair defects	Greig cephalopolysyndactyly syndrome (OMIM 175700), Pallister-Hall syndrome (OMIM 146510), polydactylies (OMIM 174200 & 174700)	Johnson (1967)
<i>Hic1</i> ^{-/-}	Hypermethylated in cancer 1	Zinc finger protein	Acrania/exencephaly, cleft palate	Limb abnormalities, abdominal body wall defects	Deleted in Miller-Dieker syndrome (OMIM 247200)	Carter et al. (2000)

Table 1.2 — *Continued*

Mutation	Gene Name	Protein Properties	Skull Phenotype	Other Major Anomalies	Human Disease	References
<i>Hspg2</i> ^{−/−}	Perlecan	Heparan sulfate proteoglycan	Reduced size or absence of frontal and parietal bones, wide sutures, exencephaly, cleft palate, hypoplastic jaws, defects in the skull base	Abnormalities of the axial and appendicular skeleton, heart anomalies	Schwartz-Jampel syndrome (OMIM 255800), dyssegmental dysplasia, Silverman-Handmaker type (OMIM 224410)	Arikawa-Hirasawa et al. (1999) Costell et al. (1999)
<i>Lama5</i> ^{−/−}	Laminin, alpha 5	Laminin	Acrania/exencephaly due to failure of neural tube closure	Appendicular abnormalities (including syndactyly) placental defects	—	Miner et al. (1998)
<i>Macs</i> ^{−/−}	Myristoylated alanine rich protein kinase C substrate	Cytoskeletal protein	Exencephaly due to failure of neural tube closure	Abdominal body wall defects, brain anomalies	—	Stumpo et al. (1995)
<i>Mmp14</i> ^{−/−}	Matrix metalloproteinase 14	Zn-dependent protease	Prominent sutures	Generalised defect in osseous and non-osseous extracellular matrix	—	Holmbeck et al. (1999)
<i>Msx1</i> ^{−/−}	Msh-like homeobox 1	Homeoprotein transcription factor	Midline defect involving the frontal bones, overlapping parietal bones, small nasal bones, cleft palate, hypoplastic jaws, tooth agenesis or retarded development, abnormalities of the auditory ossicles	—	Hypodontia with or without cleft palate/cleft lip and palate (OMIM 106600), Witkop syndrome (OMIM 189500)	Satokata and Maas (1994) Houzelstein et al. (1997)

Table 1.2 — *Continued*

Mutation	Gene Name	Protein Properties	Skull Phenotype	Other Major Anomalies	Human Disease	References
<i>Msx2</i> ^{-/-}	Msh-like homeobox 2	Homeoprotein, transcription factor	Enlarged anterior fontanelle, smaller interparietal and supraoccipital bones	Abnormalities of the axial and appendicular skeleton, hair, teeth and mammary gland defects, brain abnormalities	Enlarged parietal foramina (OMIM 168500), Boston-type craniosynostosis (OMIM 604757)	Satokata et al. (2000)
<i>Nog</i> ^{-/-}	Noggin	Secreted inhibitor of bone morphogenetic proteins	Exencephaly due to failure of neural tube closure	Abnormalities, including ectopic fusions, of the axial and appendicular skeleton, neural defects	Proximal symphalangism (OMIM 185800), multiple synostoses syndrome 1 (OMIM 186500), tarsal-carpal coalition syndrome (OMIM 186570), stapes ankylosis syndrome without symphalangism (OMIM 184460)	McMahon et al. (1998) Brunet et al. (1998)
<i>Otx2</i> ^{-/+}	Orthodenticle homologue 2	Homeoprotein, transcription factor	Acrania due to failure of neural tube closure, jaw hypoplasia	Meroanencephaly, defects of the eye and ear	—	Acampora et al. (1995) Matsuo et al. (1995) Ang et al. (1996)
<i>Pn</i>	Pugnose	—	Exencephaly, reduced size of calvarial bones, jaw hypoplasia	—	—	Kidwell et al. (1961)
<i>Runx2</i> ^{-/+}	Runt related transcription factor 2	Transcription factor Cbfa1	Midline defect involving the frontal and parietal bones, small interparietal bone	Hypoplasia of the clavicle, hypoplasia of the hyoid	Cleidocranial dysplasia (OMIM 119600)	Komori et al. (1997) Otto et al. (1997)

Table 1.2 — *Continued*

Mutation	Gene Name	Protein Properties	Skull Phenotype	Other Major Anomalies	Human Disease	References
<i>Ryk</i> ^{-/-}	Receptor-like tyrosine kinase	Divergent receptor tyrosine kinase	Reduced size of/malformed calvarial bones, cleft palate, jaw hypoplasia	Abnormalities of the appendicular skeleton	—	Halford et al. (2000)
<i>Sp7</i> ^{-/-}	Sp7 transcription factor	Zinc finger protein Osterix	Absence of osseous tissue	Global arrest in osteoblast differentiation	—	Nakashima et al. (2002)
<i>Tcfap2a</i> ^{-/-}	Transcription factor AP-2 alpha	Transcription factor	Exencephaly due to failure of neural tube closure, hypoplastic jaws	Meroanencephaly, abnormalities of sensory organs and cranial ganglia, heart anomalies, abdominal body wall defects	—	Schorle et al. (1996) Zhang et al. (1996b)
<i>Tcof1</i> ^{-/-}	Treacher-Collins Franceschetti syndrome 1, homologue	Nucleolar protein	Acrania/exencephaly due to failure of neural tube closure, nasal agenesis, malformed maxilla	Eye anomalies, brain abnormalities	Treacher-Collins syndrome (OMIM 154500)	Dixon et al. (2000)
<i>Tgfb2</i> ^{-/-}	Transforming growth factor β 2	Transforming growth factor β	Reduced size of frontal, parietal, interparietal and squamosal bones, cleft palate, abnormal mandible	Abnormalities of the axial and appendicular skeleton, eye and ear defects, urogenital anomalies	—	Sanford et al. (1997)
<i>Trp53</i> ^{-/-}	Transformation related protein 53	Tumour suppressor	Exencephaly due to failure of neural tube closure	Increased susceptibility to tumours	Various malignancies, including Li-Fraumeni syndrome (OMIM 151623)	Donehower et al. (1992) Sah et al. (1995)

Table 1.2 — *Continued*

Mutation	Gene Name	Protein Properties	Skull Phenotype	Other Major Anomalies	Human Disease	References
<i>Twist1</i> ^{-/+}	Twist gene homologue 1	Basic helix- loop-helix tran- scription factor	Overgrowth of frontal, parietal, and interparietal bones, early obliteration of calvarial sutures, persistent anterior fontanelle, delayed growth/malforma- tion of temporal bone and several facial bones	Polydactyly, rib cage defects	Sathre-Chotzen syndrome (OMIM 101500)	Bourgeois et al. (1998) Carver et al. (2002) Ishii et al. (2003)

Sources: The list was compiled mostly from secondary sources: material in Mouse Genome Informatics at the Jackson Laboratory (see appendix F), data in reviews by Wilkie and Morriss-Kay (2001) and Depew et al. (2002), and through structured literature queries.

Notes: The majority of mutations are targeted disruptions; two classical mutants with yet unidentified genetic basis are also included. Only single-gene mutations are listed. The descriptions of the abnormalities are generalisations and the phenotypes not necessarily fully penetrant. Although the developmental stage is not stated, the calvarial defects tend to be apparent at birth or in late gestation. The diseases caused by mutations in the human orthologues are usually, but not exclusively, due to loss of function as well. Calvarial defects may be a secondary feature because of impaired neural tube closure — a number of mutants with early lethality, prior to osteogenesis, due to failure of neural tube closure have been omitted.

A requirement for *Twist1* during early organogenesis results in death of homozygous null embryos around mid-gestation with pronounced neural tube closure defects, deficient cephalic mesenchyme, malformed branchial arches, and retarded limb buds (Chen and Behringer 1995). *Twist1*^{-/+} mice are viable and present a variable spectrum of abnormalities, depending on the genetic background, reminiscent, in certain aspects, of human SCS (Bourgeois et al. 1998). Amongst other features, the skull vault shows localised signs of both overgrowth and delay; there is preaxial polydactyly/thumb broadening of the hindlimb; and shifting of the nasal septum causes facial asymmetry. Focused reappraisal demonstrated a subtle yet highly penetrant midline opening in the posterior frontal region (Ishii et al. 2003) and true suture fusion (Carver et al. 2002).

1.4 Molecular Pathways in Calvarial Development

1.4.1 Introduction

In theory (Hall and Miyake 2000), the primordial condensation of the ectomeninx during intramembranous bone formation should be preceded by epithelial-mesenchymal interactions; but contrary to other comparable systems, membranous bones emerge deep within the dermis having no apparent interaction with the epidermis. The underlying neuroepithelium may fulfil this role in the skull vault. Additionally, it somehow couples late, non-allometric bone growth with brain expansion — if brain enlargement stops, bone growth also comes to a halt. Perhaps these presumed signals are communicated via the dura mater. The dura has osteogenic potential, being able to regenerate the bones, and there is evidence to suggest that it may pattern the calvaria. Its presence is required for initial induction of the sutures and it also regulates their patency afterwards, displaying positional differences — reviewed by Opperman (2000).

Appositional growth of the bones takes place at the sutural interfaces: the suture functions as a growth centre, providing cells for osteoblastic recruitment while maintaining a precursor population. The sutural core is occupied by undifferentiated mesenchyme, while the actively proliferating cells are found in the edges which, in turn, are lined by cells entering the differentiation process. The latter two populations contribute to the anatomically visible osteogenic front which is continuous with a peripheral zone around the deposited osteoid.

Signalling molecules and transcription factors linked to several major pathways, also operational during limb bud and branchial arch development, have been implicated in calvarial osteogenesis through genetic analysis, detection of local expression, and experimental manipulation of their concentrations. However, as this assembly of data has not yet matured to a coherent picture, the following account revolves around individual signalling pathways and transcription factors.

Opperman (2000) reviewed available embryological, expressional, mutational, and biochemical data and attempted a synthesis; a comprehensive discussion of the relevant signalling pathways/protein families is provided by Depew et al. (2002).

1.4.2 The Fibroblast Growth Factors

The fibroblast growth factors, Fgfs, have diverse roles in patterning, regulation of growth, differentiation, apoptosis, and migration in several tissue systems and during various phases of development, including both intramembranous and endochondral ossification — reviewed by Ornitz and Marie (2002). In mammals there are at least twenty-two *Fgf* genes whereas four genes encode the cognate receptor protein tyrosine kinases, *Fgfr1–4*. Alternative splicing of *Fgfr* genes (bar *Fgfr4*) produces two protein variants with distinct ligand binding specificities: a mostly mesenchymal c form and a mostly epithelial b form. Fgf binding, in heparan sulfate proteoglycan complexes, leads to receptor dimerisation and trans-autophosphorylation; signals are subsequently transduced via several pathways, of which the canonical Ras-Raf-MEK cascade, culminating in activation of the extracellular signal regulated kinases, and phospholipase C γ activation are believed to be important in calvarial osteogenesis.

The significance of Fgf signalling in skull vault development was uncovered through mutations in *FGFR2*, *FGFR3* and *FGFR1*, causing various craniosynostotic conditions which include the common syndromes Crouzon (OMIM 123500), Apert (OMIM 101200), Pfeiffer (101600) and Muenke (OMIM 602849) (Reardon et al. 1994; Jabs et al. 1994; Muenke et al. 1994; Wilkie et al. 1995a; Bellus et al. 1996) — reviewed by Muenke and Wilkie (2001). These mutations are dominant, non-randomly distributed in the genes, and exert their effects by gain-of-function mechanisms (Neilson and Friesel 1995; Robertson et al. 1998; Anderson et al. 1998; Yu and Ornitz 2001). Overactivation of the Fgf pathway also underlies several murine genetic models of craniosynostosis (Carlton et al. 1998; Zhou et al.

2000b; Greenwald et al. 2001; Hajihosseini et al. 2001). Analysis of loss-of-function mutations of *Fgfr1* and *Fgfr2* is hindered by early lethality (Yamaguchi et al. 1994; Deng et al. 1994; Arman et al. 1999), but ablation of *Fgfr3* does not cause skull vault defects (Deng et al. 1996; Colvin et al. 1996). Targeted inactivation of the *IIIc* spliceform of *Fgfr2* is compatible with life and reveals the positive role of this receptor variant in bone formation, causing, amongst other abnormalities, delayed calvarial ossification (Eswarakumar et al. 2002). On the other hand, of the available *Fgf* mutants only homozygous null mice for *Fgf18* (Table 1.2) manifest skull vault defects (Liu et al. 2002; Ohbayashi et al. 2002).

Several *Fgfs* and all *Fgfrs* but *Fgfr4*, mostly as the c isoforms, are present in the skeletogenic membranes and the expression patterns of *Fgfr2*, *Fgfr1*, *Fgfr3*, *Fgf2*, *Fgf9*, *Fgf18* and *Fgf20* have been sufficiently documented (Iseki et al. 1997; Kim et al. 1998; Delezoides et al. 1998; Most et al. 1998; Mehrara et al. 1998; Iseki et al. 1999; Rice et al. 2000; Johnson et al. 2000a; Hajihosseini and Heath 2002). *Fgf9* and *Fgf2* transcripts are abundant in the sutural mesenchyme, the dura mater, and at least *Fgf2* expression extends to the osteoid region; *Fgf18* and *Fgf20* activity appears more restricted, outlining the developing bones. *Fgfr2* expression correlates with the proliferative population at the osteogenic fronts and the peripheral, undifferentiated layers of the bones while transcription of *Fgfr1* precedes terminal differentiation and tends to mark a more central (with respect to the developing bone) domain, adjacent to the osteoid; *Fgfr3* shows a diffuse expression pattern.

The biological functions of *Fgfs* in osteogenic calvarial cells and tissues, assayed in numerous in vitro and in vivo studies, are seemingly bewildering and even contradictory. *Fgfs* can have mitogenic effects, supporting cell proliferation and inhibiting osteoblastic differentiation (Tang et al. 1996; Canalis and Gabbittas 1998; Kim et al. 1998; Sarkar et al. 2001; Shimoaka et al. 2002), but the commonest outcome of *Fgf* application is overt cell differentiation, induction of maturation markers, accelerated suture closure and down-regulation of *Fgfr2* (Boudreaux and

Towler 1996; Iseki et al. 1997; Debais et al. 1998; Kim et al. 1998; Most et al. 1998; Iseki et al. 1999; Rice et al. 2000; Greenwald et al. 2001; Mathijssen et al. 2001; Sarkar et al. 2001; Shimoaka et al. 2002). The latter responses simulate the altered behaviour of osteogenic cells from humans and mice with syndromic craniosynostosis — a tilting of the proliferation-differentiation balance towards the differentiation side, including overexpression of *Runx2* (Lomri et al. 1998; Fragale et al. 1999; Zhou et al. 2000b). In addition, Fgf signalling can instigate apoptosis (Mansukhani et al. 2000; Mathijssen et al. 2001). On the other hand, partial blocking of endogenous Fgf2 enhances proliferation whereas complete inhibition arrests differentiation (Moore et al. 2002). This confusing functional repertoire is comprehensible if the responses depend on the cell maturation status and the intensity and/or specificity of Fgf signalling. Iseki et al. (1999) put forward the hypothesis that a gradient of osteoblast-secreted Fgf ligand, possibly involving Fgf2, from high concentration in the osteoid matrix to low in the bone periphery and the sutural space regulates progression along the osteogenic pathway. Osteoblast precursors and pre-osteoblasts are gradually exposed to increasing levels of ligand as they move away from the suture, *Fgfr2* is down-regulated in favour of *Fgfr1*, proliferation ceases, and differentiation commences. Ornitz and Marie (2002) elaborated this model: proliferative signalling may employ low levels of Fgf18 and Fgf2 in the sutural mesenchyme; raised Fgf18 and Fgf2 levels in concert with Bmp signalling may elicit differentiation at the osteogenic fronts and the peripheral layers; and intense Fgf2 expression is associated with the terminal stage and, possibly, apoptotic death.

The altered pattern of *Fgfr2* expression in *Twist1*^{-/+} mice (Rice et al. 2000) and the relationship of *twist* and *heartless* (*htl*), an *Fgfr* homologue, in *Drosophila* (Shishido et al. 1993) encouraged the notion that *Twist1* may be upstream of *Fgfr2*.

1.4.3 The Transforming Growth Factor β Superfamily

Members of the transforming growth factor β superfamily, Tgf β , including the bone morphogenetic proteins, Bmps, and the Tgf β factors proper, have equally broad biological roles (Miyazono et al. 2001). Ligand binding to a type II receptor and association with a type I receptor elicits the serine/threonine kinase activity of the latter and leads to phosphorylation and nuclear migration of their effectors, the Smad proteins. Specific ligand inhibition, through extracellular antagonists, can also be important.

Until recently, the only relevant mouse mutant that survives beyond initiation of skeletogenesis and exhibits skull vault defects was that for *Tgfb2* (Table 1.2); *Bmp1*^{-/-} animals also show calvarial defects but, despite its name, Bmp1 is not a signalling molecule. *Tgfb2*^{-/-} neonates die with multiple skeletal abnormalities, including reduced calvarial bones and cleft palate (Sanford et al. 1997). Tissue-specific inactivation of a type II receptor gene, *Tgfb2*, overcomes the early lethality of a constitutive disruption and reveals severe defects in the frontal and parietal bones as well as in the dura mater at birth (Yoshihiro et al. 2003).

Both Tgf β and Bmp ligands have been detected in the calvaria. *Bmp2*, *Bmp4*, *Bmp7*, *Tgfb1*, *Tgfb2* and *Tgfb3* are active at the osteogenic fronts, the dura mater, the bone periphery, but not in the mid-sutural mesenchyme (Opperman et al. 1997; Roth et al. 1997; Kim et al. 1998; Rice et al. 1999; Holleville et al. 2003; Rice et al. 2003).

Bmp signalling may promote differentiation of immature calvarial cells in vitro (Hay et al. 1999; Marie et al. 2002) and localised application of Bmp4 causes a dramatic increase in bone volume but not suture closure (Kim et al. 1998). Tgf β signalling may modulate suture patency: Tgf β 2 can induce suture obliteration, accompanied by increased proliferation while Tgf β 3 has opposite effects (Opperman et al. 2000). Recently, the Bmp antagonist Noggin was highlighted as a regulatory

factor in suture fusion (Warren et al. 2003). Noggin expression is confined in the sutural mesenchyme and correlates with the open status; it is suppressed by intense Fgf signalling and its presence can prevent synostosis. Homozygous null mice die at birth with multiple defects (Table 1.2), including fusion of synovial joints and exencephaly, yet not with a grossly abnormal skull (McMahon et al. 1998; Brunet et al. 1998). Noggin may be sufficient but not necessary for maintenance of suture patency.

There is some evidence to suggest reciprocal relationships between Fgf and Tgf β signalling — Fgfs can induce Tgf β s/Bmps (Debiais et al. 1998; Holleville et al. 2003) and vice versa (Hurley et al. 1994).

1.4.4 The Hedgehogs

Despite the crucial role of indian hedgehog, Ihh, in endochondral ossification and promising initial data, the pathway has been under-investigated. Kim et al. (1998) demonstrated expression of sonic hedgehog, Shh, and the receptor patched, Ptc, at the coronal suture; Rhoda et al. (2002) confirmed these findings and also detected Ihh.

1.4.5 The Msx and Dlx Families of Homeoproteins

Mammalian homologues of the *Drosophila muscle segment homeobox (msh)*, also known as *Drop (Dr)* (Lord et al. 1995; D'Alessio and Frasch 1996), comprise a tri-genic family of chromosomally unlinked genes that encode homeoproteins distantly related to the Hox class — reviewed by Davidson (1995). Of the three paralogues, *Msx1* and *Msx2* have acquired numerous novel roles compared to the ancestral *msh* during various phases of development and in a range of systems. *Msx1* was first identified in the mouse (Hill et al. 1989; Robert et al. 1989) and *Msx2* was first isolated from quail (Takahashi and Le Douarin 1990). All *Msx* genes are compact, consisting of two exons, with seemingly uncomplicated structural organ-

isation — typified by the human and mouse *Msx2* (Bell et al. 1993; Jabs et al. 1993). However, recent data suggest that the genomics of the family may be more complex: Blin-Wakkach et al. (2001) identified an endogenous antisense transcript for *Msx1* of physiological significance and, at least in the human genome, a second intronless copy of *Msx2*, most probably a processed pseudogene, exists (Ensembl v. 16.33.1 — see appendix F). Bendall and Abate-Shen (2000) reviewed developmental and biochemical aspects of the *Msx* family and put forward a conceptual framework for their functional diversity. Generally, expression of *Msx1* and *Msx2* tends to mark proliferating or apoptotic cell populations, but not terminally differentiated cell types, and their products are strong transcriptional repressors. However, the vast majority of their *in vivo* targets remain elusive. In summary, *Msx1* and *Msx2* participate in: (1) early patterning of the cranial neural crest, limb, and branchial arches — conferring positional information in the mesenchyme; (2) epithelial-mesenchymal interactions during morphogenesis of the limb bud and the skin appendages (including teeth, hair follicles and mammary glands) — being used in signalling loops between the two tissue layers; (3) neural induction, subsequent neural tube regionalisation and specification of neuronal identity — apparently contributing to a complex homeobox code; (4) and regulation of cell proliferation, differentiation, and survival during organogenesis — controlling a delicate balance between precursor population size and differentiation rate. Their role in calvarial development is in this latter context, amongst other systems such as the skin, hair follicle, limb muscles, mammary gland, uterine epithelium, etc.

On the genetic level, the contrasting gain-of-function and loss-of-function mutations of human *MSX2* that give rise to craniosynostosis and PFM respectively were discussed in section 1.3.2.3 and the calvarial phenotype of *Msx2*^{-/-} mice was alluded to in section 1.3.3.2. Two independent attempts to reproduce the synostotic phenotype in mice by expressing either the P148H mutant or the wild-type transcript transgenically produced mixed results. Liu et al. (1995) reported bone

overlap and suture obliteration in a proportion of viable animals whereas Wino-grad et al. (1997) observed perinatal lethality with multiple abnormalities but no sign of craniosynostosis. In a refined approach, Liu et al. (1999) caused bone overgrowth and invasion of the suture by overexpressing an *Msx2* minigene under part of the native promoter at hemizygoty; this effect was accompanied by increased local proliferation and, importantly, had no detectable effect on *Fgfr2* expression. A parallel in vitro study by Dodig et al. (1999) demonstrated that forced expression of *Msx2* could suppress terminal osteoblastic differentiation whereas inhibition could decrease proliferation and accelerate differentiation. Together, these results led to the notion (Liu et al. 1999) that *Msx2* regulates the size of the osteoblast precursor pool during calvarial ossification — *MSX2*-caused craniosynostosis reflects an aberrant increase of the precursor population coupled with transient retardation of maturation which, somehow, is brought about later. At the same time, they underscore the intrinsic sensitivity of the system and indicate that the gain of function conferred by the P148H variant is rather specific. *MSX1* mutations have been identified in human disorders but the calvarial phenotype is unremarkable. A missense loss-of-function mutation was initially reported to segregate with isolated selective tooth agenesis (Vastardis et al. 1996; Hu et al. 1998), but haploinsufficiency of *MSX1* is likely to result in a wider phenotypic spectrum — mutations have been described in association with variable presentations of cleft lip and cleft palate in combination with partial tooth agenesis (OMIM 106600) (van den Boogaard et al. 2000; Jezewski et al. 2003) as well as a tooth and nail condition, Witkop syndrome (OMIM 189500) (Jumlongras et al. 2001). However, targeted disruptions in mice (Table 1.2) cause skull vault defects (Satokata and Maas 1994; Houzelstein et al. 1997): homozygous null animals exhibit cleft palate, dental defects, and a midline gap in the frontal region.

Expression of both *Msx2* and *Msx1* has been investigated in the developing calvaria with the focus on the coronal suture. During late gestation, *Msx2* transcrip-

tion is concentrated in the sutural mesenchyme, the dura mater and the osteogenic fronts, diffusing to the bone periphery, but is clearly excluded from the osteoid; after birth it shows marked down-regulation — expression disappears from the dura and becomes discontinuous around the suture (MacKenzie et al. 1992; Jabs et al. 1993; Kim et al. 1998; Liu et al. 1999). *Msx1* shows a spatially similar expression pattern but, interestingly, gene activity persists in the postnatal period (Kim et al. 1998; Orestes-Cardoso et al. 2001).

The established link between the *Msx* homeoproteins and Bmp signalling, demonstrated in other systems during vertebrate development (Davidson 1995), is likely to be operational in the skull vault as well. Exogenous Bmp4 can induce *Msx2* and *Msx1* expression (Kim et al. 1998) and Bmp activity is locally present — see section 1.4.3. There is no consensus on the action of Fgfs: Kim et al. (1998) demonstrated that application of Fgf4 triggers *Msx1* transcription while having no effect on *Msx2*; Ignelzi et al. (2003) reported responsiveness of *Msx2* to Fgfs.

The core recognition site of the Hox homeoproteins, TAAT, supports binding by the *Msx* proteins (Catron et al. 1993; Semenza et al. 1995; Catron et al. 1996) and all family members show strong repressive potential which is nevertheless independent of DNA binding (Semenza et al. 1995; Catron et al. 1995; Catron et al. 1996; Zhang et al. 1996a; Newberry et al. 1997b). Apart from direct DNA recognition, it is envisaged that *Msx* homeoproteins can exert their transcriptional effects indirectly, through protein-protein interactions which are likely to be homeodomain-mediated (Bendall and Abate-Shen 2000). Of the few well-documented target genes, *Col1a1* and especially *Bglap1*, encoding osteocalcin and jointly regulated by Fgf signalling and *Cbfa1*, are suppressed by *Msx2* (Hoffmann et al. 1994; Towler et al. 1994; Dodig et al. 1996; Hoffmann et al. 1996; Newberry et al. 1997a; Newberry et al. 1997b) — a good correlation with the observed anti-differentiation effects of *Msx2* during calvarial osteogenesis.

The mammalian *Dlx* family is related to the *Drosophila* gene *Distal-less* (*Dll*) and consists of six homeobox genes, organised in three pairs physically linked to the Hox clusters. Their functional repertoire during development is as wide as that of the *Msx* family members — reviewed by Bendall and Abate-Shen (2000) and Panganiban and Rubenstein (2002). *Dlx* and *Msx* genes show overlapping yet distinct expression patterns in numerous tissues and appear to have complementary or antagonistic functions, depending on the context. Broadly, *Dlx* expression marks cell populations where differentiation is taking place or is just about to commence; contrary to the *Msx* proteins, they are transcriptional activators. Of the six members, *Dlx5* and *Dlx6* have regulatory roles during bone development.

Dlx5^{-/-} mice (Table 1.2) present with reduced parietal and interparietal bones among other skull anomalies (Acampora et al. 1999; Depew et al. 1999) and the calvarial phenotype is exacerbated in the double *Dlx5*^{-/-} ; *Dlx6*^{-/-} mutant (Robledo et al. 2002). In humans, the only mutation identified thus far is in *DLX3*, associated with calvarial thickening in the context of the tricho-dento-osseous syndrome (OMIM 190320) (Price et al. 1998).

Dlx5 is active in the calvarial bones (Depew et al. 1999) and its expression highlights the sutural edges and the osteogenic fronts as well as the periphery of the osteoid domains, but neither the core of the suture nor the mature bone (Holleville et al. 2003). Bmp signalling up-regulates *Dlx5* (Miyama et al. 1999; Holleville et al. 2003) and among the targets of *Dlx5* are genes encoding bone matrix components — osteocalcin, collagen and bone sialoprotein (Dodig et al. 1996; Ryoo et al. 1997; Miyama et al. 1999; Benson et al. 2000). At least in the case of the *Bglap1* promoter, *Dlx* and *Msx* proteins participate in an antagonistic interplay that relies on physical interactions (Zhang et al. 1997; Newberry et al. 1998).

1.4.6 The Basic Helix-Loop-Helix Factor Twist1

Twist1 is one of the two mammalian homologues of the *Drosophila* gene *twist* (*twi*) which encodes a transcription factor of the basic helix-loop-helix type that acts through homo- or heterodimerisation, usually presenting with repressive properties. This interaction potential is likely to underpin its diverse roles during development — reviewed by O'Rourke and Tam (2002). *Twist1* is essential for mesoderm differentiation, involved in patterning and growth of the limb buds and the branchial arches, required for growth and differentiation of the head mesenchyme and proper dorso-ventral patterning of the neural tube, and also influences neural crest migration.

As mentioned in sections 1.3.2.2 and 1.3.3.2, haploinsufficiency of *TWIST1* causes SCS in humans and *Twist1*^{-/+} mice display roughly equivalent skull and limb abnormalities. The outcomes of increased gene copy number also testify towards its dosage-sensitive functions in calvarial development.

Twist1 is active in the early head mesenchyme (Chen and Behringer 1995) within and around the skeletogenic condensations. Later, its expression delineates the growing bones and decreases after birth; it is mainly restricted to the mid-sutural mesenchyme population (Rice et al. 2000; Johnson et al. 2000a). Rice et al. (2000) reported stimulation of *Twist* expression by exogenous Fgf2 but not Bmp2.

Despite the paucity of data regarding its direct targets, there is evidence that Twist1-mediated repression may involve contact and inhibition of histone acetyltransferases (Hamamori et al. 1999). Moreover, at the cell level *Twist1* protects against apoptosis (Maestro et al. 1999); functional haploinsufficiency renders the calvarial osteoblasts vulnerable to death through activation of the caspase cascade (Yusfi et al. 2002).

1.4.7 The Transcription Factors Cbfa1 and Osterix

Runx2 specifies two isoforms of one out of the three possible α subunits, Cbfa1, which complex with the common partner Cbfb to form the heterodimeric Cbf transcription factor series. It is the only member of the mammalian *Runx* group, related to *Drosophila runt (run)*, which has evolved into a specific and essential regulator of skeletogenesis — reviewed by Komori (2002) and Nakashima and de Crombrughe (2003).

Haploinsufficiency of *RUNX2* underlies human CCD (see section 1.3.2.2) and the phenotype of *Runx2*^{-/-} mice reveals its indispensable role in osteoblast determination (see section 1.3.3.2). However, Cbfa1 functions extend beyond promotion of early osteoblast differentiation. Cbfa1 is expressed in the chondrocyte lineage and positively regulates progression to terminal hypertrophy; furthermore, transgenic mice overexpressing *Runx2* are osteopenic, demonstrating a negative impact on the osteoblast status at later stages (Liu et al. 2001; Geoffroy et al. 2002).

Activation of *Runx2* in the mesenchymal condensations of the skull vault precedes considerably the onset of overt ossification (Ducy et al. 1997; Otto et al. 1997); afterwards, its expression spans the sutural mesenchyme, the osteogenic fronts and the osteoid periphery as well as the mature bone itself (Park et al. 2001).

Several signals and effectors regulate *Runx2* at the transcription level (Komori 2002); of importance, *Runx2* is up-regulated by Bmp and Fgf signalling (Ducy et al. 1997; Zhou et al. 2000b). The runt domain of Cbfa1 is used both in DNA recognition and Cbfb binding and an array of transcriptional activators, co-activators, and repressors have been shown to interact physically with Cbfa1, presumably modulating its action on numerous downstream genes, many of which encode bone-specific proteins (Komori 2002).

Osterix, a zinc finger protein encoded by *Sp7*, is a more bone-specific transcription factor compared to Cbfa1 and, similarly, essential for realisation of the

osteoblast phenotype. In *Sp7^{-/-}* mice (Table 1.2), skeletogenic condensations develop but osteoblast differentiation is globally arrested despite expression of *Runx2* (Nakashima et al. 2002). Osterix is likely to be downstream of *Cbfa1*, driving a somewhat bi-potential precursor cell population, able to give rise to either osteoblasts or chondrocytes, definitively into the osteoblastic route.

1.5 The Proximal 11p Deletion Syndrome – Enlarged Parietal Foramina and Multiple Exostoses – Points to a New Locus for Skull Defects in Humans

1.5.1 Introduction

In the search for a second locus for PFM, P11pDS, catalogued in OMIM under 601224, stands out from the clinical entities listed in Table 1.1. Originally named with the mnemonic acronym DEFECT11 and also referred to as Potocki-Shaffer syndrome (its designation has been the subject of a debate; for details see Wuyts et al. (2001) and Shaffer (2001)), it features the very rare yet distinctive combination of multiple exostoses of the long bones, PFM, and, occasionally, mental retardation and craniofacial dysostosis, inherited in an autosomal dominant fashion. Both multiple exostoses (OMIM 133700, 133701 and 600209) and PFM (OMIM 168500) occur as isolated traits and in all investigated cases combining these phenotypes heterozygous deletions, submicroscopic or cytogenetically visible, of proximal chromosome 11p have been found — facts strongly suggestive of a contiguous gene deletion syndrome. Depending on the size and location of the deletions, certain other features may be present. Very large deletions that extend distally to the critical region for the WAGR syndrome (OMIM 194072) cause, in addition, Wilms tumour, aniridia, and genitourinary anomalies.

In fact, the potential overshadowing by the prominent phenotypic spectrum of WAGR syndrome, the gradual appearance of the exostotic outgrowths dur-

ing childhood, and the incomplete penetrance and/or variable presentation of the skull defects might have obscured the syndrome in the past. Hittner et al. (1979) described an infant carrying a $\text{del}(11)(\text{pter-p14::p11.2-qter})$ – derived from a maternal intrachromosomal insertion –, affected with aniridia, PFM, agenesis of the corpus callosum, meningeal anomalies, mild facial dysmorphism, and severe psychomotor retardation. Gustavson et al. (1984) reported on a newborn with multiple defects, including wide fontanelles and sutures, ocular anomalies, and genital abnormalities, where a $\text{del}(11)(\text{p13})$ was detected. Lorenz et al. (1990) presented an atypical case of 'acrocephalosyndactyly': a combination of coronal synostosis, PFM, multiple intrasutural bones, mandibular hypoplasia, cutaneous syndactyly, micropenis, and mental retardation that resembled SCS. The first family segregating P11pDS was reported by Shaffer et al. (1993). Although a causative link between the familial deletion of proximal 11p and PFM with mental retardation was established, the presence of multiple exostoses in the pedigree was misinterpreted as a coincidental finding. Retrospectively, it is clear that McGaughan et al. (1995) described a case of P11pDS-WAGR, caused by a substantial deletion of 11p — case 6 in Figure 1.7. However, P11pDS was effectively defined by Bartsch et al. (1996). A comprehensive delineation of the syndrome was performed, based on comparative clinical and laboratory investigations of eight patients in four families, including the ones described by Lorenz et al. (1990) and Shaffer et al. (1993) — cases 1, 2, 3 and 4 in Figure 1.7. At the same time, another typical case was reported (Potocki and Shaffer 1996) — case 5 in Figure 1.7. The identification of the disease gene for isolated multiple exostoses on chromosome 11p, *EXT2* (Stickens et al. 1996; Wuyts et al. 1996), was a breakthrough in elucidating the molecular basis of the syndrome. After ascribing this facet of the phenotype to *EXT2*, the notion that P11pDS is indeed a contiguous gene deletion syndrome seemed rather plausible (Ligon et al. 1998): deletion of a closely linked yet functionally unrelated gene would account for the other consistent finding, PFM. Meanwhile, a new case was presented by

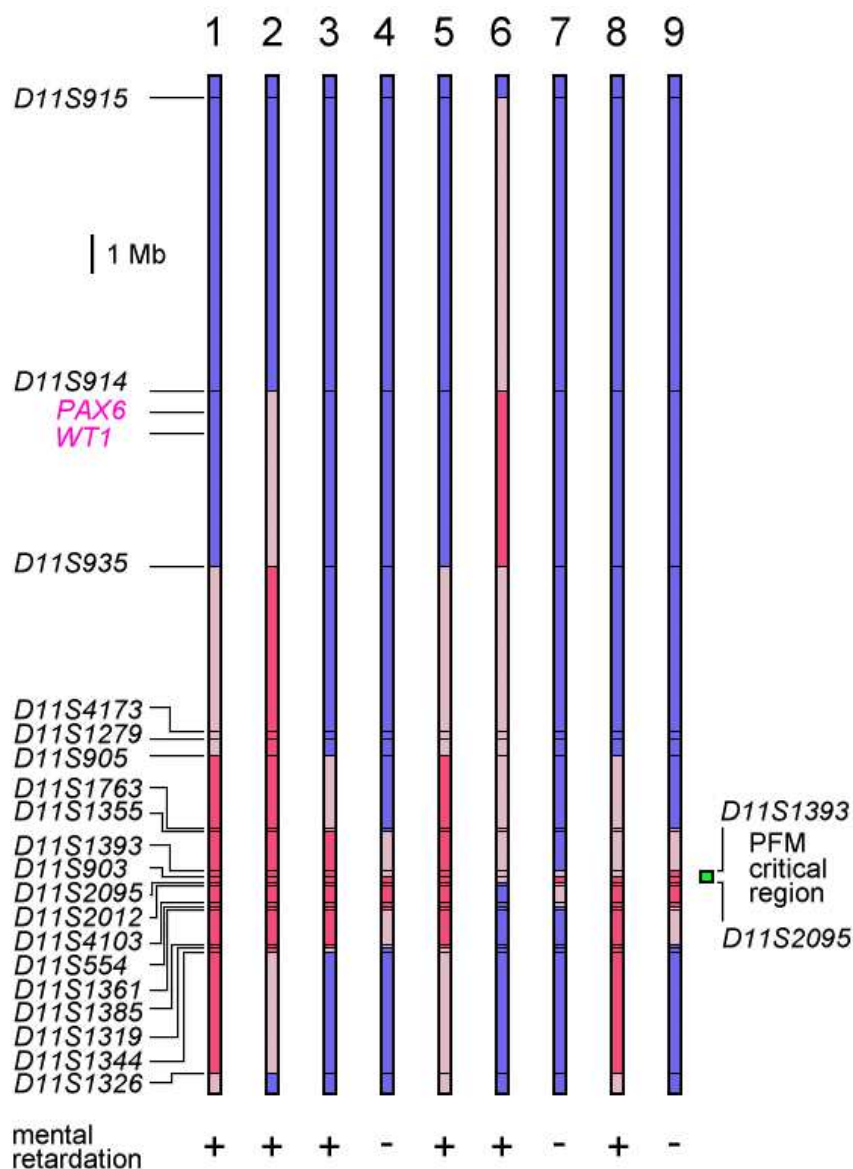


Figure 1.7: Synopsis of molecularly characterised deletions associated with proximal 11p deletion syndrome. Definitively deleted segments are shown *in red* with windows of uncertainty – because of limited marker coverage or non-informativeness – *in pink*; blue marks regions that are unambiguously present. All deletions are associated with multiple exostoses and enlarged parietal foramina whereas mental retardation is a variable feature of the syndrome. Data from published cases were collated and integrated using the local physical map (Ensembl v. 16.33.1 — see appendix F). The *PAX6* and *WT1* genes provide a useful landmark; markers run top-to-bottom in the telomere-to-centromere direction. References and cytogenetic findings: case 1, del(11)(p11.12p13) (Lorenz et al. 1990; Bartsch et al. 1996); case 2, del(11)(p11.12p13) (Bartsch et al. 1996); case 3, del(11)(p11.12p12) (Shaffer et al. 1993; Bartsch et al. 1996); case 4, normal karyotype (Bartsch et al. 1996); case 5, del(11)(p11.2p12) (Potocki and Shaffer 1996); case 6, del(11)(p11.2p14.2) (McGaughan et al. 1995; Wuyts et al. 1996); case 7, normal karyotype (Wuyts et al. 1999); case 8, der(11)(pter-p14.3::p14.2-p13::p14.3-p14.2::p13-p13::q11-cen-p11.11::p11.2-p13::q11-qter) (Yamamoto et al. 2001); case 9, normal karyotype (Hall et al. 2001). Cases 8 and 9 were published after the identification of the PFM locus on 11p.

Davies et al. (1997). Wuyts et al. (1999) characterised a small, submicroscopic familial deletion and refined the critical interval for the PFM locus — case 7 in Figure 1.7.

1.5.2 The Enlarged Parietal Foramina Locus on Chromosome 11p Is Not Allelic to *EXT2*

To envisage a disease gene for PFM distinct from *EXT2* was, by far, the favoured hypothesis in explaining P11pDS. Nevertheless, before embarking on a comprehensive gene isolation project the alternative view, i.e. PFM being allelic with multiple exostoses, had to be ruled out. Several missense and nonsense changes, frameshift-causing small deletions and insertions, as well as splice site mutations of *EXT2* have been found in constitutional samples from multiple exostoses patients (together with equivalent mutations in the related *EXT1* gene these account for approximately 70% of total cases) — reviewed by Wuyts and Van Hull (2000). The outgrowths are benign osteochondromas which emanate from the cortex of the long bones in a region adjacent to the growth plates. Growth of these lesions takes place during childhood and ultimately ceases but occasionally malignant transformation to chondrosarcomas or osteosarcomas may occur (Luckert-Wicklund et al. 1995). On this basis, the *EXT* genes were considered as a new family of tumour suppressor genes (Stickens et al. 1996). Heterozygous germline mutations encourage unregulated proliferation of chondrocytes whilst a second, somatic hit either in the same locus or elsewhere is required for tumourigenesis (Hecht et al. 1995; Raskind et al. 1995; Bovée et al. 1999). The mutational spectrum of *EXT2* strongly supported an, at least partial, loss-of-function mechanism but was not informative about the outcome of full-gene deletions as the ones encountered in P11pDS. In principle, hypomorphic alleles could cause multiple exostoses and a null allele could result in the combined phenotype — multiple exostoses and PFM. When functional data about the *EXT* proteins emerged, this speculation seemed less remote. The prod-

ucts of *EXT2* and *EXT1* are membrane-bound proteins with glycosyl transferase activity that reside in the endoplasmic reticulum and the Golgi apparatus as a functional complex essential for synthesis of the glycosaminoglycan heparan sulfate (McCormick et al. 1998; Lind et al. 1998; McCormick et al. 2000). In the extracellular matrix, glycosaminoglycans can bind, amongst other molecules, ligands for several major signalling pathways and modulate their local concentrations and availability to receptors. Importantly, the *Drosophila* homologue of the *EXT* genes, *tout-velu* (*tto*), is required for proper diffusion of hedgehog (Bellaiche et al. 1998).

However, a unique deletion event disentangled the situation. Hecht et al. (1995) described a small heterozygous deletion at proximal 11p, encompassing the marker *D11S903* in a family with multiple exostoses; one member developed a secondary chondrosarcoma and displayed loss of heterozygosity at neighbouring loci and a homozygous deletion at *D11S903*. This familial deletion was instrumental in pinpointing the *EXT2* gene and was mapped accurately (Stickens et al. 1996). It removes around 30 kb of the gene, including several 5' exons and the promoter region; hence, complete loss of function could be assumed. As no skull defects were observed in this kindred, the original hypothesis of a true contiguous gene deletion syndrome was corroborated.

1.5.3 Definition of the Minimal Critical Region for the New Enlarged Parietal Foramina Locus

The minimal critical region for the new PFM locus was defined through two informative deletions associated with P11pDS. The first deletion is very large and eliminates both the the P11pDS region and, distally, the WAGR region (McGaughan et al. 1995). Its proximal breakpoint was mapped distally to *D11S2095* (Wuyts et al. 1996) — heterozygosity was detected at *D11S2095* (case 6 in Figure 1.7). The second deletion is the smallest described so far in P11pDS. Its distal breakpoint was located proximally to *D11S1393* (Wuyts et al. 1999) — *D11S903* was deleted, but

not *D11S1393* (case 7 in Figure 1.7). Extrapolating from these deletions, Wuyts et al. (1999) proposed that *D11S1393* and *D11S2095* flank the P11pDS minimal critical region, including the PFM locus — indeed, *EXT2* had been shown to reside in this interval (Wuyts et al. 1996).

1.6 Aims

The primary goal of this work was the identification of the new gene responsible for PFM/skull vault ossification defects on chromosome 11p by detecting unequivocally pathogenic changes in compatible families. Chapter 3 describes its accomplishment. A novel homeobox gene, the human counterpart of an established mouse gene known for its involvement in limb development through both classical and engineered mutants, was discovered adjacent to *EXT2*. Structural characterisation and cDNA isolation proceeded in parallel with mutation detection. Intragenic mutations were found in five families and a new case of P11pDS was molecularly characterised. At the same time, two new mutations of *MSX2* were ascertained. These findings raised a complementary question: what is the contribution of these two homeobox genes to the mutational spectrum of craniosynostosis? Analyses of both the new gene and *MSX2* were undertaken in a panel of selected craniosynostosis patients and the results of this screen are presented in chapter 4.

The lack of detailed expression data for the new gene in the skull made a controlled expression study – in conjunction with *Msx2* – during skull development imperative. The expression of both genes, along with established markers, was followed by RNA in situ hybridisation during a wide developmental window and the results are shown in chapter 5.

Mutations in the new gene and *Msx2* cause almost identical calvarial defects in humans and result in skull phenotypes with significant overlap in mice, suggestive of a functional link. To begin to address this connection between the two homeobox genes during skull development, their interaction potential was in-

vestigated at the genetic, expression, and protein level. Chapters 6, 7, and 8 record the relevant findings.

Chapter 2

Materials and Methods

2.1 General Methods

2.1.1 Nomenclature

2.1.1.1 Gene and Protein Nomenclature

Official designations are used for human genes, if a symbol has been assigned by the nomenclature authority (HUGO Gene Nomenclature Committee — see appendix F). Otherwise, the most common non-systematic name/abbreviation is adopted.

Similarly, mouse gene symbols adhere to the official nomenclature if the gene has been listed in the relevant database (Mouse Genome Informatics at the Jackson Laboratory — see appendix F). For other species, the most familiar gene symbols and names are used.

On the other hand, the nomenclature for either human or mouse proteins is liberal: although symbols are used consistently, protein products may, or may not, share the same symbol with the respective genes — the most familiar or appropriate designations are embraced.

2.1.1.2 Mutation, Sequence Variation, and Allele Nomenclature

Mutations in human genes and sequence variants in general are described at both the DNA and protein levels, if the changes are not silent, according to the systematic nomenclature guidelines (Antonarakis and the Nomenclature Working Group 1998); recent extensions (den Dunnen and Antonarakis 2001) are also adopted (Human Gene Mutation Database — see appendix F). The only exception is the use of the arrow symbol, '→', instead of the recommended greater-than symbol, '>', to denote a nucleotide substitution. The set of reference genomic sequences for *ALX4* comprised AJ279074, AJ279075, AJ279076 and AJ279077; for *MSX2*, L22498 and L22499 were used.

The symbols for mouse alleles conform to the notation in the Jackson Laboratory gene database (Mouse Genome Informatics at the Jackson Laboratory — see appendix F).

2.1.1.3 Cytogenetic Nomenclature

Descriptions of cytogenetically visible alternations follow, usually in an abridged form, the International System for Human Cytogenetic Nomenclature (Mitelman 1995).

2.1.1.4 Disease Designations

Disease names tend to mirror the leading OMIM descriptor, although aliases are used if considered more appropriate and even renaming of an entry is proposed; the OMIM number is cited as a unique identifier (Molecular Databases at NCBI — see appendix F).

2.1.1.5 Anatomical Nomenclature

Osteological terminology for the murine skull, both at the adult state and during embryonic development, follows a reference work (Kaufman 1992; Kaufman and

Bard 1999). For the human skull, standard terms, as occurring in contemporary anatomy and embryology texts (Martini and Timmons 1997; Larsen 1997; Carlson 1999), are used.

2.1.2 Bioinformatics

The majority of interactive bioinformatic tools were accessed locally through OUBC — see appendix F. The GCG Wisconsin package v. 10.2 (Accelrys) was used for routine DNA and protein analysis; primer design was aided by Primer 3 v. 0.9; an integrating script performing multiple BLAST queries and running several exon prediction programmes simultaneously facilitated gene identification in genomic sequences. BLAST searches were carried out locally or at NCBI (see appendix F) and multiple alignments were computed by CLUSTAL X v. 1.8 (Thompson et al. 1997) at HGMP-RC — see also appendix F. Structural data were visualised with Cn3D v. 4.1, obtained from NCBI.

Additionally, information was retrieved and data were fetched from various biomedical databases; specific references can be found in the relevant sections of the text.

2.1.3 Statistics

Frequency estimates for sequence variants were calculated on the assumption that the sampling process is described by the binomial distribution. Let n represent the sample size and n_{22} , n_{12} , n_{11} the numbers of individuals with respective genotypes. The frequency estimate for the minor allele, $< q >$, is $\frac{2n_{22}+n_{12}}{2n}$ while the corresponding variance estimate equals $\frac{< q >(1-< q >)}{2n}$; translation into a confidence interval was based on F-statistics (Zar 1998). If the minor allele was not found in the test panel, a maximum estimate at $P = 0.95$ was deduced as: $0.05 = (1 - < q >)^{2n}$. In mouse crosses, observed genotypic ratios were checked against the estimated

Mendelian frequencies by the χ^2 test; minor classes were grouped. t-test calculations were performed in StarOffice spreadsheet software (Sun).

2.1.4 Reagents

2.1.4.1 Chemicals and Common Enzymes

All chemicals, unless otherwise stated, were of the Sigma, BDH, Aldrich, Fluka, Riedel de Haën, GIBCO/BRL, AnalaR, Merck, or USB brands. Restriction endonucleases and nucleic acid modifying enzymes were obtained from New England Biolabs, Roche, Promega, MBI Fermentas, or Amersham Biosciences. DNA molecular weight markers were from New England Biolabs, Kramel Biotech, or Sigma, whereas bacterial media were purchased from BIO101 or Difco. Radiochemicals were supplied by Amersham Biosciences — [α - ^{32} P] dCTP (10 mCi/ml; 3,000 Ci/mmol) and PRO-MIX [^{35} S] (14 mCi/ml; >1,000 Ci/mmol methionine).

2.1.4.2 Solutions

For all solutions the working concentrations are cited; if stocks were diluted, standard concentrates were purchased or prepared according to established recipes (Sambrook et al. 1989; Ausubel et al. 1999). Buffer pH was adjusted by adding either HCl or NaOH, unless stated otherwise. For work involving RNA, the solutions were treated with diethyl pyrocarbonate (DEPC) at a concentration of 0.05% if possible; in cases of incompatibility, the solution was prepared with DEPC-treated water and glassware. Distilled H₂O was used for the majority of solutions, unless mentioned otherwise.

2.1.4.3 Oligonucleotides

The majority of oligonucleotides were synthesised by Genosys or Interactiva; several primer pairs to amplify human polymorphic short tandem repeat loci were

purchased from ResGen. Oligonucleotides are referred to by name in the text and the sequences are tabulated in appendix C.

2.1.4.4 Clones

Human P1 Artificial Chromosome (PAC) (Ioannou et al. 1994) and Bacterial Artificial Chromosome (BAC) (Osoegawa et al. 1998) clones were purchased from ResGen. In addition, several plasmids were obtained from other laboratories or organisations under material transfer agreements, where appropriate, permitting use for research purposes. These contributions are acknowledged in the relevant sections.

2.1.4.5 Bacterial Strains

For plasmid propagation the XL1-Blue (Stratagene) and DH10B (Invitrogen) strains of *Escherichia coli* were used; protein expression was conducted in the BL21 strain (Amersham Biosciences).

2.1.5 Laboratory Hardware

2.1.5.1 Centrifuges

Centrifugation was performed in a floor-based RC5 with an SM24 rotor (Sorvall), a benchtop RT 6000B with an H1000B rotor (Sorvall), a benchtop Mark IV (Baird and Tatlock), and in the minicentrifuges Biofuge 13 (Heraeus) and 5415 series (Eppendorf).

2.1.5.2 PCR Machines

Thermocycling was carried out in either PCR Express (Hybaid) or DNA Engine (MJ) machines.

2.1.5.3 Other Basic Equipment

Shaking incubators G24 and G25 (New Brunswick) were used for bacterial cultures; nucleic acid hybridisation was performed in a Mini 10 rotisserie oven (Hybaid); spectrophotometry was carried out in an Ultraspec 2100pro ultraviolet/visible instrument (Amersham Biosciences); and for routine agarose electrophoresis, tanks and power supply units from Hybaid and Bio-Rad were used.

2.1.6 Human Cell Culture

Fibroblasts from biopsies were grown in complete RPMI medium (RPMI-1640, supplemented with fetal bovine serum to a final concentration of 15%, 60 units/ml streptomycin, 60 units/ml penicillin, 1.2 mM glutamine) in a 5% CO₂ atmosphere at 37°C. Detachment of cells for harvesting was achieved by adding a solution of 2.5% trypsin and 2.5% EDTA, followed by sedimentation in a Mark IV centrifuge (1,100 rpm at room temperature for 5 min).

Lymphocytes were immortalised by transformation with Epstein-Barr virus (Doyle 1999) and grown in complete RPMI medium (RPMI-1640, supplemented with fetal bovine serum to a final concentration of 15%, 50 units/ml streptomycin, 50 units/ml penicillin, 2 mM glutamine) in a 5% CO₂ atmosphere at 37°C.

2.1.7 Growth and Manipulation of Bacterial Hosts

Hosts (see section 2.1.4.5) harbouring ordinary plasmids were grown in standard Luria-Bertani medium (LB: 1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 0.1 mg/ml ampicillin or carbenicillin whereas PAC and BAC clones were propagated in the presence of 50 µg/ml kanamycin and 12 µg/ml chloramphenicol respectively; solid versions were prepared by adding agar at 1.5%. For protein expression work the 2x YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) with 0.1 mg/ml ampicillin or carbenicillin was employed.

Competent cells of the strain XL-1 Blue were incubated with 2-mercaptoethanol at 25 mM on ice for 30 min and subsequently mixed with the DNA preparation. Transformation was accomplished by a further incubation on ice for 30 min, a brief heat shock at 42°C for 45 sec, and immediate chilling. The cells were allowed to recover in NZY medium (0.5% yeast extract, 1% NZ amine-A casein hydrolysate, 0.5% NaCl, 0.2% hydrated MgSO₄, 3 mM NaOH) at 37°C for 1 h and, subsequently, plated onto selection LB medium. Essentially the same procedure, with omission of the 2-mercaptoethanol treatment, was applied to cells of the DH10B strain. Finally, to generate competent BL21 cells a culture was harvested during exponential growth – absorbance at 600 nm around 0.4–0.5 – by centrifugation in an RT 6000B (1,000 rpm at 4°C for 15 min) and resuspended, in $\frac{1}{10}$ of the original volume, in ice-cold transformation solution (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 10% polyethylene glycol 3350, 5% DMSO, 50 mM MgCl₂ — pH=6.5). DNA was added in 1 ml of the suspension and the mixture was incubated on ice for 45 min. Heat-pulsing was carried out at 42°C for 2 min, followed by immediate chilling and recovery in LB enriched with 20 mM glucose at 37°C for 1 h; transformants were selected on ampicillin or carbenicillin LB plates.

For long-term storage, a saturated culture of transformed cells was combined with a sterile glycerol solution to achieve a final concentration of 15% and the preparation was kept indefinitely at -70°C.

2.1.8 Isolation of Nucleic Acids

2.1.8.1 Extraction of Human Genomic DNA from Blood Samples

Genomic DNA was extracted from 3–20 ml of whole venous blood which had been collected into an EDTA tube and frozen at -20°C — the following protocol was scaled up or down as required. The method employed white blood cell lysis by

proteinase K and purification of nucleic acids by organic extraction (Sambrook et al. 1989; Ausubel et al. 1999) once the red blood cells had been selectively destroyed.

Thawed blood, approximately 10 ml, was mixed with ice cold 0.1% Nonidet P40 up to a volume of 50 ml and centrifuged in an RT 6000B (3,000 rpm at 4°C for 15 min). The supernatant was removed, the pellet vortexed with 50 ml of 0.1% Nonidet P40, and the centrifugation repeated. The supernatant was discarded and the cells were lysed by resuspension in 10 ml of lysis buffer (10 mM Tris pH=8.0, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, 0.1 mg/ml proteinase K) and incubation at 42°C for 4 h or at 37°C overnight. Following this, 1 ml of salt-EDTA-SDS solution (50 mM sodium acetate, 0.5 M NaCl, 5 mM EDTA, 2.5% SDS) was added and extraction was performed by an equal volume of 1 : 1 chloroform : phenol (equilibrated in 0.1 M Tris pH=7.5) mixture. Following centrifugation (3,000 rpm at 25°C for 15 minutes), the upper, aqueous phase was retrieved and re-extracted: once with an equal volume of chloroform : phenol, and twice with an equal volume of chloroform alone. The DNA was precipitated overnight by adding $\frac{1}{10}$ volumes of 4 M NaCl and 2 volumes of 100% ethanol. After centrifugation (3,000 rpm at 4°C for 15 min), the pellet was washed in 70% ethanol, centrifuged again, air-dried, and dissolved in 0.1–0.5 ml of Tris-EDTA (TE: 10 mM Tris pH=8.0, 1 mM EDTA). The DNA concentration was estimated by spectrophotometry (absorbance at 260 nm) and samples were stored at -20°C.

2.1.8.2 Extraction of Human Genomic DNA from Buccal Samples

Genomic DNA was extracted from buccal epithelial cells using the Puregene DNA Isolation Kit (Gentra Systems). A cytology brush (Cytosoft) that had been briefly scraped against the inside of the cheek was placed in 0.5 ml of cell lysis buffer to suspend the cells. An initial incubation at 37°C for 15 min was followed by digestion at 55°C for 1 h with 1 μ l of 50 mg/ml proteinase K and treatment with 2.5 μ l of 0.32 units/ μ l RNase A at 37°C for 30 min. The sample was cooled to room

temperature and a volume of 170 μ l of protein precipitation solution was added; it was vortexed, put on ice for 5 min, and centrifuged in a minifuge (13,000 rpm at room temperature for 3 min). The supernatant was transferred, mixed with 0.5 ml of isopropanol and 0.75 μ l of 20 mg/ml glycogen, and centrifuged (13,000 rpm at room temperature for 3 min). The DNA was washed with 0.5 ml 70% ethanol, re-centrifuged, air-dried, obtained in 50 μ l of hydration buffer, and stored at -20°C.

2.1.8.3 Extraction of Human Genomic DNA from Cell Lines

Lymphoblastoid cells or fibroblasts were harvested and the culture medium was removed by centrifugation in an RT 6000B (1,000 rpm at room temperature for 7 min), the cell pellet was washed with 25 ml of phosphate-buffered saline (PBS: 0.137 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ — pH=7.4), and re-centrifuged. PBS was discarded and the amount of material was estimated; from this point onwards the blood extraction procedure was applied (see section 2.1.8.1) with only one Nonidet P40 wash.

2.1.8.4 Extraction of Human Total RNA from Cell Lines

RNA was extracted from cultured fibroblasts and lymphoblastoid cells by the acid guanidinium thiocyanate - phenol - chloroform method (Chomczynski and Sacchi 1987). The culture suspension was centrifuged in an RT 6000B (1,000 rpm at room temperature for 7 min) and the cell pellet washed with 25 ml of PBS. After repeating the centrifugation, it was resuspended in an appropriate volume of solution D by passing it several times through a 19-gauge needle. The final RNA pellet was solubilised in 80 μ l of 1 mM EDTA at 60°C for 20 min; the preparation was quantified by absorbance at 260 nm and stored at -70°C.

2.1.8.5 Extraction of Mouse Genomic DNA

The standard method of proteinase K digestion and organic extraction (Sambrook et al. 1989; Ausubel et al. 1999) was also used for mouse tissue specimens.

Approximately 2 mm of the tail tip of an adult – more than 6 weeks old – animal was cut with surgical scissors under an approved procedure (see appendix E). The tissue, unless processed immediately, was stored at -20°C. Digestion was performed in 0.5 ml of buffer (50 mM Tris pH=7.5, 50 mM EDTA, 0.1 M NaCl, 1% SDS) supplemented with 2 μ l of 50 mg/ml proteinase K at 50°C overnight. Undigested material was removed by centrifugation in a minifuge (13,000 rpm at room temperature for 5 min) and the supernatant was subjected to one chloroform : phenol and one chloroform extraction as for human genomic DNA (see section 2.1.8.1). Precipitation followed, with 2 volumes of 100% ethanol and storage at -20°C for at least 1 h. DNA was pelleted by centrifugation (13,000 rpm at 4°C for 20 min), washed with 70% ethanol, re-centrifuged, air-dried, dissolved in 50 μ l of 10 mM Tris pH=8.0, and kept at -20°C.

The same procedure was applied on tissue samples collected from neonates and embryos; once the animal had been humanely killed, the tail tip or a piece of skin respectively was removed.

2.1.8.6 Extraction of Mouse Total RNA from Embryonic Heads

The pregnant female was sacrificed humanely by cervical dislocation, the uterus dissected out of the abdominal cavity and placed in PBS. Under a dissecting microscope (Wild) it was opened and the placental attachments of the conceptuses were cut; the embryos were freed from the extra-embryonic membranes and immediately decapitated. The isolated heads were placed in tubes, flash-frozen by immersion in liquid nitrogen, and stored at -70°C. The amount of tissue was determined and total RNA was isolated by means of the RNeasy Mini Kit (Qiagen) according to the instructions of the manufacturer. Disruption and homogenisation of the tissue was performed by forcing it several times through a 19-gauge and, afterwards, a 21-gauge needle. The concentration of the final preparation was estimated by spectrophotometry (absorbance at 260 nm) and gel electrophoresis which also checked

for degradation. It was stored at -70°C and prior to reverse transcription it was treated with RNase-free DNase (Promega).

2.1.8.7 Purification of Plasmid DNA

Small-scale purification of plasmids employed the QIAprep Spin Miniprep Kit (Qiagen); for larger amounts the column-based Plasmid Midi and Maxi Kits (Qiagen) were utilised. In either case, the protocols in the manufacturer's literature were followed and the supplied solutions were used.

2.1.8.8 Purification of P1 Artificial Chromosomes

PAC clones were isolated by a modified alkaline lysis method, on the basis of instructions provided by the supplier — the following protocol describes small-scale purification with 1.5 ml of saturated culture as starting material. Following centrifugation in a minifuge (13,000 rpm at room temperature for 5 min) the bacterial cells were resuspended in 0.3 ml of buffer supplemented with RNase (15 mM Tris pH=8.0, 10 mM EDTA, 0.1 mg/ml RNase A). Disruption was achieved by adding 0.3 ml of lysis solution (0.2 M NaOH, 1% SDS) and mixing gently. After approximately 5 min, 0.3 ml of precipitation solution (3 M potassium acetate pH=5.5 — adjusted with acetic acid) was added, shaking gently to precipitate proteins and genomic DNA, and the tube was put on ice for at least 30 min. The liquid was separated by centrifugation (13,000 rpm at 4°C for 10 min) and filtration through glass wool (Sigma). Addition of 0.8 ml ice-cold isopropanol and storage on ice for at least 5 min precipitated the DNA which was pelleted by centrifugation (13,000 rpm at 4°C for 15 min). It was washed with 70% ethanol, re-centrifuged, air-dried, and dissolved in 0.1 ml TE at 50°C . Chloroform : phenol and chloroform extractions, followed by salt re-precipitation, were introduced for higher purity at this point — performed as for human genomic DNA (see section 2.1.8.1). The PAC DNA was

obtained in 50 μ l TE and stored at -20°C . The method was also scaled-up when necessary, using an RT 6000B and an RC5 for the centrifugation steps.

2.1.8.9 Purification of Bacterial Artificial Chromosomes

The alkaline lysis method used for PAC clones (see section 2.1.8.8) was also applied to BAC clones with minor changes. The bacterial cells were resuspended in 0.1 ml of Tris-glucose buffer (50 mM glucose, 20 mM Tris pH=8.0, 10 mM EDTA) and lysed with 0.2 ml of standard alkaline solution; precipitation of debris was achieved by adding 0.15 ml of 3 M potassium acetate pH=4.8 (adjusted with acetic acid). Centrifugation was performed immediately in a minifuge (13,000 rpm at room temperature for 10 min) and the DNA was precipitated with 1 ml of 100% ethanol.

2.1.9 The Polymerase Chain Reaction

Unless otherwise stated, a typical polymerase chain reaction (PCR) was carried out using a nominal amount of 40 ng of genomic DNA in a volume of 25 μ l in tubes or plates. For DNA from blood samples and cell lines, a 20 ng/ μ l dilution was prepared and stored for several months at 4°C ; for DNA from buccal samples, the preparation was used undiluted — 0.5–1 μ l per reaction. Occasionally, certain samples were refractory to amplification, presumably due to the presence of inhibitors, and further dilution was needed. The reaction mix contained dNTPs at 0.2 mM, PCR buffer II from Applied Biosystems (10 mM Tris pH=8.3, 50 mM KCl), MgCl_2 at 1.5 mM, 0.5 μ M of each primer and 0.5–1 units of AmpliTaq Gold (Applied Biosystems); DMSO was added up to a final concentration of 10% if required. Cycling parameters for particular applications can be found in the relevant sections.

2.1.10 Reverse Transcription of RNA

First strand cDNA synthesis was performed by random priming. An amount of 10 μ g of total RNA was incubated with random hexamers (Sigma) at 17 μ M in a final

volume of 20 μ l at 70°C for 10 min. After cooling on ice, the reverse transcription reaction was set up in a volume of 40 μ l by adding: buffer (50 mM Tris pH=8.3, 75 mM KCl, 3 mM MgCl₂), dNTPs at 1 mM, DTT at 10 mM, 32 units of RNasin inhibitor (Promega), and 200 units of M-MLV reverse transcriptase (Promega). The mixture was incubated at 37°C for 1 h and the enzyme was inactivated by heating at 95°C for 5 min. The preparation was stored at -20°C and used in downstream PCR amplification reactions.

2.1.11 Restriction Endonuclease Digestion of DNA

For either genomic DNA or PCR products, digestion was performed with a three- to five-fold excess of enzyme (3–5 units per μ g of DNA) in a compatible buffer and at the recommended temperature, for at least 3 h when complete cleavage was desirable; various buffers from several suppliers were used and their details may be found in the relevant technical literature of the companies. If required, heat-inactivation of the enzyme was also carried out.

2.1.12 Gel Electrophoresis

2.1.12.1 Standard Agarose Gel Electrophoresis of DNA

Agarose gel electrophoresis for analytical or preparative purposes was carried out in either Tris-borate-EDTA (TBE: 89 mM boric acid, 89 mM Tris, 2 mM EDTA) or Tris-acetate-EDTA (TAE: 40 mM acetic acid, 40 mM Tris, 2 mM EDTA) buffer systems (Sambrook et al. 1989; Ausubel et al. 1999). Ethidium bromide was added in the gel only, at a concentration of 0.5 μ g/ml, and conventional agarose was used for gels in the 0.8–3% range; to resolve certain fragments, 4% Metaphor agarose (FMC Bioproducts) in TBE was also used. Samples were loaded alongside appropriate molecular weight markers, usually either a *Hind*III digest of phage λ or a *Hae*III digest of phage ϕ X174, after adding one volume of glycerol loading solution (30%

glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) per five volumes of DNA, and the gel was run at 2–5 V/cm. The bands were visualised by transillumination with either short- or long-wave ultraviolet light (the latter was applied when DNA was to be purified for cloning) using apparatus from Bio-Rad. Gel images were captured by means of a camera linked to Quantity 1 v. 4.1.1 software (Bio-Rad).

2.1.12.2 Field-Inversion Gel Electrophoresis of DNA

Field-inversion electrophoresis (Ausubel et al. 1999) was carried out in TBE (at half strength: 45 mM boric acid, 45 mM Tris, 1 mM EDTA) using a 1% gel prepared with low-melting agarose (BMA). The samples were loaded alongside a pulsed-field electrophoresis molecular weight marker and an appropriate programme was selected in the FIGE Mapping power supply unit (Bio-Rad); the buffer was continuously circulated between the anode and cathode by a pump.

2.1.12.3 Polyacrylamide Gel Electrophoresis of DNA

For polyacrylamide gel electrophoresis (PAGE), gels were poured between siliconised glass plates (Bio-Rad), using 0.4 mm spacers and sharks-tooth combs. Unless mentioned otherwise, a pre-made TBE-based mixture was polymerised by adding 25 μ l of TEMED (Amresco) and 400 μ l of 10% ammonium persulphate (Amresco) per 60–70 ml.

For a denaturing, sequencing-type gel (Sambrook et al. 1989; Ausubel et al. 1999) the mixture consisted of 6% acrylamide (Amresco), 0.3% bisacrylamide (Amresco), 7 M urea, and TBE. While the gel was pre-run at 70 W for at least 30 min, the samples were prepared by mixing 5 μ l of DNA with 10 μ l of formamide loading solution (0.25% bromophenol blue, 0.25% xylene cyanol in formamide); they were denatured at 96°C for 5 min, cooled on ice, loaded and run for 2–6 h at 70 W.

For a standard non-denaturing gel (Sambrook et al. 1989; Ausubel et al. 1999), urea was omitted and the final acrylamide concentration was variable, de-

pending on the size range of the fragments. The samples were prepared by mixing 4 μ l of DNA with 1 μ l of loading solution (10% Ficoll 400, 50 mM EDTA, 0.5% SDS, 0.25% bromophenol blue, 0.25% xylene cyanol); they were loaded and run at 10–20 W for several h.

2.1.12.4 Gel Extraction of DNA

The band of interest was excised from an agarose gel and the DNA was retrieved using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions; it was eluted in 30–50 μ l of 10 mM Tris pH=8.0 and stored at -20°C.

2.1.12.5 Polyacrylamide Gel Electrophoresis of Proteins

Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a discontinuous vertical minigel system (Bio-Rad), using 0.5 mm spacers (Sambrook et al. 1989; Ausubel et al. 1999). A 10% separation gel consisted of 0.375 M Tris pH=8.8, 0.1% SDS, 10% acrylamide and 0.264% bisacrylamide; it was polymerised by adding 50 μ l of ammonium persulphate and 5 μ l TEMED per 10 ml of solution. Gels in the 8%–12% range were also employed and prepared by adjusting proportionally the amount of the acrylamide-bisacrylamide mix. The composition of the stacking layer was: 0.125 M Tris pH=6.8, 0.1% SDS, 4% acrylamide and 0.106% bisacrylamide; it was polymerised by adding 25 μ l of ammonium persulphate and 5 μ l TEMED per 5 ml of solution. Five volumes of protein sample were combined with one volume of loading buffer (0.35 M Tris pH=6.8, 10.28% SDS, 36% glycerol, 0.6 M DTT, 0.012% bromophenol blue; kept in aliquots at -70°C) and denaturation was achieved by heating at 95°C for 2 min. A readily detectable molecular weight marker – Precision Broad Range pre-stained marker (Bio-Rad) – was included and the gel was run at 2–5 V/cm for the required length of time in a Tris-glycine buffer (25 mM Tris, 0.192 M glycine, 0.1% SDS).

2.1.13 DNA Sequencing

2.1.13.1 Sequencing of PCR Products

PCR products were directly sequenced by means of the BigDye termination chemistry (Applied Biosystems) and run either on an ABI PRISM 377 gel-based sequencer or an ABI PRISM 3100 capillary sequencer (Applied Biosystems).

Following purification by gel extraction, 40 μ l of DNA were mixed with 10 μ l of alkaline solution (2 M NaOH, 2 mM EDTA) and heated at 37°C for 30 min. The denatured DNA was precipitated by adding 5 μ l of 3 M sodium acetate pH=5.2 (adjusted with acetic acid) and 0.275 ml of 100% ethanol and storing at -70°C for at least 30 min. It was retrieved by centrifugation in a minifuge (13,000 rpm at 4°C for 20 min), washed with 0.5 ml 70% ethanol, re-pelleted (13,000 rpm at 4°C for 5 min), air-dried, and dissolved in 10–20 μ l of H₂O. A standard sequencing reaction was set up with 5 μ l of denatured DNA, 1 μ l of primer dilution (3.3 μ M), 4 μ l of BigDye reagent, 4 μ l of sequencing diluent (0.2 M Tris pH=9.0, 5 mM MgCl₂), and 6 μ l of H₂O; occasionally, for templates with strong secondary structure and/or high GC content, 10% DMSO was included. The reaction mixture was overlaid with mineral oil and the tube placed on a pre-heated, at 96°C, block. Cycling parameters were as follows: 96°C for 15 sec; 96°C for 45 sec, 50°C for 45 sec, and 60°C for 3 min 30 sec (25 cycles). Post-reaction purification involved ethanol precipitation with or without salt, depending on the sequencer employed. For ABI PRISM 377 runs, 2 μ l of 3 M sodium acetate pH=4.8 (adjusted with acetic acid) and 70 μ l of 100% ethanol were added; for ABI PRISM 3100, the reaction was combined with 16 μ l of H₂O and 64 μ l of 95% ethanol. In either case, the mixture was incubated at room temperature for 15 min, followed by centrifugation (13,000 rpm at room temperature for 20 min). The supernatant was discarded, the – usually invisible – pellet was washed with 0.15 ml of 70% ethanol, and re-precipitated (13,000 rpm at

room temperature for 5 min). Ethanol was aspirated and the pellet was dried at 50°C. If required, the desiccated reaction was stored at -20°C for several days.

For analysis on the ABI PRISM 377 instrument, the pellets were dissolved in 4 µl of loading solution (TBE, 5 mM EDTA, 5 mg/ml dextran blue in formamide), denatured at 90°C for 2 min, and 1.5 µl was loaded on a denaturing gel (6 M urea, 4.5% acrylamide, 0.228% bisacrylamide, TBE — de-ionised with Amberlite MB-150 and filtered through a 0.2 µ filter (Nalgene); polymerised by adding 40 µl of TEMED and 0.45 ml of 10% ammonium persulphate in 40 ml of solution) using a 0.2 mm comb (Applied Biosystems). The sequencer was pre-run prior to sample application and electrophoresis was performed under appropriate conditions as recommended by the manufacturer. The gel file was processed with ABI Sequence Analysis v. 3.3 (Applied Biosystems) and the extracted sequence data were analysed in the Sequencher v. 3.1.1 (GeneCodes) package. For analysis on the ABI PRISM 3100, the pellets were dissolved in formamide, denatured at 90°C for 2 min, and automatically loaded on capillaries filled with proprietary polymer (Applied Biosystems). Suitable run parameters, as suggested by the manufacturer, were specified through the 3100 Data Collection software v. 1.0.1 (Applied Biosystems) and primary traces were then processed using ABI Sequence Analysis v. 3.7. Final sequence analysis also employed Sequencher.

2.1.13.2 Sequencing of Plasmids

The protocol for PCR products (see section 2.1.13.1) was slightly modified when plasmid DNA was sequenced. The sequencing reaction was set up with 0.5–2 µg of purified plasmid and the clean up and running steps were identical. Denaturation was achieved by mixing 15 µl of DNA solution with 10 µl of TE and 5 µl of 1.2 M NaOH on ice, followed by heating at 85°C for 5 min. The denatured DNA was precipitated by adding 75 µl of ice-cold 100% ethanol and 3 µl of 2 M ammonium acetate pH=4.5 (adjusted with acetic acid) and storing at -70°C for at least 10 min.

It was obtained by centrifugation in a minifuge (13,000 rpm at 4°C for 20 min), washed with 75% ethanol, re-pelleted, and dissolved in 6 μ l.

2.1.14 Ligation of DNA Fragments

When not readily compatible, the ends of the molecules were modified by the Klenow fragment of DNA polymerase I (New England Biolabs) in a suitable solution – either the supplier's buffer: 10 mM Tris pH=7.5, 5 mM MgCl₂, 7.5 mM DTT; or any restriction enzyme buffer – as described (Sambrook et al. 1989): using 1–2 units, a protruding 3' terminus was removed in the absence of dNTPs, whereas a recessed 3' terminus was filled in their presence at 33 μ M. PCR products were cloned directly in the 5' T-overhang vector pGEM-T Easy (Promega).

A standard ligation reaction was carried out in a volume of 20 μ l, employing T4 DNA ligase (New England Biolabs) in the manufacturer's buffer (50 mM Tris pH=7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 μ g/ml BSA). The amount of enzyme used, in the range of 400–2,000 units, depended on the nature of the ligation — i.e. blunt or cohesive ends. The reaction was incubated at room temperature for at least 3 h; if required, it was stopped by heating and stored at -20°C. For fragment cloning, 10–50 ng of linearised vector and an appropriate amount of insert were used — aiming for a molecular excess of two- to ten-fold in favour of the latter. For intramolecular circularisation, the DNA concentration was reduced in the range of pg/ μ l.

2.1.15 Radiolabelling of DNA

2.1.15.1 Labelling of Double-Stranded Fragments

The purified fragment was labelled by random priming employing the Megaprime Labelling Kit (Amersham Biosciences). An amount of DNA in the range of 20–100 ng was mixed with 5 μ l of random nonamers (concentration unspecified) and the

volume was made up to 32 μl ; following denaturation at 95°C for 5 min, 3 μl of [α -³²P] dCTP, 10 μl of the manufacturer's buffer with unlabelled nucleotides (containing dATP, dGTP and dTTP in a Tris pH=7.5, MgCl₂, and 2-mercaptoethanol solution; particulars unspecified), and 2 μl of the Klenow fragment of DNA polymerase I (1 unit/ μl) were added and the mixture was incubated at 37°C for at least 10 min. The labelled probe was enriched by gel filtration chromatography through a spin column of G-50 Sephadex (Roche): the beads were packed by centrifugation in a minifuge (3,000 rpm at room temperature for 1 min), the reaction was applied, and the flow-through was collected after re-centrifugation (3,000 rpm at room temperature for 2 min). The labelled probe was denatured by heating at 95°C for 5 min, put on ice, and used immediately.

2.1.15.2 Labelling of Oligonucleotides

Oligonucleotides were 3' end-labelled with [α -³²P] dCTP using the deoxynucleotidyl terminal transferase (Roche). A reaction was set up by combining: 1 μl of oligonucleotide (10 μM), 4 μl of the supplier's buffer (1 M potassium cacodylate, 0.125 M Tris pH=6.6, 1.25 mg/ml BSA), 0.6 μl of CoCl₂ (25 mM), 3 μl of [α -³²P] dCTP, and 0.5 μl of terminal transferase (400 units/ μl) in a total volume of 20 μl . Following incubation at 37°C for 30 min, the labelled oligonucleotide was purified by passing the reaction through a column of G-25 Sephadex (Roche) – spin-column chromatography conditions were as for double-stranded DNA (see section 2.1.15.1) – and used immediately.

2.1.16 Blotting and Hybridisation of DNA or RNA

2.1.16.1 Southern Blot Hybridisation Using Double-Stranded DNA Probes

Blotting was performed by capillary transfer from an agarose gel onto a nylon membrane under a neutral high-salt solution (Ausubel et al. 1999). After electrophoresis,

the gel was soaked for 1 h in a denaturing solution (0.5 M NaOH, 1.5 M NaCl) and subsequently in a neutralising solution (0.5 M Tris pH=7.0, 1.5 M NaCl) for 1 h. When fragments larger than 10 kb were present, as for field-inversion electrophoresis gels, the treatment was preceded by exposure to short-wave ultraviolet light on a trans-illuminator for approximately 1 min. Meanwhile, a sponge was placed in a tray half-filled with NaCl-sodium citrate buffer (SSC, at 6-fold strength: 0.9 M NaCl, 90 mM sodium citrate pH=7.0) and covered with two sheets of SSC-moistened 3M paper (Whatman). The gel was placed on the paper and covered with a piece of water-moistened Zeta-probe GT Genomic Tested blotting membrane (Bio-Rad). It was overlaid with two wet pieces of 3M paper and the stack was completed by absorbent paper towels and a weight. Following overnight transfer, the membrane was rinsed in SSC (at 2-fold strength: 0.3 M NaCl, 30 mM sodium citrate pH=7.0), air dried, and the DNA was covalently immobilised by brief exposure to ultraviolet light in a Stratalinker 2400 (Stratagene) and by heating at 80°C for 2 h. The membrane was stored indefinitely at room temperature.

Hybridisation was performed in roller tubes. The membrane was pre-wet with SSC (at 2-fold strength: 0.3 M NaCl, 30 mM sodium citrate pH=7.0), rolled-up, and placed in the tube with the DNA-bound side facing inwards. It was allowed to pre-hybridise at 65°C for at least 4 h in 15–25 ml of phosphate buffer (0.5 M NaHPO₄ pH=7.2, 7% SDS, 1% BSA, 1 mM EDTA). The denatured probe was then added in fresh pre-warmed buffer and hybridisation was carried out overnight at 65°C.

The first wash solution consisted of SSC (at 2-fold strength: 0.3 M NaCl, 30 mM sodium citrate pH=7.0) and 0.1% SDS; the membrane was incubated for at least 10 min under shaking at room temperature. Gradually, the wash stringency was increased by lowering the SSC strength down to 0.1-fold (15 mM NaCl, 1.5 mM sodium citrate pH=7.0); if background noise persisted, the temperature was increased up to 50°C. The wet membrane was then wrapped in cling wrap and exposed to film X-OMAT LS (Kodak) at -70°C.

If necessary for further use, the probe was removed by pouring a hot (70–95°C) solution of 0.1% SDS on the membrane.

2.1.16.2 Southern Blot Hybridisation Using Oligonucleotide Probes

DNA to be hybridised with oligonucleotide probes was transferred from either agarose or denaturing acrylamide gels. For the latter, alkaline blotting with concurrent fixation of the DNA was employed (Ausubel et al. 1999). The gel was overlaid with Zeta-probe GT Genomic Tested blotting membrane, pre-wet in alkaline solution (0.5 M NaOH, 1.5 NaCl); two pieces of 3M paper, soaked in the same solution, were placed on top and pressure was applied for at least 1 h. Following transfer, the membrane was neutralised by immersion in SSC (at 2-fold strength: 0.3 M NaCl, 30 mM sodium citrate pH=7.0) for 10 min.

Hybridisation was carried out in roller tubes using 15–25 ml of a polyethylene glycol-SDS solution (7% polyethylene glycol 8000, 10% SDS). The membrane was rolled-up, placed in the tube with the DNA-bound side facing inwards, and the probe was added. For standard applications it was let to hybridise at 42°C for at least 3 h; unbound probe was removed by a single wash with SSC (at 2-fold strength: 0.3 M NaCl, 30 mM sodium citrate pH=7.0) and 0.1% SDS at room temperature. For sensitive applications, like typing with allele-specific oligonucleotides, all critical parameters – hybridisation temperature as well as composition and temperature of the final wash solution – were optimised empirically and can be found in the relevant sections. The wet membrane was covered with cling wrap and exposed to film X-OMAT LS at room temperature.

When required, stripping was achieved by heating the membrane up to 70°C in a solution of 0.1% SDS.

2.1.16.3 Colony Blot Hybridisation

To identify transformants likely to harbour a desired construct, bacterial colonies were lifted onto nylon membrane, lysed, and the DNA was fixed in situ according to standard methods (Sambrook et al. 1989). Round pieces of Hybond-N membrane (Amersham Biosciences) were used while the hybridisation itself was performed as described either in section 2.1.16.1 or in section 2.1.16.2, depending on the nature of the probe.

2.1.16.4 Northern Blot Hybridisation

Commercially available RNA blots were purchased and hybridised with double-stranded DNA probes in Ultrahyb solution (Ambion) within roller tubes. After pre-hybridising the membrane in 10–15 ml of buffer at 42°C for at least 30 min, the denatured probe was added and hybridisation was allowed to proceed overnight at the same temperature.

The initial wash consisted of SSC (at 2-fold strength: 0.3 M NaCl, 30 mM sodium citrate pH=7.0) and 0.1% SDS and was carried out at 42°C; for the final wash the SSC concentration was reduced (0.1-fold strength: 15 mM NaCl, 1.5 mM sodium citrate pH=7.0). The wet membrane was covered with cling wrap and exposed to film X-OMAT LS at -70°C for several days.

2.1.17 Genotyping of Short Tandem Repeat Loci

Details for the human short tandem repeat loci of interest were accessed through The Genome Database — see appendix F. They consisted of polymorphic (CA)_n elements, (GATA)_n elements, or complex repeats and the majority have been reported in the context of human genetic or radiation hybrid maps (Gyapay et al. 1994; Murray et al. 1994; James et al. 1994; Dib et al. 1994; Stewart et al. 1997); gradually, all have been integrated in the human genome sequence and are accurately localised (Ensembl v. 16.33.1 — see appendix F).

PCR was performed in plates in a volume of 25 μ l either under the standard method (see section 2.1.9) or under the Généthon (Gyapay et al. 1994) recipe (10 mM Tris pH=9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.01% gelatin, 0.125 mM dNTPs, 1 μ M of each primer, and 0.5–1 units of AmpliTaq (Applied Biosystems)). Optimal conditions for amplification are specified for each marker. Unless otherwise mentioned, a uniform cycling programme was used: 94°C for 4 min; 94°C for 1 min, cooling to 55°C at a rate of 1°C per sec and incubation for 1 min (35 cycles); and 72°C for 5 min.

PCR products were combined if the size ranges of the loci were compatible and resolved by denaturing PAGE. After electrophoresis, the gel was blotted and hybridised with an appropriate radiolabelled probe: a (CA)₁₀ oligonucleotide, a (GATA)₄ oligonucleotide, one of the PCR primers, or a mixture thereof was used.

2.1.18 Denaturing High-Performance Liquid Chromatography Analysis of DNA

PCR-amplified DNA fragments were screened for heterozygous variants using automated denaturing high-performance liquid chromatography (DHPLC) analysis (Underhill et al. 1997) as implemented in the WAVE DNA Fragment Analysis System (Transgenomic). In principle, under partially denaturing conditions all different homoduplexes and heteroduplexes in a DNA population would exhibit distinct elution profiles when subjected to ion-pair reverse-phase chromatography; in practice, the majority of heteroduplex species produced patterns which ranged from strikingly different to subtly dissimilar when compared to normal homoduplexes.

A 30- μ l oil-free PCR reaction was performed in skirted 96-well plates (ABgene), covered with adhesive film (ABgene) under a heated lid. To minimise noise from polymerase-introduced changes, a combination of 0.9 units of AmpliTaq Gold and 0.15 units of Pwo (Roche) was used. Heteroduplexes were formed by melting

and letting the PCR products reanneal gradually: 95°C for 5 min; cooling to 68°C at a rate of 0.03°C per sec; 68°C for 5 min; and cooling to 20°C at a rate of 0.02°C per sec. A volume of 5 μ l was injected onto the solid phase which consisted of alkylated poly(styrene-divinylbenzene) particles, packed in a column, and eluted by a gradient of acetonitrile in 0.1 M triethylammonium acetate (Transgenomic) — ultra pure H₂O (resistance: 18 M Ω /cm) was used in solution preparation. The output was monitored by plotting its absorbance at 260 nm. Column temperature and liquid phase parameters were optimised for each individual fragment. Melting profiles were calculated by the WAVE Maker software v. 3.4.4 (Transgenomic) and, usually, two temperatures were selected — the local helical fraction was always above 60%, but below 100% in the regions of interest. The gradient rate was manually adjusted in order to centre the peaks in the elution time window.

The chromatograms were assessed subjectively. A gross initial comparison of all experimental waveforms against the pattern of the normal homoduplex and the patterns of controls representing frequent polymorphisms at heterozygosity, run in the same batch, resulted in a preliminary classification — there was some flexibility to accommodate PCR artefacts and/or poor yield. A second round of evaluation followed: experimental and control patterns were superimposed and inspected for even fine differences. Samples with aberrant or suspicious patterns were then short-listed for sequencing.

2.1.19 Single-Strand Conformation Polymorphism Analysis of DNA

PCR amplimers were screened for variants by manual, gel-based, single-strand conformation polymorphism (SSCP) analysis using [³²P] dCTP. A standard 25 μ l PCR reaction was performed in the presence of 0.1 μ l [α -³²P] dCTP. A 5 μ l aliquot was mixed with 10 μ l of SSCP loading solution (20 mM EDTA, 5 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol in formamide), denatured at 96°C for 5 min, and kept on ice until loading.

Electrophoresis was carried out in non-denaturing polyacrylamide gels under two different protocols, based on TBE (at half strength: 45 mM boric acid, 45 mM Tris, 1 mM EDTA) and Tris-Mes-EDTA (TME: 35 mM Mes, 30 mM Tris, 1 mM EDTA) buffers (Hayashi et al. 1998). The TBE gel (7% acrylamide, 0.186% bisacrylamide, half strength TBE) was polymerised by adding 0.1 ml of TEMED and 0.2 ml of 10% ammonium persulphate in 60–70 ml of solution and run under regulated conditions: constant power of 20–30 W at 10°C, by using a power supply unit linked to a temperature sensor (Bio-Rad) within a cold room with nominal temperature of 4°C. Following a pre-run of at least 30 min to achieve temperature uniformity across the gel, the samples were loaded and allowed to migrate for 4 to 10 h, depending on the size of the fragments. The TME gel was prepared as above with TME replacing TBE and run at a higher temperature: under constant power of 80 W, a gel temperature of 22°C was reached in an environment of approximately 4°C. Following electrophoresis, the gel was transferred onto 3M paper, dried on a vacuum drier at 80°C (Bio-Rad), and exposed to film X-OMAT LS.

2.2 Patients

All laboratory and clinical investigations for research purposes were performed under projects authorised by the local clinical ethics committee. Details, including invitation letters and information sheets, are presented in appendix E.

Families 1–20, comprising the PFM/skull defects panel, were recruited through three channels. Clinicians who had published reports of relevant phenotypes were invited to approach these subjects and, upon observance of the ethical guidelines, obtain specimens for analysis. In other cases, the laboratory was contacted directly by clinicians wishing to arrange mutational analyses for their patients. In addition, certain families were ascertained locally. Skull phenotypes were assessed by plain radiography, three-dimensional computed tomography (CT) scanning, or magnetic resonance imaging (MRI). With the exception of fam-

ily 8, no individuals had radiological manifestations suggestive of craniosynostosis or CCD, and at least one affected person from each family had a normal G-banded karyotype. Family 6 demonstrated the hallmark clinical features of P11pDS — PFM with multiple exostoses of the long bones. With the exception of family 4, available clinical descriptions of families segregating *ALX4* mutations did not indicate any associations with dysmorphic features, abnormalities of the limbs, abdominal wall, male genitalia, skin, hair, or teeth. On the other hand, families 21 and 22 were singled out from a panel of 211 individuals with craniosynostosis where the common mutations in *FGFR2*, *FGFR3*, *TWIST1*, and *FGFR1* had been previously excluded. A full list of the families, summarising the clinical and molecular findings, is affixed in appendix B.

Family 1 (Figure 3.6) resides in Spain and a clinical account has been previously published (Salamanca et al. 1994). PFM were noted in a young boy, III-5, and a large posterior fontanelle was detected prenatally in his sister, III-6, by ultrasonography at 15 weeks; radiography upon birth confirmed a midline defect. Both their father, II-6, and the paternal grandmother, I-2, showed typical bilateral foramina but otherwise the family history is unremarkable. The feature could have arisen *de novo* as there was no evidence of skull defects in the previous generation. The underlying mutation has been published (Mavrogiannis et al. 2001).

Family 2 (Figure 3.7) is of Slovakian origin and has been partially described (Kutilek et al. 1997). However, the kindred was re-assessed and the clinical information was revised and enriched following comprehensive skull radiography. Originally, locally thin parietal bones in individual IV-4 in early childhood were misinterpreted as a sign of rickets. Radiography at the age of 19 months revealed well-defined symmetrical PFM while the anterior and posterior fontanelles had closed. There were no dysmorphic features or neurological findings. Her younger sister, IV-6, was born with a midline defect whereas her older brother, IV-5, had an ambiguous presentation — abnormal skull modelling yet no apparent defects. The

trait was traced to the paternal line: PFM were ascertained in the father, III-6, the paternal grandmother, II-3, and the great-grandmother, I-2. A paternal great-aunt, II-4, exhibited only increased bone thickness in an otherwise normal skull radiograph, but was inferred to be an obligate carrier — both her offspring, III-7 and III-8, manifested PFM. On review of the family history, there were apparently coincidental associations with other medical conditions, yet two members with skull defects had learning difficulties of unknown cause. The underlying mutation has been published (Mavrogiannis et al. 2001).

Family 3 (Figure 3.8) is British. A midline skull defect and a patent vitello-intestinal duct were ascertained in IV-1 as a neonate. Subsequently, PFM were identified on skull radiography in his father, III-4, and great uncle, II-1, neither of whom were previously aware of their condition. Individual III-2, the daughter of II-1, was born with a midline skull defect and had unexplained drop attacks during childhood. A CT brain scan revealed an enlarged, cerebrospinal fluid-filled space in the posterior fossa. The underlying mutation has been published (Mavrogiannis et al. 2001).

Family 4 (Figure 3.9) is from Spain. A diagnosis of familial PFM was established in III-2 during childhood on recurrence of the defects seen in previous generations which, however, did not require any surgical intervention. Her dentition included only two upper incisors and she exhibited slightly deviant facial features. On inspection of the limbs, short and broad thumbs with bilateral fifth finger brachydactyly were noticeable. The rest of the skeleton was unremarkable and there was no evidence of neurological problems. Apart from the skull defects, her younger brother, III-3, presented with microcephaly and a similar combination of other abnormalities — absence of two upper incisors, mild facial dysmorphism, as well as short and broad thumbs. His global skeletal development was lagging behind his age and, as previously, there was no indication of neurological complications. The elder brother, III-1, also had microcephaly – but normal psychomotor

development – in addition to PFM and a comparable spectrum of minor abnormalities: malpositioned teeth, similar facial presentation with his siblings, as well as short and broad thumbs and halluces. On skull radiography, PFM were evident in both their father, II-3, and the paternal grandfather, I-2, who had also witnessed the trait in the previous generation. Microcephaly is likely to be a coincidental finding as it was seen in the spouse of II-3, II-4.

Family 5 (Figure 3.10) resides in Brazil. The family was ascertained through individual III-2 who presented with a severe, generalised skull defect in infancy. His mother, II-2, had a wide opening in the posterior parietal region while the maternal grandfather, I-2, had typical PFM. All three affected members exhibited a wide hiatus of the tentorium cerebelli associated with a large cerebrospinal fluid-filled space on brain MRI scans. However, there was no evidence of cerebellar hypoplasia. Apart from benign neonatal convulsions in II-2 and III-2, no other clinical abnormalities were noted; moreover, an electroencephalogram in III-2 was normal. The underlying mutation has been published (Mavrogiannis et al. 2001).

Certain clinical and molecular aspects of family 6 (Figure 3.12), segregating P11pDS, have been reported previously (Davies et al. 1997). Apart from individuals III-1, III-2, II-1, and II-2 the initial study included the grandfather I-1 as well as two other affected members from a different branch of the pedigree. The combination of PFM and exostotic outgrowths was a consistent finding in affected members but there were no dysmorphic features and, importantly, no signs of mental retardation. The familial microdeletion has been grossly characterised (Davies et al. 1997): the deleted region was found to extend from *D11S905* distally to *D11S903* proximally, thus confirming hemizyosity for *EXT2*.

Family 7 (Figure 3.14) is from the Netherlands. A congenital skull defect in individual II-1 was closed by bone grafting during infancy. Although the medical history regarding the previous generation was, allegedly, negative, familial incidence of the trait was confirmed upon birth of his son, III-1. A wide posterior

fontanelle was observed and surgical intervention was also considered necessary. Apart from the skull defects the medical histories of II-1 and III-1 were unremarkable.

Family 8 (Figure 3.15) is British. PFM were first noticed in the proband, III-1, during childhood and the diagnosis of PFMCCD was established at the age of 18 years after an incidental radiological examination because of head injury. His father, II-1, had palpable defects and reported that a chest radiograph taken as part of a routine Army medical examination had revealed short clavicles. The paternal grandmother, I-1, was reputed to have sloping shoulders but was not available for examination. The proband has three children, two of whom share similar defects; both also have broad foreheads with frontal bossing, but their head circumferences are within normal limits. The elder son, IV-1, presented with well-defined PFM and hypoplastic clavicles whereas the younger son, IV-3, had wide posterior and anterior fontanelles in infancy. No other medical problems were notable. The underlying mutation has been published (Garcia-Miñaur et al. 2003).

The clinical and molecular data regarding family 9, of British origin, have been previously published (Wilkie et al. 2000). A deletion encompassing the entire *MSX2* gene segregates in the pedigree and clinically affected members showed typical PFM with no other noteworthy conditions. However, there is at least one case of non-penetrance in the family, represented by individual II-2.

The underlying mutation – *MSX2* 515G→A (*MSX2* R172H) – in family 10, of Polish origin, has been previously reported (Wilkie et al. 2000). Apart from PFM, macrocephaly and a small Wormian bone were noticed in the proband, II-1; in addition, headaches were mentioned and abnormalities were seen in an electroencephalogram. No PFM were observed in his father, I-2, but soft swellings on the skull during childhood were reported.

Family 11 is also of Polish origin and the causative mutation along with a clinical description have been published (Wilkie et al. 2000) — a two-amino acid

deletion within the homeodomain of *MSX2*: *MSX2* 475_480delCGCAAG (*MSX2* R159_K160del). In the proband, II-1, and her mother, I-1, apart from PFM, dental abnormalities were seen. In II-1, eruption of primary teeth was delayed and the order was reversed while the permanent dentition also appeared late. Furthermore, advanced caries were evident. I-1 had false upper teeth at the age of 27 years. Other family members were subsequently recruited: the maternal grandfather and grandmother, two sisters and a brother of I-1, as well as two nephews. None of them had a history of skull defects but all three sibs of I-1 had dental problems — advanced caries or a need for artificial teeth.

Family 12 is also of Polish origin and a clinical account has been published (Chrzanowska et al. 1998). The proband presented with multiple skeletal anomalies – including parietal bone defects –, facial dysmorphism, branchial and auricular fistulae, genital abnormalities, sensorineural hearing loss, and teeth and hair anomalies upon birth. The family history was negative and the syndromic associations suggest either a new branchial syndrome (Chrzanowska et al. 1998) or the FG syndrome (Rauch et al. 1998) — OMIM 305450.

The proband in family 13, of British origin, exhibited a skull defect in the superior occipital region during infancy which was confirmed by imaging. Bulging of tissue was also noticeable and a diagnosis of haemangioma with an associated osseous defect was suggested. There were no neurological findings and the family history was negative.

A radiological case report of giant parietal foramina in the proband of family 14, of German origin, has been previously published (Solymosi et al. 1987); his mother was, also, affected.

The proband of family 15, of British origin, was originally erroneously diagnosed with PFM. The skull defect is in the occipital region and a number of other abnormalities have pointed towards the VATER association (OMIM 192350).

Family 16 is Australian. The proband presented with multiple skeletal abnormalities, including PFM, as well as language and motor delay. Abnormal radiological findings were reported for her mother but there was no indication of PFM.

Limited information exists for family 17, of British origin. The only member available for analysis was the allegedly unaffected father of the proband.

Family 18 resides in Poland. Skull defects with hair agenesis in the overlying skin, leaving two oval, hairless spots on the parietal bone were seen in the proband. However, the family history appeared negative.

Family 19 is British. Samples from a couple with a negative history on both sides were analysed following a termination of pregnancy — the fetus exhibited multiple anomalies, including PFM.

Family 20 is of German origin. PFM and unilateral foot hexadactyly were noticed in the proband upon birth, although the skull defects healed spontaneously soon afterwards. Delayed skull development as well as partial tooth agenesis was reported for his father. There was no indication of skull defects in the paternal grandparents.

Only the proband, diagnosed with Crouzon syndrome, was available for analysis from the consanguineous family 21 — parents are first cousins. However, review of the medical history indicated a rather atypical condition with craniosynostosis and connective tissue abnormalities reminiscent of Marfan syndrome (OMIM 182212). Her brother also had marfanoid features and subtle craniosynostosis.

In the nuclear family 22, the proband presented with uni-coronal synostosis; both parents were asymptomatic.

2.3 Mice

Mice colonies were maintained in an animal house with a constant light cycle environment — 10 h dark : 14 h light period. Animals, allowed to mate overnight, were inspected for a vaginal plug in the morning of following days; when found, that day was designated as day 0 of the gestation period. When sacrificed for analysis, only non-regulated 'Schedule-1' methods of humane killing (Wolfensohn and Lloyd 1998) were employed and details can be found in the relevant sections. Nevertheless, genotyping of live animals involved a minor amputation and was covered by personal and project licences — particulars are included in appendix E.

Heterozygous male and female mice for the *Alx4^{tm1Rwi}* allele (Qu et al. 1997b) in the 129 background were a gift from Dr Ron Wisdom. A male mouse heterozygous for the *Msx2^{tm1Rilm}* allele (Satokata et al. 2000) in a complex background was provided by Dr Robert Maxson. By history, the background was estimated as 80% : 20% C57BL/6 : BALB/c with a minor 129 contribution. Breeding colonies were established by mating the mutants with corresponding wild-type Sv129 and C57BL/6J strains (Harlan) for at least five generations.

2.4 Linkage Analysis

Two-point lod scores were calculated using MLINK of the FASTLINK package v. 4.0P (Cottingham et al. 1993) at HGMP-RC — see appendix F.

Linkage of four chromosome 11p short tandem repeat loci, *D11S1393*, *D11S903*, *D11S2095*, and *D11S554*, to non-syndromic PFM was investigated in families 2 and 1 (see section 2.2 and appendix B). The disorder was considered as an autosomal dominant trait with a mutant allele frequency of 10^{-5} and three liability classes with different penetrance values in heterozygotes were defined. Class 1 included affected individuals and normal, unrelated, spouses; by definition, the penetrance value equaled 1; class 2 accounted for radiologically nor-

mal individuals at 50% prior risk; arbitrarily, a penetrance value of 0.8 was assigned. Class 3 covered apparently normal individuals at 25% prior risk but without radiographic evidence; to recognise this uncertainty, the penetrance value was relaxed to 0.5. In compiling pedigree data files for analysis, all individuals from family 1 were allocated in class 1 whereas certain members of family 2 fell into class 2 (II-1, II-4, III-4) and class 3 (III-1, III-2, III-3, IV-2, IV-3). The phenotypic status was specified unambiguously for all persons with the exception of individual IV-5 in family 2 where it was considered as unknown.

Genotyping data for the markers employed along with the aforementioned assumptions can be found on inspection of the input files in appendix D; the output tables are also affixed.

2.5 Isolation and Characterisation of Human *ALX4*

2.5.1 Mapping

After consulting publicly available web pages at the Southwestern Medical Center, Texas, USA, displaying a contig alleged to cover the *D11S903–D11S2095* interval – apparently assembled during the course of *EXT2* cloning (Stickens et al. 1996) – the PAC clones RPCI-3 404c10, RPCI-3 526d1, RPCI-1 189f14, RPCI-3 366b4, RPCI-3 511i10, RPCI-3 404m15, RPCI-1 189p12, and RPCI-3 368b4 were obtained for independent evaluation. Preliminary analysis indicated that RPCI-3 404c10 and RPCI-1 189p12 were unlikely to map around *EXT2* and further work on them was abandoned. On the other hand, the BAC clones RPCI-11 70a24 and RPCI-11 706a13 as well as the cosmid clone cSRL101h11 were identified through BLAST queries starting with *EXT2*- and RPCI-3 404m15-derived sequences respectively; at a later stage of the project, limited experimental analysis of the two BACs was carried out. Mapping employed a variety of techniques to allow a degree of redundancy. A technical account is provided below while the results are embodied in Figure 3.1.

2.5.1.1 Sequence Content

RPCI-3 404m15 was found to have been sequenced in its entirety (accession number: AC124062) and overlapped at one end with the fully-sequenced cosmid cSRL101h11 (accession number: U73628). Within this continuum, *D11S1393*, *D11S578*, as well as the previously developed STSs 102d9T7 and 109d12T3 (Stickens et al. 1996) that map immediately upstream of the first exon of *EXT2*, were identified. On the other side of *EXT2*, as the sequence cover provided by the sampled BAC clones RPCI-11 70a24 (accession number: AC025533) and RPCI-11 706a13 (accession number: AC090163) improved, several of the 3' exons of the gene, the marker *D11S2095*, and all four exons of *ALX4* were gradually recognised within unordered fragments.

2.5.1.2 PCR for Sequence-Tagged Sites

The panel of promising PAC clones – RPCI-3 526d1, RPCI-1 189f14, RPCI-3 366b4, RPCI-3 511i10, RPCI-3 404m15, and RPCI-3 368b4 – was checked against a set of STSs marking sequences within and around *EXT2*.

Two STSs were developed to highlight the ends of clone RPCI-3 404m15: 404m15-1, defined by the primer pair 404m15-1-F and 404m15-1-R, and 404m15-3, defined by 404m15-3-F and 404m15-3-R. As mentioned, 102d9T7 was expected to map just upstream of *EXT2*; it was amplified using the primers 102D9T7-F and 102D9T7-R (Stickens et al. 1996). The exons of *EXT2* provided six definitively-ordered STSs, designed on available genomic sequences (Clines et al. 1997): EXT2-EX3 for exon 3 (primers EXT2-EX3-F and EXT2-EX3-R); EXT2-EX8 for exon 8 (primers EXT2-EX8-F and EXT2-EX8-R); EXT2-EX9 for exon 9 (primers EXT2-EX9-F and EXT2-EX9-R); EXT2-EX10 for exon 10 (primers EXT2-EX10F and EXT2-EX10R); EXT2-EX11 for exon 11 (primers EXT2-EX11-F and EXT-EX11-R); and EXT2-3GN for exon 14 (EXT2-3GN-F and EXT2-3GN-R). With the exception of 102d9T7 and EXT2-EX3 all were amplifiable under standard conditions using

a $\frac{1}{100}$ dilution of PAC DNA as template; the cycling programme was: 94°C for 4 min; 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec (35 cycles); 72°C for 7 min. The EXT2-EX3 PCR failed to work and for 102d9T7 the annealing temperature was lowered to 50°C.

Moreover, the well-ordered short tandem repeat loci *D11S1393*, *D11S903* (sequence-mapped adjacent to exon 5 of *EXT2*), and *D11S2095* (Wuyts et al. 1996) were incorporated in the analysis — amplification conditions can be found in 2.6.1.

2.5.1.3 Hybridisation

PCR-typing of the PAC clones was complemented by hybridisation. All clones in the mapping panel were digested with either *KpnI* or *EcoRI*, blotted, and hybridised with labelled probes for all the STSs mentioned in section 2.5.1.2. Either PCR products (for non-tandem repeat sites) or one of the primers (for tandem repeat loci) were employed as probes under routine conditions.

Once the exons of *ALX4* were defined they were also mapped onto the contig: PCR products representing the four exons (see section 2.6.2) were used to probe the PAC blots.

Furthermore, the BAC clones RPCI-11 70a24 and RPCI-11 706a13 were examined, through an equivalent approach, for presence of *D11S2012*.

2.5.1.4 Isolation of Clone Ends

The clones RPCI-3 366b4 and RPCI-3 368b4 were fine-mapped by means of end-fragment isolation. Following a digest with *ApaI*, the vector pCYPAC2 with the residual insert was re-circularised and used to transform XL-1 Blue cells. Positive isolates were obtained and the two ends per original clone were released by a combined *ApaI* and *NotI* digest, purified, and utilised as probes against the full panel of PAC clones — see section 2.5.1.3.

2.5.1.5 Restriction Mapping

All PAC clones in the mapping panel were cut with *Mlu*I and the resulting fragments were separated by field-inversion electrophoresis. This digest offered sizing data for the PAC clones and, following blotting, was used as a resource to investigate the orientation and spacing of the *EXT2* and *ALX4* transcription units. Successive probing with the amplimers EXT2-3GN-F–EXT2-3GN-R, ALX4-F5–ALX4-R5, ALX4-F2–ALX4-R2, and ALX4-F3–ALX4-R3 as well as with one of the primers for *D11S2095* demonstrated common bands for certain probe combinations.

A more focused approach employed various digests – *Sac*II, *Eco*RV, *Bss*SI, *Nae*I, *Aat*II, and *Xho*I – of RPCI-3 526d1. The fragments were resolved by field-inversion electrophoresis and a blot was prepared which was, similarly, subjected to sequential hybridisation with all the aforementioned probes; common bands were then identified and sized.

Finally, by exploiting unique recognition sites for *Mlu*I and *Asc*I in the insert of RPCI-3 511i10, the large first intron of *ALX4* was sized. Single digests and a combined digest were performed, electrophoresed in a field-inversion gel, blotted, and probed with the ALX4-F3–ALX4-R3 product.

2.5.1.6 Long-Range PCR

The orientation of *ALX4* relative to *EXT2* was verified and the intergenic interval estimated precisely by long-range PCR using the clones RPCI-3 526d1 and RPCI-3 511i10 as templates. The same approach on RPCI-3 511i10 enabled accurate sizing of all but the first intron of *ALX4*. The Expand Long Template PCR System (Roche) was used following the recommendations of the manufacturer: PCR reactions were set up in 50 μ l and contained the proprietary buffer 3 with 2.25 mM MgCl_2 (details unspecified), 5% DMSO, 0.5 mM dNTPs, 0.3 μ M of each primer, 2.5 units of the polymerase mix, and a $\frac{1}{100}$ dilution of PAC DNA. The cycling programme included a incremental elongation step: 94°C for 2 min; 94°C for 10 sec, 50°C for 30

sec, 68°C for 10 min (10 cycles); 94°C for 10 sec, 50°C for 30 sec, 68°C for 10 min (20 cycles, with a 20 sec increment per cycle); 68°C for 7 min. The primer combinations EXT2-3GN-F and ALX4-F5, EXT2-3GN-F and ALX4-R3 were initially assessed; subsequently, the *EXT2*–*ALX4* interval was split by testing all possible combinations of EXT2-3GN-F and ALX4-F5 with *D11S2095* primers. Sizing reactions for the introns of *ALX4* were attempted with the combinations ALX4-F3 and ALX4-R2, ALX4-F2 and ALX4-R4, ALX4-F4 and ALX4-R5.

2.5.1.7 Miscellaneous

The final contig (Figure 3.1) includes one reasonable assumption from the existing literature. The distance between the first exon of *EXT2* and the experimentally mapped STS 102d9T7 was inferred from a previous physical map of the region (Stickens et al. 1996) — estimated to be in the range of few kb.

2.5.2 Assessment of Copy Number

Digests of normal human genomic DNA with *Kpn*I, *Hind*III, *Bam*HI, *Eco*RI, and *Nco*I were resolved in a 1% agarose TAE gel, blotted and hybridised with the PCR products ALX4-F3–ALX4-R3 or ALX4-F5–ALX4-R5.

2.5.3 Detection of mRNA

Ready-to-hybridise filters with poly(A)+ RNAs from several human fetal tissues – Real Human Fetal mRNA Blot (Invitrogen) and Human Fetal Multiple Tissue Northern Blot II (Clontech) – were probed with the PCR products ALX4-F3–ALX4-R3 or ALX4-F5–ALX4-R5.

2.5.4 cDNA Isolation by Reverse Transcription-PCR

Using the primer combination ALX4-F3 and ALX4-R5, an approximately 1.6 kb-long RT-PCR product could be obtained from normal human fibroblast RNA preparations — PCR conditions were as for amplification of *ALX4* exonic segments

(see section 2.6.2) but with 38–42 cycles. The amplicon was ligated successfully with pGEM-T Easy but on propagation in the XL-1 Blue host, rearrangements were taking place. Instead, and to circumvent potential PCR errors, the product was subjected to direct sequencing with an array of specific primers — ALX4-F3, ALX4-F7, ALX4-F10, ALX4-F8, ALX4-R7, ALX4-R10, ALX4-R8, and ALX4-R5. This sequence was assigned the accession number AJ404888 and an annotated version is shown in appendix A.

An independent cDNA sequence (accession number: AB058691), derived from the clone KIAA1788 (Nagase et al. 2001), is essentially identical — there are four differences but at the polymorphic positions 104, 304, 729, and 879.

2.6 Molecular Analysis of *ALX4*

2.6.1 Linked Genetic Markers

A set of four polymorphic markers in the vicinity of *ALX4*, *D11S1393*, *D11S903*, *D11S2095* and *D11S2012*, were typed for linkage analysis purposes, served as landmarks during contig construction, and provided evidence of heterozygosity in the region. They were localised experimentally relative to *ALX4* (Figure 3.1) and map, in any case, less than 300 kb away from the gene. Other sets comprising more distant short tandem repeat loci both telomeric-to-*ALX4* – *D11S4173*, *D11S1330*, *D11S1279*, *D11S905* – and centromeric-to-*ALX4* – *D11S4103*, *D11S554*, *D11S1361* – were used to map the deletion in family 6. Particulars are listed in Table 2.1.

2.6.2 PCR Amplification

The coding portion of *ALX4* was amplifiable in four fragments under uniform conditions, in the presence of 10% DMSO: 94°C for 4 min; 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec (35 cycles); 72°C for 7 min. The PCR for the coding part of exon 1 used the primer pair ALX4-F3 and ALX4-R3, producing a 651 bp fragment;

Table 2.1: Genetic Markers around *ALX4*

Marker	Repeat Type	Distance from <i>ALX4</i>	Amplification Reaction	Cycling Programme	Reference
<i>D11S1393</i>	(GATA) _n	~220 kb (t)	Standard	Standard	Murray et al. (1994)
<i>D11S903</i>	(CA) _n	~90 kb (t)	Généthon	Standard	(Dib et al. 1994)
<i>D11S2095</i>	(CA) _n	<10 kb (t)	Standard	Standard	James et al. (1994)
<i>D11S2012</i>	(GATA) _n	~60 kb (c)	Standard	Standard	Murray et al. (1994)
<i>D11S4173</i>	(CA) _n	~4.0 Mb (t)	Généthon	Standard	Dib et al. (1994)
<i>D11S1330</i>	(CA) _n	~3.9 Mb (t)	Généthon	Standard	Dib et al. (1994)
<i>D11S1279</i>	(CA) _n	~3.8 Mb (t)	Standard	Standard	Stewart et al. (1997)
<i>D11S905</i>	(CA) _n	~3.3 Mb (t)	Généthon	Standard	Dib et al. (1994)
<i>D11S4103</i>	(CA) _n	~0.5 Mb (c)	Généthon	Standard	Dib et al. (1994)
<i>D11S554</i>	Compound	0.6 Mb (c)	Standard	95 °C for 2 min; 95 °C for 15 sec, 60 °C for 30 sec, 72 °C for 90 sec (35 cycles); 72 °C for 5 min	Phromchotikul et al. (1992)
<i>D11S1361</i>	(CA) _n	0.6 Mb (c)	Généthon	Standard	Dib et al. (1994)

Notes: *D11S1393*, *D11S903*, *D11S2095* and *D11S2012* are tightly linked to *ALX4*; *D11S4173*, *D11S1330*, *D11S1279* and *D11S905* comprise a telomeric marker set while *D11S4103*, *D11S554* and *D11S1361* map on the centromeric side. Following the marker name, the nature of the repeat element and the approximate physical distance from *ALX4* (t: telomeric side; c: centromeric side) are cited. Distances were either estimated experimentally or reflect the current human genomic assembly (Ensembl v. 16.33.1 — see appendix F). For the standard and Généthon amplification protocols as well as the standard cycling parameters see section 2.1.17.

exon 2 was obtained as a 417 bp fragment using the primers ALX4-F2 and ALX4-R2; exon 3 PCR employed the primers ALX4-F4 and ALX4-R4, generating a 283 bp fragment; and the coding part of exon 4 was amplified by the pair ALX4-F5 and ALX4-R5 as a 645 bp fragment. In addition, internal primers were used under the same conditions to amplify specific segments. The primers ALX4-F7 and ALX4-R7 not only flank the poly(P/Q) repeat but also in combination with ALX4-R3 and ALX4-F3 respectively split exon 1 in two overlapping fragments. In an equivalent design, exon 4 was obtained in two overlapping fragments by means of the pairs ALX4-F5–ALX4-R8 and ALX4-F8–ALX4-R5.

2.6.3 Mutation Screening

Initially, screening was carried out by SSCP and, at a later stage, by DHPLC; in addition, size variation in the poly(P/Q) tract was investigated by plain electrophoresis.

For SSCP, exons 2 and 3 were amplified as ALX4-F2–ALX4-R2 and ALX4-F4–ALX4-R4 fragments respectively. The larger exons 1 and 4 were divided in two overlapping fragments per exon — defined by the primer pairs ALX4-F3–ALX4-R7 and ALX4-F7–ALX4-R3 for exon 1; and ALX4-F5–ALX4-R8 and ALX4-F8–ALX4-R5 for exon 4 (see section 2.6.2). Two different SSCP protocols were adopted, based on the TBE and TME buffers, in order to increase the overall sensitivity of the approach, yet several variants were not detectable in the subset of samples analysed under these conditions. The mutations 653G→A and 418C→T generated SSCP shifts but the change 736C→T was invisible; the mutation 620C→A was not assayed. From the repertoire of polymorphisms, the changes 104G→C, 594C→A, 729G→A, 879C→T, 1074C→T, and 1464C→T were picked up whereas the variants 304C→T and 778-11G→A were missed. The rare polymorphisms 63C→T, 69G→C, 906+32C→T, 1282G→A, and 1392G→A, as well as the ambiguous variants 605T→G and 314_325delCGCAGCCGCAGC were not assessed by SSCP.

For DHPLC, one PCR amplicon was generated per exon — defined by the primer pairs ALX4-F3–ALX4-R3, ALX4-F4–ALX4-R4, ALX4-F2–ALX4-R2, and ALX4-F5–ALX4-R5 (see section 2.6.2). The analyses were performed at 65°C and 67°C for exon 1, at 63°C and 65°C for exons 2 and 3, and at 65°C only for exon 4. Appropriate homozygous normal reference samples and heterozygous controls for the following detectable polymorphisms were included: 104G→C and 304C→T (in the compound state), 594C→A, 729G→A, 778-11G→A, 879C→T, and 1464C→T. However, the mutation 620C→A did not produce a distinct waveform and the frequent polymorphism 1074C→T was, also, undetectable. On the other hand, several minor polymorphisms (63C→T, 69G→C, 906+32C→T, 1282G→A, and 1392G→A) as well

as the ambiguous variants 605T→G and 314_325delCGCAGCCGCAGC were ascertained. All other mutations (653G→A, 418C→T, and 736C→T) were not assessed by DHPLC.

The poly(P/Q) element within exon 1 was obtained as a 117 bp product using the primer pair ALX4-F7–ALX4-R7 and following standard denaturing PAGE and blotting it was detected by hybridisation using a [³²P]-labelled ALX4-F7 oligonucleotide.

2.6.4 Sequencing

The four amplimers (see section 2.6.2) representing the entire coding region of *ALX4* were routinely sequenced for variant detection purposes with the original PCR primers: ALX4-F3 and ALX4-R3 for exon 1, ALX4-F2 and ALX4-R2 for exon 2, ALX4-F4 and ALX4-R4 for exon 3, ALX4-F5 and ALX4-R5 for exon 4. This approach offered full bi-directional coverage for the small exons 2 and 3 and partial coverage for the larger exons 1 and 4. Although this limitation was rarely a problem, the internal primers ALX4-F7 and ALX4-R7 for exon 1 as well as ALX4-F8 and ALX4-R8 for exon 5 were occasionally utilised. At the same time, reliable reference sequences were established from normal individuals — available under accession numbers: AJ279074, AJ279075, AJ279076, and AJ279077. For the variant 314_325delCGCAGCCGCAGC, the change could not be deduced unambiguously from the trace of a heterozygous sample. A homoduplex was isolated from the mixed ALX4-F3–ALX4-R3 product by cloning into pGEM-T Easy and subsequent size selection; it was sequenced using appropriate internal primers, as above, and the generic plasmid primers pUC-F and pUC-R.

2.6.5 Allele-Specific Restriction Digests and Oligonucleotides

Diagnostic restriction digests were developed for all *ALX4* mutations and one of the ambiguous variants while allele-specific oligonucleotides were designed for three of them. This was also the assay of choice for the numerous common polymorphisms.

The mutation 653G→A removes a site for *MspI*. The ALX4-F2–ALX4-R2 amplicon is normally split by *MspI* in three fragments of 75, 162, and 180 bp; the mutant sequence produces two fragments of 180 and 237 bp. In addition, the change was detectable by hybridisation with the oligonucleotide ALX4 R218Q: discrimination was achieved by a final wash with SSC (at 0.1-fold strength: 15 mM NaCl, 1.5 mM sodium citrate pH=7.0) and 0.1% SDS at 30°C.

The mutation 418C→T generates a new site for *BfaI*. The normally intact 651 bp-long ALX4-F3–ALX4-R3 amplicon is divided into two fragments of 120 and 531 bp. Moreover, the oligonucleotide ALX4 Q140X could detect the change under routine hybridisation conditions.

The mutation 736C→T abolishes a site for *PvuII*. Three fragments of 63, 76, and 278 are normally produced upon digestion of the amplicon ALX4-F2–ALX4-R2; the mutation results in two fragments of 139 and 278 bp. Alternatively, the oligonucleotide ALX4 Q246X could detect the substitution by hybridisation: specificity was achieved by a final wash with SSC (at 0.2-fold strength: 30 mM NaCl, 3 mM sodium citrate pH=7.0) and 0.1% SDS at room temperature.

The mutation 620C→A was tested by restriction digestion only. The normally intact 417 bp-long ALX4-F2–ALX4-R2 amplicon generates two fragments of 222 and 195 bp upon digestion with *BfaI*.

The variant 605T→G creates a new site for *NcoI*. Thus, the normally intact 417 bp-long ALX4-F2–ALX4-R2 product is divided into two fragments of 206 and 211 bp.

Table 2.2: Allele-Specific Oligonucleotides for *ALX4* Polymorphisms.

Variant	PCR product	Oligonucleotide Name	Hybridisation Temperature	Wash Stringency
104G→C	ALX4-F3-ALX4-R3	ALX4 R35T	42° C	0.2× SSC, 0.1% SDS at 25° C
304C→T	ALX4-F3-ALX4-R3	ALX4 P102S	42° C	0.1× SSC, 0.1% SDS at 55° C
594C→A	ALX4-F2-ALX4-R2	ALX4 708C-A	42° C	0.1× SSC, 0.1% SDS at 50° C
729G→A	ALX4-F2-ALX4-R2	ALX4 842G-A	30° C	0.1× SSC, 0.1% SDS at 40° C
778-11G→A	ALX4-F4-ALX4-R4	ALX4 892-11G-A	30° C	1× SSC, 0.1% SDS at 25° C
879C→T	ALX4-F4-ALX4-R4	ALX4 996C-T	42° C	0.1× SSC, 0.1% SDS at 25° C
1074C→T	ALX4-F5-ALX4-R5	ALX4 1188C-T	42° C	0.1× SSC, 0.1% SDS at 45° C
1464C→T	ALX4-F5-ALX4-R5	ALX4 1578C-T	30° C	0.1× SSC, 0.1% SDS at 40° C

Notes: For each variant the relevant PCR amplimer and allele-specific oligonucleotide are mentioned. Blotting, hybridisation, and washing steps were carried out at the specified conditions that enable allele discrimination. 20× SSC is 3 M NaCl, 0.3 M sodium citrate pH=7.0.

Details of detection methods for all other variants are summarised in Table 2.2.

2.6.6 Fluorescence In Situ Hybridisation on Chromosomes

The BAC clones RPCI-11 58k22 and RPCI-11 193f22, which have been sequence-mapped proximally to *D11S2012*, were labelled with digoxigenin and used to probe metaphase chromosomes from a lymphoblastoid cell line derived from individual II-1 in family 6. Slide pre-treatment, probe labelling and blocking, washing and detection were performed according to established methods (Kearney and Buckle 1992). Three layers of antibodies were employed, the latter two being FITC conjugates. To mark chromosome 11, a probe specific for the 11q telomere was used alongside the BAC clones. Microscopy, image capture, and manipulation were carried out on a BX51 fluorescence microscope equipped with a camera (Olympus) using Macprobe v. 4.3 software (Applied Imaging).

2.6.7 Quantitative Southern Blotting

A standard Southern blot with *Eco*RI digests was co-hybridised with probes specific for exons 1 and 4 of *ALX4*. The product ALX4-F3–ALX4-R3 represents exon 1 and detects a band at approximately 5 kb while the exon 4-derived amplicon ALX4-F5–ALX4-R5 detects a band at approximately 7.5 kb. Gross quantitation relied on visual inspection.

2.7 Molecular Analysis of *MSX2*

2.7.1 Linked Genetic Markers

A panel of five flanking short tandem repeat loci – all (CA)_n repeats (Dib et al. 1994) amplifiable with the Généthon buffer under routine cycling parameters (see section 2.1.17) – and the intragenic polymorphic element *MSX2*-CA were utilised for linkage exclusion analysis and detection of heterozygosity.

D5S425 maps approximately 2.7 Mb away from *MSX2* on the centromeric side. *D5S394* is located more distally, approximately 1.6 Mb away from the gene, while *D5S2058* is less than 0.6 Mb away. *D5S498* is even closer, at approximately 0.3 Mb. On the telomeric side, *D5S2034* resides approximately 0.9 Mb away from *MSX2*.

MSX2-CA has been described before (Jabs et al. 1993). It is a polymorphic (CA)_n repeat located in the intron of *MSX2*, immediately upstream of exon 2. For amplification, the primers *MSX2*-CA-F and *MSX2*-CA-R were used with the standard buffer and the following cycling programme: 94°C for 4 min; 94°C for 15 sec, 62°C for 30 sec, 72°C for 1 min (30 cycles); 72°C for 5 min.

2.7.2 PCR Amplification

The coding region of *MSX2* was amplified from genomic DNA using previously published primers (Wilkie et al. 2000). Exon 1 PCR employed the primer pair

MSX2-E1FB and MSX2-E1RB and generated a 541 bp fragment; 10% DMSO was included in the reaction and the cycling profile was as follows: 94°C for 4 min; 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec (35 cycles); 72°C for 7 min. Exon 2 PCR employed the pair MSX2-E2F and MSX2-E2R and produced a 496 bp fragment under the following conditions: 94°C for 4 min; 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec (35 cycles); 72°C for 7 min.

2.7.3 Mutation Screening

Exon 1 (MSX2-E1FB–MSX2-E1RB) and exon 2 (MSX2-E2F–MSX2-E2R) fragments (see section 2.7.2) were screened for variants using DHPLC. The analyses were performed at 65°C and 68°C for exon 1, and at 62°C and 63°C for exon 2 alongside a homozygous normal reference sample and heterozygous controls for the readily detectable polymorphisms -17C→G and 386C→T. Both the 417_418delCA and 505_508dupATTG mutations were associated with aberrant patterns while a third polymorphism, 379+59A→G, was also detectable by DHPLC.

2.7.4 Sequencing

The two amplimers covering the entire coding region of *MSX2* (see section 2.7.2) were sequenced for variant detection purposes using the original PCR primers — MSX2-E1FB and MSX2-E1RB for exon 1, MSX2-E2F and MSX2-E2R for exon 2. Satisfactory bi-directional coverage was usually achieved and the internal primers MSX2-E1FC and MSX2-E1RC for exon 1 and MSX2-E2FB and MSX2-E2RB for exon 2 were rarely used. Previously published sequences were used (Jabs et al. 1993) for reference purposes — accession numbers: L22498 and L22499. Both mutations identified, 417_418delCA and 505_508dupATTG, cause frameshifts and produced complex patterns of overlap when PCR fragments of heterozygous individuals were sequenced. Mutant homoduplexes were resolved through cloning in pGEM-T Easy and selected by means of a restriction digest or oligonucleotide hybridisation (see

section 2.7.5). Inserts were sequenced using appropriate internal primers, as above, and the generic polylinker primers pUC-F and pUC-R.

2.7.5 Allele-Specific Restriction Digests and Oligonucleotides

As the two novel mutations in *MSX2* either remove or insert nucleotides they would be readily detectable by hybridisation; in addition, a diagnostic restriction digest was also a possibility for one of them. Assays for the mutations 515G→G and 475_480delCGCAAG, as well as for the two frequent polymorphisms, -17C→G and 386C→T, have been documented before (Wilkie et al. 2000). The rare third variant 379+59A→G was confirmed by a restriction digest.

The mutation 417_418delCA does not result in loss or gain of a restriction site and was confirmed by hybridisation: the amplicon MSX2-E2F–MSX2-E2R was probed with the oligonucleotide MSX2 417-418DELCA which recognises specifically the mutant sequence under routine conditions.

The mutation 505_508dupATTG eliminates a site for *Bsr*DI. The 496 bp-long amplicon MSX2-E2F–MSX2-E2R is normally cleaved, generating two fragments of 154 and 342 bp; in the mutant, the full-size product persists. Alternatively, the oligonucleotide MSX2 505-508DUPATTG would detect the mutant sequence under routine conditions.

The mutation 475_480delCGCAAG binds specifically the oligonucleotide #719: the amplicon MSX2-E2F–MSX2-E2R was probed and washed under standard conditions.

Finally, the variant 379+59A→G generates a new site for *Aci*I. This would result in a distinct pattern upon digestion of amplicon MSX2-E2F–MSX2-E2R and fragment separation by native PAGE.

2.7.6 Quantitative Southern Blotting

The design exploited a paralogous, intronless copy of *MSX2* on chromosome 17 as internal control (Ensembl v. 16.33.1 — see appendix F). Following digestion with *EcoRI*, blotting, and probing with the exon 1-derived amplicon MSX2-E1FB–MSX2-E1RB, two distinct bands were visualised: one at approximately 6.5 kb was in agreement with the genomic sequence around the functional gene; hence, the second, at approximately 10.5 kb, was assigned to the pseudogene.

2.8 In Situ Hybridisation to mRNA

Detection of *Alx4*, *Msx2*, *Runx2*, *Col2a1*, and *Spp1* transcripts during development of the mouse skull relied on non-radioactive in situ hybridisation, employing digoxigenin-labelled probes and immunohistochemical visualisation. Different protocols were followed for whole embryos around mid-gestation and frozen sections of head tissue, both representing slight variations of established methods (Sharpe and Mason 1999; Ausubel et al. 1999). Wild-type embryos of the C57BL/6 strain were collected after sacrificing the pregnant female; embryos up to E11–11.5, destined for whole-mount hybridisation, were killed by immersion in fixative while older embryos were killed by decapitation.

2.8.1 Preparation of Digoxigenin-Labelled Riboprobes

For all five genes analysed, suitable fragments for probe usage were obtained in vectors supporting in vitro transcription. The *Alx4*-specific probe was provided by Dr Ron Wisdom and consisted of an approximately 650 bp fragment derived from the 3' UTR of the gene (Qu et al. 1997b), cloned into the *EcoRI* site of pBluescript II KS⁺ (Stratagene). To generate the antisense transcript, the plasmid was cut with *EcoRV* and the insert was transcribed from the T7 promoter; the sense transcript was produced from the T3 promoter after linearisation with *BamHI*. The *Msx2* plasmid was a gift from Dr Richard Maas. Approximately 1 kb of the *Msx2* cDNA, spanning

the entire coding region, was cloned into the *Bam*HI and *Eco*RI sites of pBluescript II SK⁻; once digested with *Xba*I the antisense strand was transcribed by the T7 promoter, whereas the sense strand was produced by the T3 promoter after linearisation with *Eco*RV. The *Runx2* plasmid was contributed by Dr Georg Schwabe and the insert consisted of an approximately 430 bp cDNA fragment in pBluescript II SK⁻; to produce the antisense probe it was cut with *Xba*I and transcribed from the T7 promoter. The *Col2a1*-specific probe was obtained from Prof. Benoit de Crombrughe and the 405 bp insert, cloned into the *Eco*RV site of pBluescript II KS⁺, covered mostly 3' UTR sequence (Metsäranta et al. 1991). Linearisation with *Eco*RI and transcription from the T3 promoter generated the antisense probe. Finally, the 984 bp *Spp1* fragment (Iseki et al. 1999) represented almost the entire coding portion of the cDNA, cloned into the *Hind*III site of pGEM1 (Promega); after digestion with *Bam*HI, it was transcribed from the SP6 promoter to give the antisense probe.

A transcription reaction was set up in a final volume of 20 μ l with approximately 1 μ g of linearised template: the DNA was incubated with 20 units of T7, T3, or SP6 polymerase (Roche) and 20 units of RNasin inhibitor (Roche) in the manufacturer's buffer (40 mM Tris pH=8.0, 6 mM MgCl₂, 10 mM DTT, 2 mM spermidine), supplemented with the digoxigenin labelling mix (1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP, 0.35 mM DIG-11-UTP) from the same provider. RNA accumulated during incubation at 37°C for 2 h and was selectively precipitated by adding 80 μ l of TE, 10 μ l of 4 M LiCl, and 0.25 ml of 100% ethanol. Following storage at -20°C for at least 30 min it was pelleted by centrifugation in a minifuge (13,000 rpm at 4°C for 10 min), air-dried, and obtained in 20 μ l of TE; the procedure was often repeated to minimise the amount of DNA carried over. The preparation was subsequently fragmented down to an average size of 200 bp by controlled alkaline hydrolysis. The RNA pellet was dissolved directly in a weak alkaline solution (60 mM Na₂CO₃, 40 mM NaHCO₃ — freshly prepared) and incubated at 60°C for 20–35 min, depending on the starting length; degradation was stopped by adding 1 μ l

of pure acetic acid. Standard precipitation with 20 μ l of 3 M sodium acetate pH=5.2 and 0.5 ml ethanol concentrated the probe. After storage at -20°C for at least 30 min, centrifugation (13,000 rpm at 4°C for 20 min), washing with 70% ethanol, and re-centrifugation, the RNA was dissolved in 0.1 ml TE and found to be stable at -70°C for at least 2–3 months.

2.8.2 In Situ Hybridisation in Frozen Tissue Sections

Dissected skulls were put in fixative (4% paraformaldehyde in PBS) and stored at 4°C overnight. Once washed twice with PBS at room temperature for 10 min each time, sucrose infusion was carried out: the solution was replaced with 30% sucrose in PBS and the specimens were allowed to equilibrate — i.e. until sinking. Embedding in Oct compound (Sakura) was achieved by rapid freezing in isopentene on dry ice; the frozen specimen was stored at -70°C for few weeks.

Serial sections were cut in a JM3000 (Jung) cryostat at a width of 20 μ m and mounted onto coated slides (BDH). Within few hours the slides were flooded with fixative (4% paraformaldehyde in PBS) at room temperature for 5 min and subsequently washed three times by immersion in PBS at room temperature for 5 min each time. Before storage, dehydration was performed through an ethanol gradient: 30 : 70 ethanol : PBS; 60 : 40 ethanol : PBS; 80 : 20 ethanol : PBS; 95 : 5 ethanol : PBS; pure ethanol — each step was carried out at room temperature for 2 min. After a final wash with 100% ethanol the slides were allowed to air-dry and stored desiccated at -70°C for several weeks.

Treatment for hybridisation commenced with digestion (10 μ g/ml proteinase K in PBS) at room temperature for 5 min; the reaction was stopped by immersion in a freshly-made glycine solution (2 mg/ml glycine in PBS) for 30 sec and two washes in PBS for 30 sec each time. The tissue was then stabilised by re-fixation – incubation in fixative (4% paraformaldehyde in PBS) at room temperature for 5 min – and washed three times with PBS for 3 min each time. Acetylation

followed by submerging the slides in a solution of 0.25% acetic anhydride and 0.1 M triethanolamine – while the slides were submerged, the anhydride was added dropwise, under stirring, in the triethanolamine solution already acidified with few drops of concentrated HCl – at room temperature for 10 min. The slides were ready for hybridisation after three washes with PBS at room temperature for 5 min each time and dehydration through an ethanol gradient (as above).

The hybridisation buffer consisted of 50% formamide, 0.6 M NaCl, 10 mM Tris pH=7.6, 0.25% SDS, 1 mM EDTA, 10% dextran sulphate, Denhardt's reagent (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% BSA) and 0.2 mg/ml bacterial tRNA. The working areas of the slides were demarcated by liquid rubber (Weldtide), flooded with hybridisation buffer, and incubated at room temperature for 2 h over a tray filled with 50% formamide, SSC (at 4-fold strength: 0.6 M NaCl, 60 mM sodium citrate pH=7.0). After pre-hybridisation, the slides were covered with a minimal volume of hybridisation buffer containing the digoxigenin-labelled ribo-probe at 0.2–0.4 $\mu\text{g}/\text{ml}$, pre-heated at 80°C. A piece of parafilm was placed over the liquid and the slides were incubated overnight within a tightly sealed container, humidified with 50% formamide, SSC (at 4-fold strength: 0.6 M NaCl, 60 mM sodium citrate pH=7.0) at 70°C.

All washings were carried out at 70°C. The first wash solution consisted of SSC (at 5-fold strength: 0.75 M NaCl, 75 mM sodium citrate pH=5.0) and was applied for 5 min to remove the parafilm and the rubber; in the second solution the strength dropped to 0.2-fold and the slides were left submerged for 2–3 h.

2.8.3 In Situ Hybridisation in Whole Mouse Embryos

Whole embryos were submerged in fixative (4% paraformaldehyde in PBS) and stored at 4°C overnight. The paraformaldehyde was removed by three washes in PBS with Tween-20 (PBT: PBS, 0.1% Tween-20) on ice for 30 min each time and dehydration through an ethanol gradient followed: 25 : 75 ethanol : PBT; 50 : 50

ethanol : PBT; 75 : 25 ethanol : PBT; and pure ethanol — each step was carried out on ice and lasted 1 h. At this stage the specimens were stored in 100% ethanol at -20°C for several weeks.

Processing for hybridisation started with re-hydration through a reverse ethanol gradient: 75 : 25 ethanol : PBT; 50 : 50 ethanol : PBT; 25 : 75 ethanol : PBT; pure PBT — each step was performed on ice and lasted 5 min. The PBT solution was replaced once prior to protease treatment, involving incubation with 10 µg/ml proteinase K in PBT at room temperature for 5–10 min. Washing in a freshly-made glycine solution (2 mg/ml glycine in PBT) on ice for 5 min stopped the digestion and after two washes with PBT on ice for 5 min the specimens were re-fixed in a freshly-prepared mix of cross-linkers (0.2% glutaraldehyde, 4% paraformaldehyde in PBT) on ice for 20 min. Following three washes with PBT on ice for 5 min each time, endogenous enzymatic activities that could interfere with subsequent steps were inactivated by incubation in PBT at 70°C for 30 min. The pre-hybridisation manipulations ended with bleaching by hydrogen peroxide (6% H₂O₂ in PBT) at room temperature for 1 h and three subsequent washes with PBT, also at room temperature, for 5 min each.

The hybridisation buffer consisted of 50% formamide, SSC (at 5-fold strength: 0.75 M NaCl, 75 mM sodium citrate pH=5.0), 1% SDS, 50 µg/ml heparin, and 50 µg/ml yeast tRNA. After pre-hybridisation at 70°C for 1 h, fresh buffer with approximately 0.35 µg/ml of digoxigenin-labelled riboprobe preparation was added and hybridisation took place at 70°C overnight under shaking on a rocking platform.

Washing was carried out at 70°C, allowing approximately 30 min for each step. The first wash solution consisted of 50% formamide, SSC (at 5-fold strength: 0.75 M NaCl, 75 mM sodium citrate pH=5.0), 1% SDS and was applied twice; in the second solution the SSC strength dropped to 3-fold.

2.8.4 Immunohistochemical Detection

After hybridisation and probe washing the slides were transferred to Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris pH=7.5) at room temperature for five minutes. The working areas of the slides were marked using a PAP pen (Daido Saugyo) and flooded with 1% of blocking reagent (Roche) in TBS. Following incubation at room temperature for 1 h the anti-digoxigenin alkaline-phosphatase-conjugated antibody (Roche) was applied, diluted $\frac{1}{5,000}$ in TBS. Binding took place at 4°C overnight within a humidified container. Unbound antibody was removed by rinsing the slides in TBS three times in total over 15 min and the buffer was changed for the alkaline phosphatase reaction (0.1 M NaCl, 0.1 M Tris pH=9.5, 50 mM MgCl₂). The chromogens nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were added at 0.34 mg/ml and 0.18 mg/ml respectively and the reaction progressed in a humidified chamber in the dark. Phosphate removal from BCIP yields an indolyl derivative that is oxidised to an indigo dye while NBT is reduced to a formazan; together, these two chemical species form a purple-coloured precipitate. When sufficient colour intensity was achieved, after 2–72 h depending on the probe, the reaction was stopped by washing in TE. The sections were mounted into 90% glycerol in PBS under a coverslip, sealed with nail varnish, and stored at room temperature protected from light.

For whole-mount embryos, post-hybridisation procedures began with transfer to TBS with Tween (TBST: 0.15 M NaCl, 0.1 M Tris pH=7.5, 0.1% Tween-20) at room temperature. The buffer was replaced twice over 15 min and was followed by the blocking solution – 1.5% of blocking reagent in TBST – at room temperature for 60 min. Meanwhile, the anti-digoxigenin alkaline-phosphatase-conjugated antibody was prepared: the antibody itself was diluted $\frac{1}{333}$ in 0.015% of blocking reagent in TBST and incubated on ice for 60 min; 4 volumes of 1.5% blocking in TBST were then added to give the working solution. The specimens were placed in

the antibody solution and incubated under shaking at 4°C overnight. Three quick washes with TBST at room temperature for 15 min in total were followed by five long washing sessions lasting 1 h each, all under continuous shaking. The buffer was changed for the alkaline phosphatase reaction (0.1 M NaCl, 0.1 M Tris pH=9.5, 50 mM MgCl₂, 0.1% Tween -20) which employed, as before, the NBT/BCIP chemistry. It was stopped by three washes with pure reaction buffer over 30 min and the specimens were stored in PBT at 4°C. Prior to photography they were mounted in 50% glycerol in PBT.

2.8.5 Photography

Sections were photographed on a DMRBE microscope (Leica) equipped with an MPS 60 camera (Leica) using a colour-reversal film – Fujichrome 64 type II film (Fujifilm). After development, individual frames were scanned on a slide scanner – Coolscan III (Nikon) – and the images were assembled, enhanced, and colour-adjusted in Photoshop v. 6.0 (Adobe) or Gimp v. 1.2.2. An equivalent procedure was followed for whole-mount embryos using a dissecting microscope (see section 2.10.2).

2.9 Genotyping of Mice for *Alx4* and *Msx2* Alleles

DNA was extracted from tail tips of adult mice, to be characterised for breeding purposes, and from tissue specimens of neonates and embryos, to be typed for analytical work, and diluted down to $\frac{1}{100}$. For each locus, two separate diagnostic PCR reactions were set up in plates under standard conditions but in a final volume of 50 μ l using 2 μ l of the diluted DNA preparation. Irrespective of their origin all specimens were typed for both *Alx4* and *Msx2* at least twice; if the PCR performance of a particular sample was consistently poor, the respective animal was discarded or destroyed.

2.9.1 *Alx4*

The two alleles, the mutant *Alx4*^{tm1Rwi} and the wild-type were detected by two PCR reactions employing distinct primer pairs, as described (Qu et al. 1997b): the mutant allele is amplifiable by the combination mALX4 TD-F and mALX4 TD-R, yielding a 400 bp fragment, while the wild-type allele produces a 350 bp fragment with mALX4 WT-F and mALX4 WT-R. Identical cycling parameters were used: 96°C for 8 min; 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min (35 cycles); 72°C for 7 min.

2.9.2 *Msx2*

The two alleles, the mutant *Msx2*^{tm1Rilm} and the wild-type were detected by two PCR reactions employing three primers in total, as described (Satokata et al. 2000): the mutant allele is amplifiable by the combination SXG-2 and SXG-5, yielding a 650 bp fragment, while the wild-type allele produces a 350 bp fragment with SXG-4 and SXG-5. A two-stage cycling programme was used for both reactions: 96°C for 8 min; 94°C for 1 min, 62°C for 1.5 min, 72°C for 2 min (3 cycles); 94°C for 1 min, 60°C for 2 min, 72°C for 2 min (30 cycles); 72°C for 5 min.

2.10 Skeletal Staining of Mice

Newborn animals were killed humanely by an overdose of pentobarbitone (Rhône Merieux), administered intraperitoneally, eviscerated, and skinned. At the same time, a piece of tail tissue was removed for genotyping.

2.10.1 Dual Bone and Cartilage Staining Using Alizarin Red and Alcian Blue

The carcass was fixed in 95% ethanol for 12–72 h and skeletal staining was performed sequentially. Initially, the specimen was immersed in the Alcian Blue solution (76% ethanol, 20% acetic acid, 0.015% Alcian Blue 8GX) for 24–48 h, followed by

differentiation in 95% ethanol for 7 days — the ethanol was replaced several times as required. Transparency of the soft tissues was accomplished by maceration in 1% KOH — until the cartilages became visible — and extensive washing under running tap water for 12–18 h. Subsequently, the specimen was put in the Alizarin Red solution (0.1% Alizarin Red S dissolved with few drops of 1% KOH — the pH was then gradually lowered until the characteristic bright red colour was attained) for 3–12 h. Due excess was removed by washing under running tap water for 30 min and differentiation was carried out by means of a decolourising solution (20% glycerol, 1% KOH) for 10–50 days — as before, the solution was replaced regularly. Final dehydration was carried out through an ethanol gradient: 7% ethanol, 20% glycerol; 14% ethanol, 20% glycerol; 21% ethanol, 30% glycerol; 28% ethanol, 40% glycerol; and 35% ethanol, 50% glycerol. Cleared specimens were stored indefinitely at room temperature in 35% ethanol, 50% glycerol.

2.10.2 Photography

A dark-field image of the stained and cleared specimen was obtained by conventional photography, using an MPS 60 camera on a dissecting microscope (Wild) and a colour-reversal — Fujichrome 64 type II — film. After development, individual frames were scanned on a slide scanner — Coolsan III — and the images were subjected to limited manipulation and enhancement in Photoshop v. 6.0 or Gimp v. 1.2.2

2.11 Semi-Quantitative RT-PCR Analysis of Gene Expression in Mouse Embryos

Suitable crosses between single heterozygotes were set up to obtain all three genotypes per locus: $Alx4^{+/+}$, $Alx4^{+/-}$, and $Alx4^{-/-}$, as well as $Msx2^{+/-}$, $Msx2^{+/-}$, and $Msx2^{-/-}$. Embryos were collected at E11.5–12 and, following genotyping, total RNA was isolated from the entire heads. DNase-treated RNA preparations were

equalised by spectrophotometry and electrophoresis. Reverse transcription was carried out with a starting amount of approximately 5 μ g. Once completed, the reaction was diluted to a volume of 80 μ l and 6 μ l were used per downstream PCR.

For all five transcripts that were assessed by semi-quantitative PCR – *Alx4*, *Msx2*, *Twist1*, *Runx2*, and *Gapd* – the amplification primers were designed to map in different exons in order to circumvent problems related to any residual genomic DNA. The pair mALX4-F and mALX4-R amplified a 672 bp product; the pair mMSX2-F and mMSX2-R a 534 bp product; the pair mTWIST-F and mTWIST-R a 449 bp product; the pair mRUNX2-F and mRUNX2-R a 795 bp product; and the pair mGAPDH-F and mGAPDH-R a 983 bp product. Standard PCR conditions were employed with one exception: 1 unit of AmpliTaq was used in all reactions. A two-step programme was used for all reactions: 94°C for 4 min; 94°C for 30 sec, 68°C for 1 min (variable number of cycles); and 72°C for 5 min. For the abundant *Twist1* transcript 30 cycles were sufficient while 33 were needed for *Gapd*. Optimal amplification of *Alx4* and *Msx2* required 36 cycles and a product from the *Runx2* transcript was visible after 39 cycles. Prior to the experimental reactions all designs were checked against preparations of normal mouse cDNA and genomic DNA: a sharp band was obtained without any sign of amplification from genomic DNA — only a very faint smear for the *Gapd*-specific PCR was noticed.

2.12 The Glutathione-S-Transferase Pull-down Assay

2.12.1 Constructs

Because of the stability problems encountered during propagation of a cloned cDNA fragment of human *ALX4*, an independently isolated almost full-length *ALX4* cDNA clone (Nagase et al. 2001), KIAA1788, was obtained from Dr Nobumi Kusuvara and found to be stable in the DH10B host. The 5,654 bp cDNA fragment

with a poly(A) stretch begins just upstream of the first putative initiation codon and apparently contains the full 3' UTR of the gene, enabling production of the larger, 411 amino acid-long, ALX4 isoform. It has been inserted between the *SalI* and *NotI* positions of pBluescript II SK⁺ by means of a 5' *SalI* adaptor and a 3' oligo(dT) primer with a *NotI* site.

On the other hand, the coding part of the human *MSX2* cDNA was derived by RT-PCR from a normal fibroblast cell line using the primers MSX2-E1FB and MSX2-E2R. The PCR employed buffer 3 and the polymerase mix of the Expand Long Template PCR System and was set up according to the instructions of the manufacturer; the cycling parameters were as for exon 1 of *MSX2* (see section 2.7.2) but the number of cycles was increased to 40. The 924 bp-long product was cloned into pGEM-T Easy and several isolates were sequenced. Despite the use of a proof-reading polymerase, errors had been introduced in all clones analysed. To obtain at least the genuine coding portion of *MSX2* an internal *NcoI*–*NarI* fragment was swapped between two mutant clones; this approach produced a cDNA species with only one residual – and presumably irrelevant as far as protein production in vitro is concerned – change, namely 812C→T at the 3' UTR. The construct was sequenced extensively with the internal primers MSX2-E1FC, MSX2-E1RC, MSX2-E2FB, MSX2-E2RB, as well as the external ones pUC-F and pUC-R and would direct synthesis of the full, 267 amino acid-long, *MSX2* protein.

The mouse *Maged1* cDNA was a gift from Dr Ken Watanabe. The plasmid pF-Dlxin-1 (Masuda et al. 2001; Sasaki et al. 2002) contained the entire coding region of *Maged1* with an engineered FLAG epitope, inserted in the pcDNA3 vector (Invitrogen) — although no size information and cloning details were made available the construct encodes the full, 775 amino acid-long, *Maged1* protein.

Finally, the human *HOXD13* cDNA was kindly provided by Mr Shih-Hsin Kan. A 1,008 bp-long RT-PCR product covering the coding region of *HOXD13* was inserted in pGEM-T Easy and then subcloned into pRD67:HA after introducing

EcoRI and *XhoI* sites at either end of the fragment. The full, 335 amino acid-long, protein could be produced.

The coding part of *ALX4* cDNA was excised from KIAA1788 as an approximately 2.1 kb-long *SalI*–*SmaI* fragment (a controlled partial digest with *SalI* was necessary because of an internal *SalI* site) and fused in-frame with the sequence encoding the GST protein in the pGEX-4T-3 vector (Amersham Biosciences) which had been cut with *SalI* and *NotI* — the latter site had been blunt-ended to enable directional cloning. Recombinants were identified by colony hybridisation and a pGEX-*ALX4* isolate was sequenced extensively using both the internal primers *ALX4*-F7, *ALX4*-R7, *ALX4*-F10, *ALX4*-R10, *ALX4*-F8, *ALX4*-R8, and the external ones pUC-F and pUC-R. The predicted fusion protein would include all 411 amino acids of the large isoform of *ALX4*, linked to the GST part by an artificial peptide derived from adaptor and vector polylinker sequences.

To obtain the GST-*MSX2* fusion the coding part of *MSX2* cDNA was removed from the previously described construct as an approximately 0.9 kb-long *TaiI*–*NotI* fragment. Trimming of the 3′ overhang at the *TaiI* end enabled directional in-frame cloning into the *SmaI* and *NotI* positions of the pGEX-4T-3 vector. As before, clones were selected by colony hybridisation and a pGEX-*MSX2* isolate was fully sequenced using the aforementioned set of primers. The chimeric protein would include all 267 amino acids of *MSX2*, fused to the GST part by an artificial peptide encoded by 5′ UTR and vector polylinker sequences.

2.12.2 Purification of Glutathione-S-Transferase Fusion Proteins from Bacterial Extracts

BL21 hosts transformed with either the pGEM-4T-3 vector, encoding the GST protein itself, and the pGEX-*MSX2* construct were grown to saturation overnight. An exponential culture was initiated by $\frac{1}{100}$ dilution into 200 ml of fresh medium and allowed to grow at 37°C for approximately 2 h — reaching absorbance values at 600

nm in the range of 0.4–0.6. Induction with 1 mM of isopropyl- β -D-thiogalactoside (IPTG) followed and the incubation continued for 2 h. The cells were pelleted in an RT 6000B (3,000 rpm at 4°C for 15 min) and obtained in 10 ml PBS. The suspension was split in 1–1.5 ml aliquots and lysed using a sonicator (Lucas Dawe Ultrasonics) at minimal power. Debris were separated by centrifugation in a minifuge (13,000 rpm at 4°C for 10 min).

Bacterially expressed fusion proteins were affinity-purified by glutathione sepharose 4B beads (Amersham Biosciences) on the basis of the supplier's literature. To minimise degradation during manipulations the PBS solution used had been supplemented with the Complete protease inhibitor cocktail (Roche). The original 75% slurry was washed and resuspended at 50% in PBS prior to use: 1.33 volumes of slurry were sedimented in an RT 6000B (1,000 rpm at 4°C for 5 min) and washed with 10 volumes of ice-cold PBS; following re-centrifugation the beads were obtained in 1 volume of PBS and stored for several days at 4°C. Approximately 0.2 ml of 50% glutathione beads were added to the lysate and following binding at room temperature for 30 min under gentle agitation they were sedimented by centrifugation (1,500 rpm at 4°C for 10 min), washed three times with 1 ml PBS, and finally mixed with 0.1 ml of PBS. An initial attempt to express the GST-ALX4 chimera in BL21 cells was encouraging but, despite the satisfactory yield, the fusion protein was not purifiable under the conditions applied. On the other hand, the GST-MSX2 fusion was produced at a lower level but enriched preparations were readily obtained.

To balance the amounts of GST and GST-MSX2, the preparation of the former was diluted in the range of $\frac{1}{100}$ – $\frac{1}{500}$ and aliquots from both were removed, boiled in electrophoresis buffer, and checked for purity by SDS-PAGE while the remaining amount was stored at 4°C for not more than 48 h. After separation the gel was stained by Gelcode Blue reagent (Pierce) at room temperature for 30 min and rinsed in water. The GST product was visible as a pure 29 kDa band whereas

the purity of the GST-MSX2 preparation was rather variable — however, a major band around 48 kDa was consistently present and was in good agreement with the theoretical estimate. Depending on the test gel, the inputs for the pull-down experiments were adjusted accordingly — on average, 0.5–1 μ g of protein was used.

2.12.3 Synthesis of [^{35} S]-Labelled Proteins

The TNT Coupled Reticulocyte Lysate System (Promega) was used for vitro production of radiolabelled proteins, according to the manufacturer's instructions. Standard 50 μ l reactions were set up as follows: 25 μ l of rabbit reticulocyte lysate was mixed with 2 μ l of concentrated proprietary buffer, 1 μ l of amino acid mixture (minus methionine) at 1 mM, 2 μ l of PRO-MIX [^{35} S], 1 μ l of RNasin inhibitor at 40 units/ μ l, 1 μ l of appropriate polymerase as provided, and approximately 2 μ g of plasmid DNA. Both the *ALX4* and *Maged1* cDNAs were transcribed by the T7 polymerase whereas the SP6 polymerase was used for *HOXD13*. The reaction was scaled up for *ALX4* and *HOXD13* to compensate for the lower yields. After an incubation at 30°C for 90 min the reaction was immediately checked by SDS-PAGE. Aliquots were electrophoresed while the remaining amount was stored at 4°C for not more than 48 h. After separation the gel was soaked in fixative (10% acetic acid, 50% methanol) at room temperature for 30 min, dried, exposed to a Phosphor screen (Molecular Dynamics) and analysed using a Storm 860 instrument (Molecular Dynamics) and the software package Image Quant v. 5.1 (Molecular Dynamics). The *ALX4* product migrated at approximately 50 kDa as a doublet – reflecting the dual initiation site at the cDNA – while *HOXD13* was sized at approximately 38 kDa; *Maged1* was sized around 75 kDa. The relative quantities of the labelled products were estimated empirically and the amounts used for the pull-down experiments were adjusted accordingly.

2.12.4 Pull-down Conditions

Stringent pull-down conditions (Ausubel et al. 1999) employed a relatively high concentration of competitor and incubation at 4°C for few hours. Appropriate amounts of [³⁵S]-labelled proteins were mixed with 0.2 ml of bacterial protein extracts at 10 mg/ml in binding buffer A (50 mM KH₂PO₄ pH=7.5, 0.15 M KCl, 1 mM MgCl₂, 10% glycerol, 1% Triton X-100) supplemented with the Complete protease inhibitor cocktail. The procedure was repeated for the bead-bound GST fusion proteins and the two mixtures were combined. Binding was allowed to take place at 4°C for 1–4 h under shaking. Subsequently the beads were sedimented in a minifuge (1,500 rpm at 4°C for 10 min), washed three times with an excess of binding buffer A, and re-pelleted. Any traces of liquid were removed and the bound proteins were released by boiling in electrophoresis buffer. Trapping was assessed by SDS-PAGE followed by autoradiography.

Relaxed pull-down conditions have been used in interaction assays for Maged1 (Sasaki et al. 2002) without a substantial decrease in specificity and were, consequently, adopted. The protein preparations were combined as before but in the presence of binding buffer B (20 mM Tris pH=7.5, 0.15 M KCl, 0.5% Nonidet P40, 0.5% BSA) supplemented with the Complete inhibitors and incubated at 4°C overnight. The beads were pelleted and any unbound material was removed by three washes with PBS, performed as before.

Chapter 3

Molecular Genetics of Human Skull Defects: the *ALX4* & *MSX2* Genes

3.1 Introduction

The working hypothesis that P11pDS, being a true contiguous gene deletion syndrome, predicts a second major locus for enlarged parietal foramina in close proximity to the *EXT2* gene, could be tested experimentally. A preliminary analysis of families segregating isolated, non-syndromic PFM where mutations in *MSX2* had been excluded, provided positive evidence of linkage to 11p11–p12 and concentrated efforts on the construction of a physical contig covering the minimal critical region along with comprehensive bioinformatic investigations. This combined approach – which benefited from resources and data that had accumulated during the isolation and structural characterisation of *EXT2* (Stickens et al. 1996; Wuyts et al. 1996; Clines et al. 1997) and from the ongoing public human genome sequencing project (International Human Genome Sequencing Consortium 2001) – identified a novel homeobox gene of the paired class, the human orthologue of mouse *Alx4* (Qu et al. 1997a; Hudson et al. 1998), and detected apparently pathogenic changes in six families with PFM/skull ossification defects. At the same time, the mutational repertoire of *MSX2* was enriched: two new mutations were ascertained, including one associated with the rare syndrome of PFMCCD.

3.2 The *ALX4* Locus

Following an account of the positional cloning and characterisation of *ALX4*, this section elaborates on the individual families, presenting the clinical and molecular findings.

3.2.1 Confirmation That a Second Locus for Isolated Enlarged Parietal Foramina Maps to Chromosome 11p

Linkage to *MSX2* was excluded in two families with radiologically documented PFM and a segregation pattern strongly supportive of autosomal dominant inheritance. Recombination events were detected in families 1 and 2, originally described by Salamanca et al. (1994) and Kutilek et al. (1997) respectively (Figures 3.6 and 3.7; see appendix B), between the disease and at least two linked markers out of six tested — *MSX2*-CA (an intragenic short tandem repeat), *D5S2058*, *D5S498*, *D5S425*, *D5S394* and *D5S2034*. Sequencing of the coding region of *MSX2* also failed to reveal any abnormal variants and heterozygosity for *MSX2*-CA in several affected individuals from both families ruled out hemizyosity.

Family 2 (Figure 3.7) included seven affected individuals in four generations but also a case of complete non-penetrance (obligate carrier II-4) and an individual with an ambiguous presentation (IV-5). Two other persons with normal skull radiographs were at 50% prior risk (II-1 and III-4) although the existence of reputedly unaffected offspring, after accounting for reduced penetrance, reduced this figure below 25%. Assuming eight fully informative meioses once the phase had been established, a maximum LOD score around 2.4 in favour of linkage would have been achievable with a closely linked marker from 11p11–p12; in combination with the smaller family 1, the cumulative LOD score could, potentially, increase by approximately 0.6.

After adjusting for radiologically normal individuals at 50% and 25% prior risk and classing the phenotype of IV-5 in family 2 as unknown, a slightly lower

value was obtained — a two-point LOD score of 2.93 at $\theta=0.0$ with *D11S2095*. The datasets for three other loci that map within or around the minimal critical interval, *D11S1393*, *D11S903* and *D11S554* were less informative (see appendix D).

3.2.2 Construction of a Physical Contig and Bioinformatic Approaches — *ALX4* Is the Prime Positional Candidate

Initial BLAST searches uncovered one fully sequenced genomic clone mapping within the minimal critical interval, defined distally by *D11S1393* and proximally by *D11S2095*, and indicated that others were being sequenced. Yet, the high degree of sequence fragmentation and the poor coverage downstream of *EXT2* discouraged an approach that would have relied solely on the flow of raw data from the genome sequencing projects and, subsequently, on gene prediction by bioinformatic tools. Instead, this line of investigation was backed by the construction of a physical contig, spanning the *D11S1393*–*D11S2095* region. The primary aim was to enable accurate independent positioning of newly-identified clones and/or sequenced fragments as they emerged from the databases relative to established landmarks in the region; this would accelerate any further structural characterisation of positional candidates. Secondly, had this strategy been unfruitful, it would have served as a resource for experimental gene identification — e.g. by direct cDNA selection.

A publicly available resource listing genomic clones alleged to cover the *EXT2* region (Southwestern Medical Center, Texas, USA — these particular web pages have now been discontinued), apparently selected during the course of *EXT2* cloning (Stickens et al. 1996), served as a starting point for contig construction. The PAC clones RPCI-1 189f14, RPCI-3 366b4, RPCI-3 368b4, RPCI-3 404m15, RPCI-3 511i10, and RPCI-3 526d1 were confirmed as true positives and assembled into a contig using: the framework of *EXT2* exons (Clines et al. 1997), namely exons 3, 8, 9, 10, 11 and 14; the well-ordered markers *D11S1393*,

D11S903 (located adjacent to exon 5 of *EXT2*), and *D11S2095* (Wuyts et al. 1996); the STSs 102d9T7, 109d12T3, and 61a2T3 that map just upstream of *EXT2* (Stickens et al. 1996); two experimentally-developed STSs marking the ends of clone RPCI-3 404m15, 404m15-1 and 404m15-3; and end-fragment isolation from clones RPCI-3 368b4 and RPCI-3 366b4. RPCI-3 404m15 had been fully sequenced (accession number: AC124062) and the order of markers *D11S1393* and *D11S578* relative to *EXT2* contrasted with a former mapping (Wuyts et al. 1996). The fully sequenced cosmid cSRL101h11 (Stickens et al. 1996) (accession number: U73628) was identified through BLAST searches as a match against RPCI-3 404m15 and the sequence-sampled BAC clones RPCI-11 70a24 (accession number: AC025533) and RPCI-11 706a13 (accession number: AC090163) emerged as BLAST hits when the databases were queried with the 3' exons of *EXT2*. cSRL101h11 had been reported as positive for *D11S2095* (Stickens et al. 1996), yet overlapped with RPCI-3 404m15, while the two BAC clones clearly extended downstream of *EXT2*. Figure 3.1 integrates these data. Jointly, the clones RPCI-3 404m15 and cSRL101h11 provided approximately 135 kb of continuous sequence upstream of *EXT2*, outflanking *D11S1393*. The gap between the first exon of *EXT2* and this sequenced stretch was inferred to be negligible (Stickens et al. 1996), in accordance with the sizes of the relevant clones. On the other hand, the size of the *EXT2* transcription unit had been estimated to be 108 kb (Clines et al. 1997). When considered along with the size range for the PAC clones, these data predicted that the *D11S1393*–*D11S2095* interval is less than 300 kb.

Although a compact gene might be embedded in any of the large exons of *EXT2*, this scenario was judged unlikely and the readily available sequence upstream of *EXT2* was comprehensively analysed both by manual BLAST searches and by gene prediction software. Three putative genes were found to lie between *D11S1393* and *EXT2*: a gene of unknown function represented by the Unigene cluster Hs. 98649 (Molecular Databases at NCBI — see appendix F), the human homo-

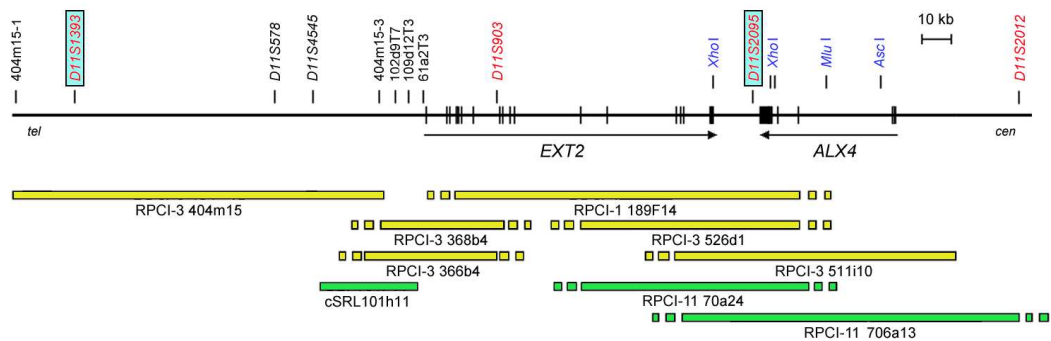


Figure 3.1: Physical map around the P11pDS minimal critical region, encapsulating the principal structural features of the *ALX4* and *EXT2* transcription units: exon organisation, orientation, and localisation relative to defined landmarks. Above the line, several relevant STSs (polymorphic short tandem repeat loci *in red*; all others *in black*) and restriction enzyme sites (*in blue*) are shown. The *D11S1393* and *D11S2095* loci, believed to define the telomeric and centromeric boundaries respectively of the minimal interval for P11pDS are framed and highlighted *in cyan*. Below the line, the clone contig is outlined: the PAC clones – *in yellow* – were evaluated experimentally, whereas positioning of the two BAC clones and the cosmid – *in green* – is based on sequence content — however, the presence of the most proximal marker, *D11S2012*, in RPCI-11 706a13 was verified experimentally. The scheme integrates information from a variety of sources — see section 3.2.2 for details. The *PHACS* gene which maps upstream of *EXT2* is not shown.

logue of a *Fugu rubripes* gene with significant similarity to the plant gene family coding for 1-aminocyclopropane-1-carboxylate synthase (ACS) (Peixoto et al. 2000), and a paralogue or pseudogene related to the latter. The lack of functional data and the absence of salient protein domains in the available sequence for Hs. 98649 (InterPro database at EBI — see appendix F) discouraged further work at this stage. The existence of sequences encoding, potentially, an ACS was enigmatic as the enzyme participates in ethylene biosynthesis, a chemical mediator confined to plants (Van der Straten et al. 1992); whatever its function, an enzymatic deficiency (assuming loss-of-function underlying mutations) was hardly compatible with the dominant mode of inheritance observed for PFM. Consequently, the focus shifted to the region downstream of *EXT2*.

An equivalent analysis of the highly unordered fragments from RPCI-11 706a13 revealed a sequence island encoding a putative homeodomain of the paired class (Bürglin 1994; Banerjee-Basu and Baxeavanis 2001). More specifically, it featured the distinctive amino acid residues of the paired-like subclass, including glutamine at position 50 (Galliot et al. 1999). An adjacent segment of non-homeobox sequence assigned the novel gene to a small group of vertebrate genes related to *Drosophila aristaless (al)* (Schneitz et al. 1993), consisting of *Alx4*, *Cart1*, and *Alx3* (Meijlink et al. 1999). Despite potential sequence errors and the limited cDNA coverage, it was apparent that the available sequence was not identical to those of human *CART1* (Gordon et al. 1996) or *ALX3* (Wimmer et al. 2002); in fact, high similarity with murine *Alx4* and conservation of synteny between human proximal chromosome 11p and the region on mouse chromosome 2 where *Alx4* resides (Mouse Genome Informatics at the Jackson Laboratory — see appendix F) strongly suggested that this was the human orthologue of *Alx4*.

Mouse *Alx4* cDNA had previously been trapped twice in two-hybrid screens. Although the initial isolation was fortuitous as *Alx4* turned out to be a spurious interactant of FosB (Qu et al. 1997a), it may be a potential partner for the retinoblastoma family protein p130 (Hudson et al. 1998); at the same time, it was cloned as a cross-hybridising species to mouse *Rax* cDNA and the chick orthologue was also reported (Takahashi et al. 1998). Mice heterozygous for a targeted disruption, *Alx4^{tm1 Rwi}*, are reportedly normal but homozygotes exhibit preaxial polydactyly, skull ossification defects, and – usually lethal – gastroschisis (Qu et al. 1997b). Soon afterwards it was realised that *Alx4* is allelic to an established locus for murine polydactyly which had been identified in a random mutagenesis project, *Strong's luxoid* (Strong and Hardy 1956; Strong 1961), and a missense mutation within the homeodomain, R206Q, was identified in the original strain (Qu et al. 1998) — this allele is now designated *Alx4^{lst}*. *Alx4^{lst}* behaves semidominantly as heterozygous animals manifest variable degrees of polydactyly

whereas homozygotes present a constellation of patterning and growth defects. The most conspicuous features in homozygotes are severe preaxial polydactyly, reduction and/or duplication of the radius, calvarial defects and overall distortion of the skull, partial alopecia, open eyelids at birth, and weakening of the abdominal body wall — a detailed description can be found in chapter 6. A third allele that arose spontaneously, *Alx4*^{lst-J}, was also found to harbour a mutation — a small intrahomeobox deletion causing a frameshift (Qu et al. 1998; Takahashi et al. 1998). Finally, a chemically-induced large deletion encompassing *Alx4*, *Alx4*^{lst-Alb}, is also associated with characteristic polydactyly, indicating that loss of *Alx4* function underlies at least part of the mutant phenotype (Qu et al. 1998).

In terms of its expression, *Alx4* typifies a developmental regulator involved in pattern formation and control of growth. Transcription is limited, with few exceptions, in the embryonic period and is initially relatively intense in certain structures such as the limb buds and the head primordia. Gradually, activity is extinguished or dramatically down-regulated and *Alx4* transcripts become confined in specific domains and cell groups. Gross assessment in whole-mount embryonic mouse specimens indicated that *Alx4* transcripts are detectable as early as at E8.25, prior to 'turning' and full closure of the neural tube, in the mesenchyme of the anterior end adjacent to the neural folds (Qu et al. 1997a; Qu et al. 1997b). At E9, expression was observed not only in the head primordia but also in the flank mesenchyme that would give rise to the limb buds and the ventral body wall (Qu et al. 1997a; Qu et al. 1997b). Between E9.5–11.0, *Alx4* expression showed progressive restriction in the frontonasal process (and subsequently, in the medial and lateral nasal processes), the distal part of the first branchial arch (and later, in both the maxillary and mandibular processes), and the anterior aspect of the limb buds (eventually defining a narrow anteroproximal domain) (Qu et al. 1997a; Qu et al. 1997b; Beverdam and Meijlink 2001). After E11.5, *Alx4* expression was reported to decline significantly both in the developing head and the limbs, although a new

focus of expression was seen at the fingertips (Qu et al. 1997a; Qu et al. 1997b). An equivalent pattern of expression was noted for the chick orthologue during limb development (Takahashi et al. 1998).

Finer evaluation in sectioned specimens revealed expression of *Alx4* in specific mesenchyme populations during late embryonic development, marking sites of epithelial-mesenchymal interactions in the skin appendages and in the dermal skull bones (Hudson et al. 1998). Between E12.5 and E15.5, apart from the limb buds, *Alx4* transcription highlighted condensations associated with the primitive ectomeninx, several developing skull bones, hair and whisker follicles, teeth, ribs, and the ventral body wall. There was a negative correlation with maturation and by E18.5, *Alx4* activity was just detectable in the whisker follicles. Individual mesenchymal cells around the ducts of the mammary glands were a noticeable exception as *Alx4* expression was observed after birth.

In summary, the skull defects associated with a null *Alx4* allele in mice, the expression pattern of *Alx4*, and the case of *MSX2* as a homeobox gene crucially implicated in skull development, made the human orthologue of *Alx4* the prime positional candidate for the new PFM locus at 11p11–p12. Mutational analysis of available coding segments was launched along with experimental characterisation of the new gene.

3.2.3 Characterisation of the *ALX4* Gene

As the sequence cover of the region improved, mostly from the RPCI-11 706a13 BAC clone, more parts, albeit unordered, of the new homeobox gene emerged and solidified the initial conclusion: the human orthologue of mouse and chick *Alx4*, *ALX4* (name approved by the HUGO Gene Nomenclature Committee — see appendix F), maps downstream and in close proximity to *EXT2*. Pairwise alignments between genomic sequences from RPCI-11 706a13 (accession number: AC090163) and the mouse *Alx4* cDNA (accession number: AF001465) indicated that the human

gene, like its murine counterpart (Qu et al. 1997b; Qu et al. 1998), consists of four exons; in fact, sequences from the putative exons 3 and 4 were identified within the same genomic fragment. Overall, an open reading frame of 1,233 bp was deduced, with the homeodomain split between exons 2 and 3; such an arrangement is common for members of the paired class (Bürglin 1994). Long-range PCR using the PAC clones RPCI-3 526d1 and RPCI-3 511i10 suggested that the terminal exons of *EXT2*, i.e. exon 14, and *ALX4*, i.e. exon 4, are separated by approximately 16 kb, in reverse orientation (Figure 3.1); an equivalent analysis between exon 14 of *EXT2* and exon 1 of *ALX4* was negative. Furthermore, the marker *D11S2095* was pinpointed in the intergenic interval, approximately 3.5 kb away from *ALX4* and 12.5 kb away from *EXT2* (Figure 3.1). The relationship of the *ALX4* and *EXT2* transcription units was corroborated by *Xho*I digestion of clone RPCI-3 526d1: probes corresponding to the terminal exons of each gene and *D11S2095* all detect the same, approximately 18 kb-long, fragment. The sizes of introns 2 and 3 were estimated by long-range PCR to be in the range of 7.5 and 2.3 kb respectively. The size of intron 1 was inferred from clone RPCI-11 706a13-derived sequences in combination with mapping of RPCI-3 511i10, the only PAC clone positive for exon 1, with rare-cutting restriction enzymes. Inspection of available intronic sequences, indicated that recognition sites for *Mlu*I and *Asc*I map 10.9 kb upstream of exon 2 and 5 kb downstream of exon 1 respectively which, by single digests, turned out to be unique in the clone insert. Probing of a combined digest with an exon 1-specific probe indicated that the *Mlu*I–*Asc*I interval in intron 1 is approximately 17.5 kb. Subsequent analysis mapped the polymorphic locus *D11S2012* centromeric to *ALX4*. The 3' end of *ALX4* was precisely located following identification of two ESTs (accession numbers: AW071529 and AW613995) containing a putative poly(A) signal and mapping approximately 4 kb downstream from the termination codon (Figures 3.1 and 3.2). Genomic sequences covering the coding parts of all four exons were obtained independently from normal DNA samples (accession numbers: AJ279074–AJ279077).

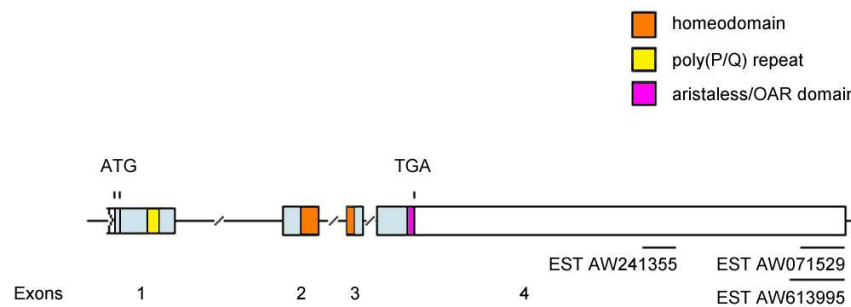


Figure 3.2: Exon-intron organisation of the *ALX4* gene and major domains at the protein level. Alignments with the mouse *Alx4* cDNA and presence of canonical splice junctions demarcated the four exons of human *ALX4* in genomic sequences; their coding parts are marked in colour. Intron sizes were estimated by long-range PCR and restriction mapping: intron 1, ~33.4 kb; intron 2, ~7.5 kb; intron 3, ~2.3 kb. The transcription start site was not defined, but the polyadenylation site in the long 3' UTR was inferred from two ESTs. A dual translation initiation codon may encode two products in vivo — see Figure 8.1. A poly(P/Q) repeat is present in the N-terminus, the paired-like homeodomain is split between exons 2 and 3, and the signature aristaless/OAR domain occupies the very end of the coding frame. Exons are drawn to scale and references to the 3' ESTs are by accession number. An annotated version of the *ALX4* cDNA sequence (accession number: AJ404888) can be found in appendix A.

To ensure that mutation detection procedures were targeting the functional copy of *ALX4*, its representation in the human genome was assessed by hybridisation. There was no evidence of paralogous sequences and therefore *ALX4* appears to be a single-copy gene on chromosome 11p (Figure 3.3). Consistent with the deduced genomic organisation, a single *ALX4*-specific transcript of 5.5–6.0 kb was detected in human fetal muscle and liver (Figure 3.4); hence, the 5' untranslated region (UTR) is predicted to be in the range of few hundred bp.

Exploiting apparently illegitimate transcription of *ALX4* in adult fibroblasts, a cDNA fragment covering the entire coding region of the gene was isolated by RT-PCR and subjected to direct sequencing (accession number: AJ404888; see appendix A). An attempt to clone the *ALX4* cDNA failed as the constructs exhib-

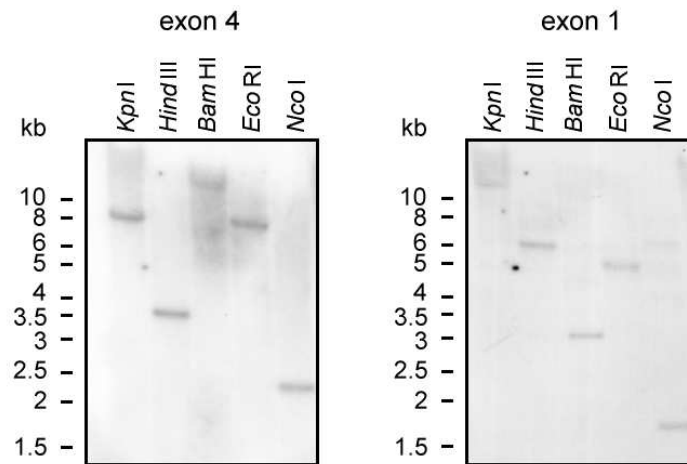


Figure 3.3: The *ALX4* transcription unit is uniquely represented in the human genome. Southern blot analysis using the non-homeobox exons 1 (*right*) and 4 (*left*) of *ALX4* as probes against various digests of normal human genomic DNA. No extra specific bands which could indicate the presence of paralogues and/or pseudogenes are seen.

ited marked instability during propagation. A dual translation initiation site may encode two products that differ in length by 16 amino acid residues, as documented for mouse *Alx4* (Qu et al. 1997a; Hudson et al. 1998) — see Figure 8.1. The longer protein, of 411 residues and with a predicted molecular weight of approximately 44 kDa, shows 93.5% similarity to the corresponding murine isoform. Apart from the paired-like homeodomain (Bürglin 1994; Galliot et al. 1999; Banerjee-Basu and Baxeavanis 2001), it features a conserved domain at the C-terminus, known as aristaless/OAR domain – the OAR acronym standing for *Otp*, *aristaless*, *Rx* –, and a striking poly(P/Q) stretch in the N-terminus (InterPro database at EBI — see appendix F). The former is a common auxiliary domain in paired-like homeoproteins (Galliot et al. 1999) but the latter is not matched, to such extent, in the mouse or chick orthologues (Figure 3.5). Database searches using the entire predicted protein (Molecular Databases at NCBI — see appendix F) confirmed that *ALX4* belongs to the aristaless-related family of paired-like homeoproteins, prototyped by the product

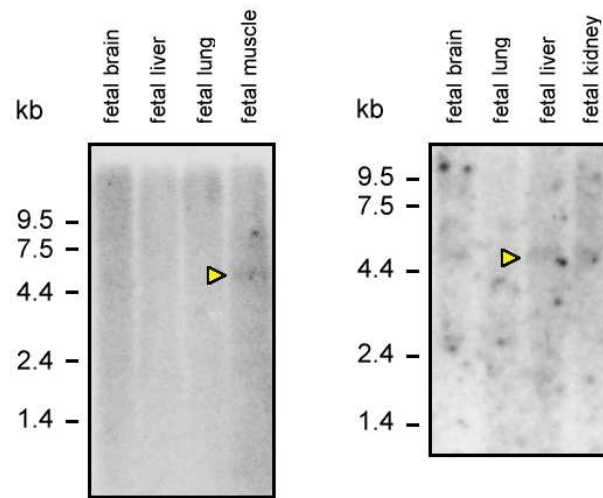


Figure 3.4: *ALX4* encodes an apparently single mRNA species of 5.5–6.0 kb in human fetal tissues. Commercial poly(A)+ Northern blots from different suppliers were hybridised with probes from exon 4 (*left*) and exon 1 (*right*) of *ALX4*. A low-abundance transcript of ~5.5–6.0 kb is detected in fetal muscle and liver (*yellow arrowheads*). The discrepancy between the liver samples in the two blots is probably attributable to the different age of the tissues.

of the *Drosophila* gene *al*. Within this branch at least three groups may be distinguished (Galliot et al. 1999): the Al group proper, comprising solely invertebrate proteins; the Arx group that includes the mouse Arx and its deuterostome orthologues; and the most coherent Alx group, consisting of the Alx4, Cart1 and Alx3 deuterostome proteins. Its members are structurally closely related, harbour an aristaless/OAR domain, and display overlapping expression patterns during development suggestive of functional links (Meijlink et al. 1999; Beverdam and Meijlink 2001); ALX4 joined the Alx group as the missing third human member (Figure 3.5).

3.2.4 Identification of Mutations

Excluding three kindreds (families 9, 10, and 11) where mutations in *MSX2* had previously been found (Wilkie et al. 2000) all other 17 families in the research panel (see

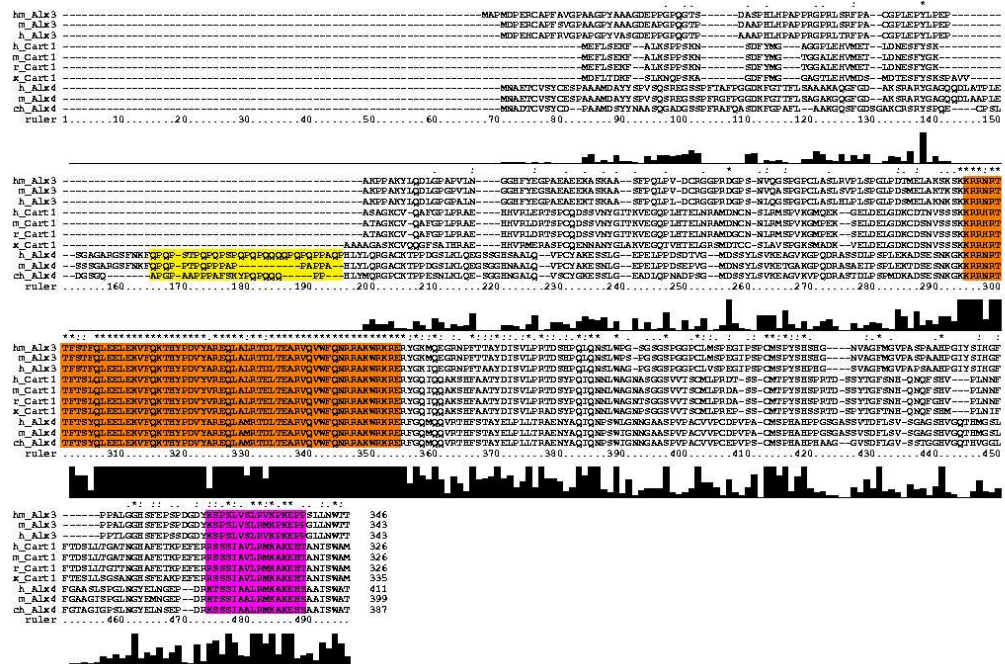


Figure 3.5: Human ALX4 belongs to a small group within the aristaless-related family of paired-like homeoproteins, comprising the Alx4, Cart1 and Alx3 proteins. The group appears to be confined in the deuterostomes. Above the multiple sequence alignment conservation of specific groups of amino acid residues is shown: a *star* denotes presence of a single invariant residue; a *colon* indicates conservation of a 'strong' group (STA; NEOK; NHOK; NDEQ; QHRK; MILV; MIFL; HY; FYW); and a *full stop* indicates conservation of a 'weak' group (CSA; ATV; SAG; STNK; STPA; SGND; SNDEQK; NDEQHK; NEQHRK; FVLIM; HFY). Below the alignment, the bar height reflects the value of an overall conservation score at a particular point. The readily recognisable domains are highlighted: the homeodomain (red); the aristaless/OAR domain (magenta); and the poly(P/Q) element (yellow). The latter is absent from Cart1 and Alx3, and, in general, outside of the homeodomain and the aristaless/OAR box the sequences have diverged considerably. Multiple sequence alignment by CLUSTAL X, incorporating the majority of vertebrate members: hm_AlX3, *Mesocricetus auratus* Alx3 (accession number: CAA57161); m_AlX3, *Mus musculus* Alx3 (accession number: O70137); h_AlX3, *Homo sapiens* Alx3 (accession number: O95076); h_Cart1, *Homo sapiens* Cart1 (accession number: NP_008913); m_Cart1, *Mus musculus* Cart1 (accession number: XP_125879); r_Cart1, *Rattus norvegicus* Cart1 (accession number: Q63087); x_Cart1, *Xenopus laevis* Cart1 (accession number: Q91574); h_AlX4, *Homo sapiens* Alx4 (accession number: Q9H161); m_AlX4, *Mus musculus* Alx4 (accession number: O35137); ch_AlX4, *Gallus gallus* Alx4 (accession number: AAC61772). Recently, homologues in sea urchins were reported (not shown).

appendix B and section 2.2) were examined for mutations in the coding region of *ALX4*. Several were analysed by screening techniques and sequencing of the coding parts of all four exons was carried out in at least one affected member per family, with the exception of families 7, 8, 19, and 20. Overall, five different heterozygous *ALX4* mutations were identified in six families: a missense change – encountered twice – which alters a critical residue in the homeodomain (653G→A, encoding R218Q); three nonsense substitutions (418C→T, resulting in Q140X; 736C→T, resulting in Q246X; and 620C→A, resulting in S207X); and a new submicroscopic deletion of proximal 11p that causes hemizyosity for *ALX4*.

3.2.4.1 Family 1 — 653G→A (R218Q) and Enlarged Parietal Foramina

In this previously published family by Salamanca et al. (1994) (Figure 3.6), a diagnosis of PFM was established prenatally in the proband, III-6, who presented with a palpable, wide defect in the posterior parietal region after birth (skull radiograph at the age of 9 years). Her older brother, III-5, was also affected (skull radiograph at the age of 12 years) and the phenotype was traced to the paternal line. Both the father, II-6, (skull radiograph at the age of 39 years) and the paternal grandmother, I-2, (skull radiograph at the age of 70 years) exhibited typical bilateral parietal foramina. The four affected individuals and the unaffected spouse, II-7, were analysed by SSCP. A shift in exon 2 segregated with the skull defects and sequencing of the sample from III-6 revealed a heterozygous G→A transition at nucleotide 653; at the protein level, it replaces an arginine residue at the fifth position of the homeodomain with a glutamine residue (R218Q). The change was confirmed by allele-specific restriction digest and shown to be present in the four affected members analysed. Furthermore, it was not detected in 96 chromosomes from normal subjects by means of allele-specific hybridisation. This family was reported by Mavrogiannis et al. (2001).

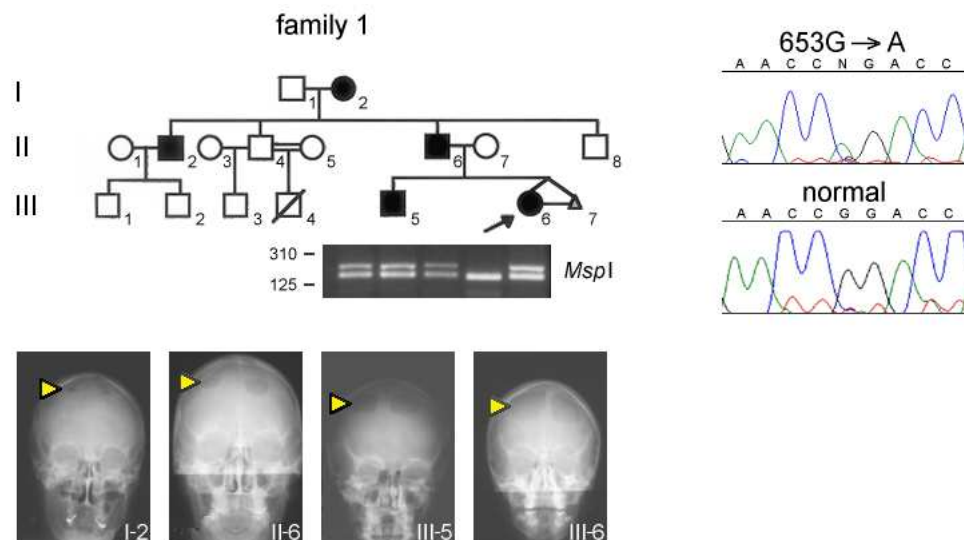


Figure 3.6: The heterozygous 653G→A missense substitution, encoding R218Q, segregating in a three-generation family with enlarged parietal foramina. The mutation was identified by sequencing (trace from individual III-6) and confirmed by restriction digest: the second exon of *ALX4* was amplified and cut with *MspI*. Normally, the two recognition sites split the 417 bp product in three fragments of 75, 162, and 180 bp. One site is abolished by the substitution, resulting in a larger fragment of 237 bp. The 75 bp fragment is not shown and the 162 and 180 bp fragments have not resolved, appearing as a single band. Skull radiographs, courtesy of Dr Alberto Salamanca, demonstrate typical bilateral enlarged parietal foramina (yellow arrowheads); the defects are more severe in younger family members. The pedigree depicts family 1 (see appendix B and section 2.2).

3.2.4.2 Family 2 — 418C→T (Q140X) and Enlarged Parietal Foramina

Part of family 2 (Figure 3.7), the largest in the panel, has been described (Kutilek et al. 1997) but the affection status of certain individuals was revised after accessing further skull radiographs and obtaining a comprehensive family history. Defined enlarged parietal foramina were noticed in skull radiographs of the proband, IV-4, (not shown) whereas a midline gap, essentially mild cranium bifidum, was apparent in her younger sister, IV-6, (skull radiograph at the age of 3 months). No osseous defects were obvious in their brother, IV-5, (skull radiograph at the age of 5 years) yet overall skull modelling was abnormal. The father, III-6, (skull radiograph at the age of 28 years) and the paternal grandmother, II-3, (skull radiograph at the age of 49 years) had narrower bilateral foramina which were just visible in the great-grandmother, I-2, (skull radiograph at the age of 72 years). The trait segregates in another branch of the pedigree, founded by the non-manifesting individual II-4 (skull radiograph at the age of 43 years): both her children, III-7 and III-8, exhibited bilateral PFM (skull radiographs at the age of 20 and 17 years respectively). Two other individuals at 50% prior risk, II-1 and III-4, were assessed as normal. Following detection of an SSCP variant in exon 1 which showed association with the skull defects, sequencing was undertaken in the sample from III-6. A heterozygous C→T transition at nucleotide 418 was uncovered which is predicted to create a termination codon at triplet 140 (Q140X). A diagnostic restriction digest detected the change not only in all affected members but also in the obligate carrier II-4 and in IV-5. On the other hand, individuals II-1 and III-4 do not carry the variant which was also not found in 96 chromosomes from normal subjects, checked by allele-specific hybridisation. This family was reported by Mavrogiannis et al. (2001).

3.2.4.3 Family 3 — 736C→T (Q246X) and Enlarged Parietal Foramina

Family 3 is unpublished and was clinically ascertained by Prof. Andrew Wilkie (Figure 3.8). The proband, IV-1, presented as a neonate with a midline skull de-

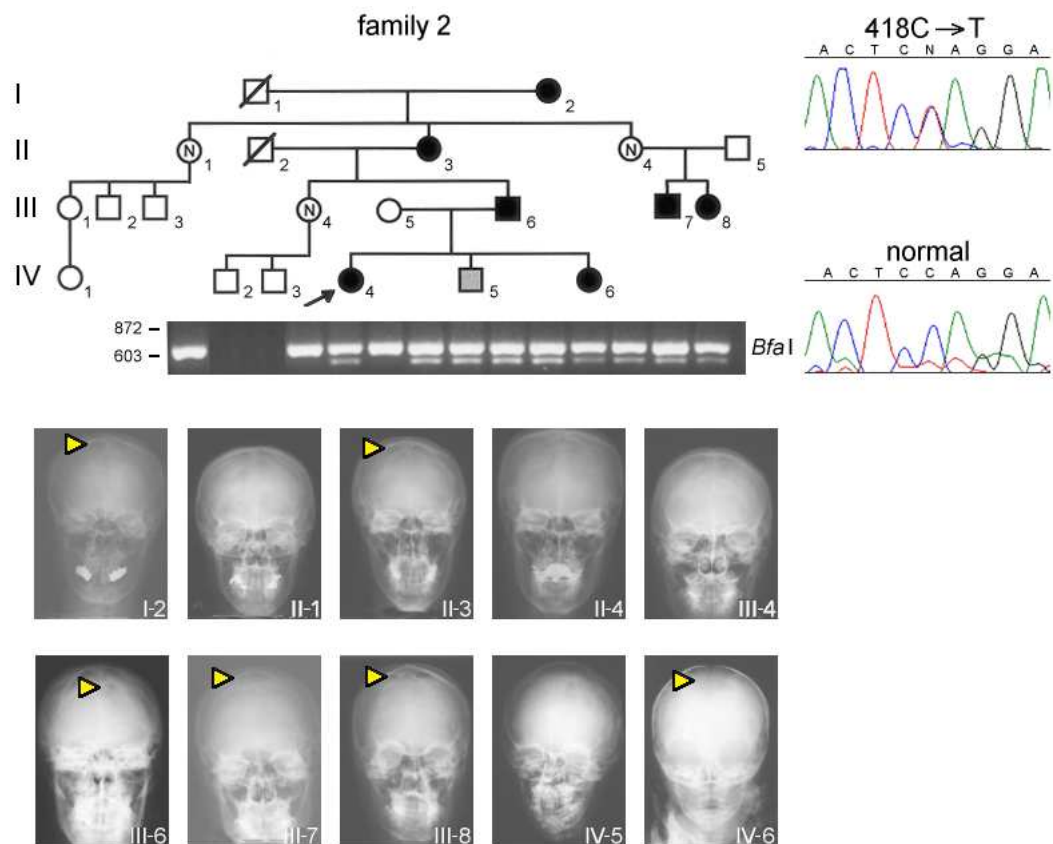


Figure 3.7: The heterozygous 418C→T nonsense mutation, predicted to create a termination codon at triplet 140 (Q140X), segregating in a four-generation family with enlarged parietal foramina. The change was identified by sequencing (trace from individual III-6) and verified by restriction digest: the first exon of *ALX4* was amplified and cut with *BfaI*. No recognition site is normally present and the 651 bp product is left intact. The substitution generates a site that divides the product into two fragments of 120 and 531 bp. Only the larger mutant band is shown, just below the full-length product; its reduced intensity reflects trapping of mutant strands into undigestible heteroduplexes. Skull radiographs, courtesy of Drs Alica Baxová and Stepan Kutilek, show enlarged parietal foramina in most manifesting mutation carriers – with the exception of IV-6 where a midline defect is noticeable – (yellow arrowheads) and reveal two cases of incomplete/non-penetrance. Although skull modelling is abnormal in IV-5 (greyed), no defects are seen. Moreover, II-4, an obligate mutation carrier, is assessed as normal. The proband also presented with classical enlarged parietal foramina (not shown). N, radiologically normal skull. The pedigree summarises family 2 (see appendix B and section 2.2).

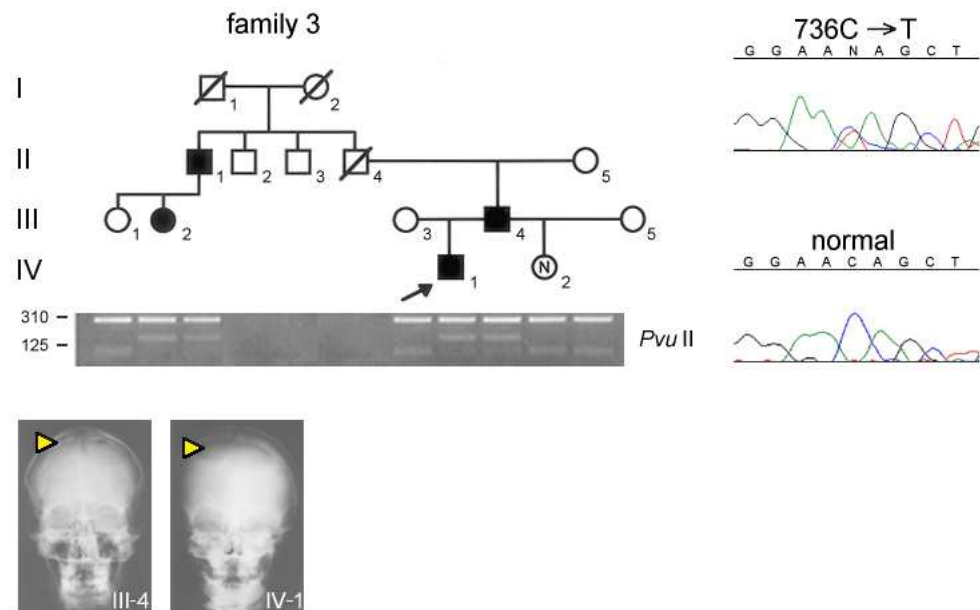


Figure 3.8: The heterozygous 736C→T nonsense substitution which results in a stop codon at triplet 246 (Q246X), segregating in a three-generation family with enlarged parietal foramina. The mutation was identified by sequencing (trace from individual IV-1) and confirmed by restriction digest: the second exon of *ALX4* was amplified and cut with *PvuII*. Normally, two recognition sites split the 417 bp product in three fragments of 63, 76, and 278 bp. One site is abolished by the substitution, producing an intermediate fragment of 139 bp. The smallest fragments appear as a single band. Available skull radiographs show a wide defect in IV-1 which is being bridged along the midline; his father, III-4, exhibits typical enlarged parietal foramina (yellow arrowheads). N, radiologically normal skull. The pedigree depicts family 3 (see appendix B and section 2.2).

fect (skull radiograph at the age of $1\frac{1}{2}$ years) and bilateral PFM were identified in his father, III-4, (skull radiograph at the age of 40 years) and great uncle, II-1. Individual III-2, the daughter of II-1, was also born with a midline skull defect and CT brain scan showed an enlarged cerebrospinal fluid-filled space in the posterior fossa (not shown). Screening by SSCP failed to reveal any variants, but sequencing, performed in the sample from IV-1, indicated a heterozygous C→T transition at nucleotide 736 which generates a termination codon at triplet 246. The change was verified by restriction digestion and was found in all affected members, while being absent from two unaffected individuals at 50% prior risk, IV-2 and III-1. In addition, it was not represented in a panel of 96 chromosomes from normal subjects, assayed by allele-specific hybridisation. This family was also reported by Mavrogianis et al. (2001).

3.2.4.4 Family 4 — 620C→A (S207X) and Enlarged Parietal Foramina

Family 4 has not been described before and was referred by Dr Feliciano Ramos (Figure 3.9). Wide PFM were seen in the proband, III-2, (skull radiograph at the age of 13 years) and less extensive bilateral defects were observed in her siblings, III-1 and III-3 (skull radiographs at the ages of 14 and 9 years respectively). Even narrower openings were present in the father, II-3 (skull radiograph at the age of 39 years); nevertheless, they were substantial in the affected grandfather, I-2 (skull radiograph at the age of 68 years). No variants were evident in DHPLC analysis but subsequent sequencing, carried out in the sample from III-2, revealed a heterozygous C→A transversion at nucleotide 620. The change is located just upstream of the homeobox and creates a termination codon at triplet 207 (S207X). A diagnostic restriction digest confirmed the change and showed co-segregation of the variant with the skull defects.

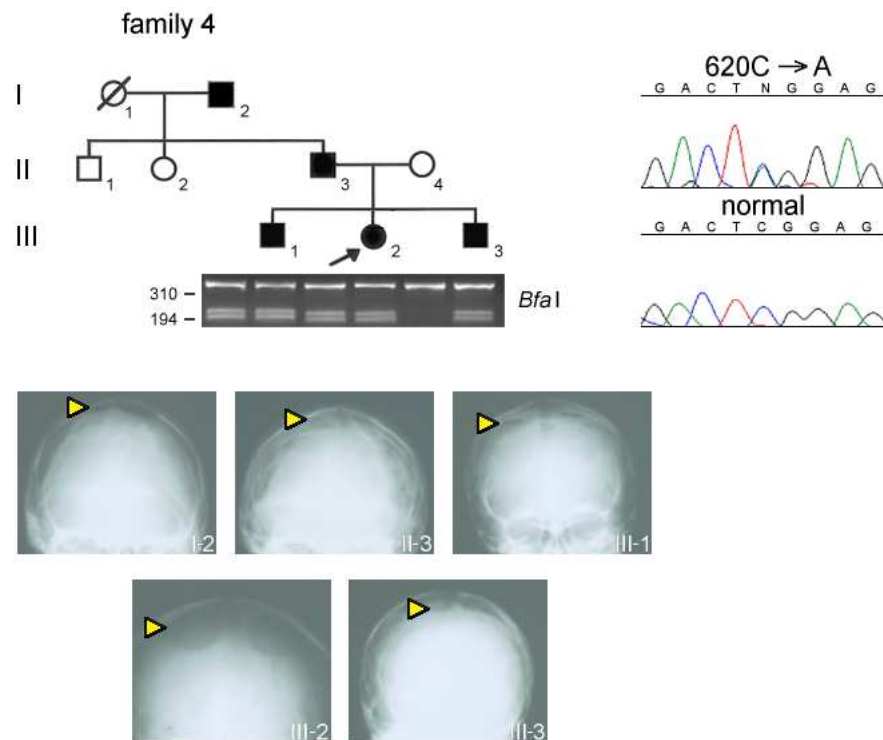


Figure 3.9: The heterozygous 620C→A nonsense change which generates a stop codon at triplet 207 (Q207X), segregating in a three-generation family with enlarged parietal foramina. The mutation was identified by sequencing (trace from individual III-2) and confirmed by restriction digest: the second exon of *ALX4* was amplified and digested with *Bfa*I. No recognition site is normally present and the 417 bp product persists. The mutation introduces a site which splits the product in two fragments of 195 and 222 bp. Skull radiographs, courtesy of Dr Feliciano Ramos, demonstrate enlarged parietal foramina in all mutation carriers (*yellow arrowheads*); the defects are wider in the proband, III-2. The pedigree illustrates family 4 (see appendix B and section 2.2).

3.2.4.5 Family 5 — 653G→A (R218Q) and Enlarged Parietal Foramina/Cranium Bifidum with Meningeal Anomalies

Family 5 is, also, unpublished and was referred by Dr Chong Ae Kim (Figure 3.10). Individual III-2 manifested cranium bifidum, presenting as a generalised radiolucency (skull radiograph at the age of 1 year), whereas his mother, II-2, had a large ellipsoid defect in the parietal region (skull radiograph at the age of 23 years). The maternal grandfather, I-2, demonstrated classical bilateral foramina (skull radiograph at the age of 50 years). CT and MRI brain scans of individuals I-2, II-2, and III-2 (Figure 3.11) not only outlined precisely the osseous defects but also revealed a wide hiatus of the tentorium cerebelli; this localised meningeal anomaly was associated with a large space posteriorly, occupied by cerebrospinal fluid, but without concomitant cerebellar hypoplasia. On SSCP analysis, all three samples produced a shift, indistinguishable from the one observed in family 1, and sequencing, performed in the sample from II-2, identified the same change — a heterozygous 653G→A substitution, encoding R218Q, which was verified by the already established diagnostic digest. The analysis of this family was reported by Mavrogiannis et al. (2001).

3.2.4.6 Family 6 — a New Case of Proximal 11p Deletion Syndrome

The preliminary characterisation of a heterozygous deletion of proximal 11p was previously reported in an abstract form for this family (Davies et al. 1997). The family displayed the hallmark features of P11pDS: multiple exostoses and PFM, inherited in an autosomal dominant fashion over four generations, without any sign of associated mental retardation. The deleted interval was reported to contain the loci *D11S905*, *D11S1355*, and *D11S903* and to be bordered distally by *D11S1360* and proximally by *D11S4103*. A comprehensive analysis was performed in four affected members of this kindred (Figure 3.12) in order to fine-map the deletion boundaries; this, in turn, could facilitate further delineation of P11pDS. All four affected dele-

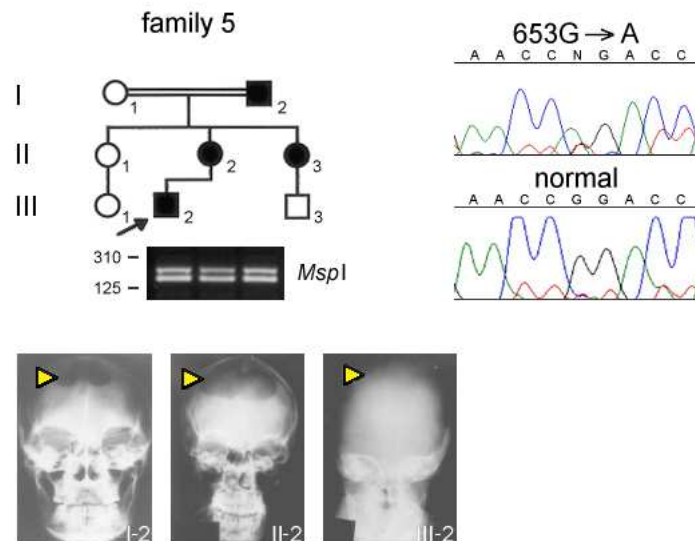


Figure 3.10: The heterozygous 653G→A missense substitution, encoding R218Q, segregating in a three-generation family with enlarged parietal foramina/cranium bifidum and abnormal patterning of the meninges. The mutation was identified by sequencing (trace from individual II-2) and confirmed by restriction digest: the second exon of *ALX4* was amplified and cut with *MspI*. Normally, the two recognition sites split the 417 bp product in three fragments of 75, 162, and 180 bp. One site is abolished by the substitution, resulting in a larger fragment of 237 bp. The 75 bp fragment is not shown and the 162 and 180 bp fragments have not resolved, appearing as a single band. Skull radiographs, courtesy of Dr Chong Ae Kim, demonstrate bilateral, relatively wide, enlarged parietal foramina in I-2 and a pronounced oval defect in his daughter, II-2 (yellow arrowheads); a generalised radiolucency is observed in her son, III-2, indicative of severe calvarial underossification. A CT evaluation of the osseous defects along with brain MRI scans, revealing the meningeal anomalies, is presented in Figure 3.11. The pedigree displays family 5 (see appendix B and section 2.2).

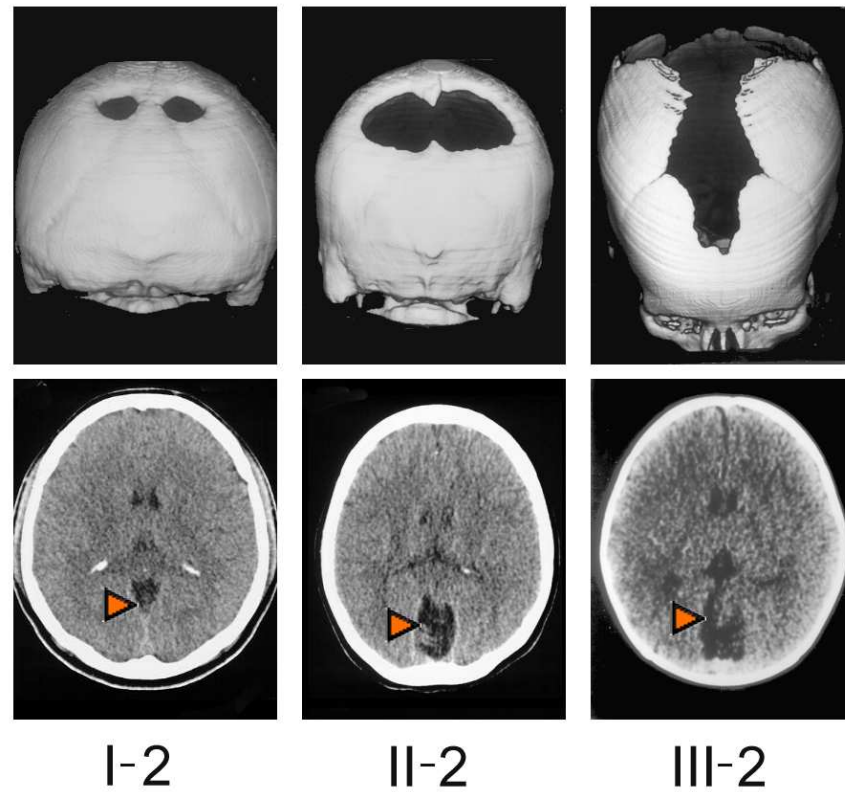


Figure 3.11: Skull ossification defects and abnormal patterning of the meninges in family 5 — CT and MRI scans. Three-dimensional reconstructions of the skulls of III-2, II-2, and I-2 (see Figure 3.10) by CT illustrate the evolution of the osseous defect (*upper row*): the 1-year-old proband (antero-superior view) shows cranium bifidum — split skull; his 23-year-old mother (posterior view) has a wide defect nevertheless confined in the posterior parietal region with two bony projections developing along the midline; in the 50-year-old grandfather (posterior view) the gap has been bridged, marking off the symmetric enlarged parietal foramina. Transverse MRI sections through the brain (*lower row*) disclose a wide opening of the tentorium cerebelli (*red arrowhead*) associated with a large space, filled with cerebrospinal fluid, in all three individuals. There is no indication of cerebellar hypoplasia. Images courtesy of Dr Chong Ae Kim.

tion carriers – II-1, II-2, III-1, and III-2 – showed typical, bilateral PFM — skull radiographs obtained at the ages of 42, 40, 12, and 6 years respectively. In addition, bone-covered cartilaginous projections at the juxta-epiphyseal regions of the long bones were a consistent finding (not shown; a representative radiograph from another family member, also a documented deletion carrier, is displayed). Typing data for the set of short tandem repeat loci used – *D11S4173*, *D11S1330*, *D11S1279*, *D11S905*, *D11S903*, *D11S2095*, *D11S2012*, *D11S4103*, *D11S554* and *D11S1361*, in telomere-to-centromere order – indicated that the region defined by *D11S1279* telomerically and *D11S2012* centromerically is missing, thus confirming hemizyosity for *ALX4* (Figure 3.1). Based on the current genomic assembly (Ensembl v. 14.31.1 — see appendix F) a minimum size estimate for the deletion would be in the range of 3.8 to 3.9 Mb. *D11S1330* and *D11S4103* were uninformative, falling within the grey zones, whereas *D11S4173* and *D11S554* showed heterozygosity in at least one affected individual and are predicted to flank the deletion. Consequently, the maximum size estimate would be 4.1–4.2 Mb. The centromeric breakpoint was pinpointed by FISH analysis: hemizyosity was seen for the sequences in the BAC clone RPCI-11 58k22 (accession number: AC010768) which maps approximately 100 kb proximal to *D11S2012*; on the contrary, the BAC clone RPCI-11 193f22 (accession number: AC092733), located further proximally (approximately 400 kb away from *D11S2012*) and containing *D11S4103*, was found not deleted (Figure 3.13).

3.3 The *MSX2* Locus

3.3.1 Novel Mutations

The *MSX2* mutations in families 9, 10, and 11 had been reported previously (Wilkie et al. 2000): a deletion eliminating the entire gene in family 9; a 515G→A missense substitution, encoding R172H, in family 10; and a 475_480delCGCAAG deletion, removing two amino acid residues – R159_K160del – in family 11. Certain

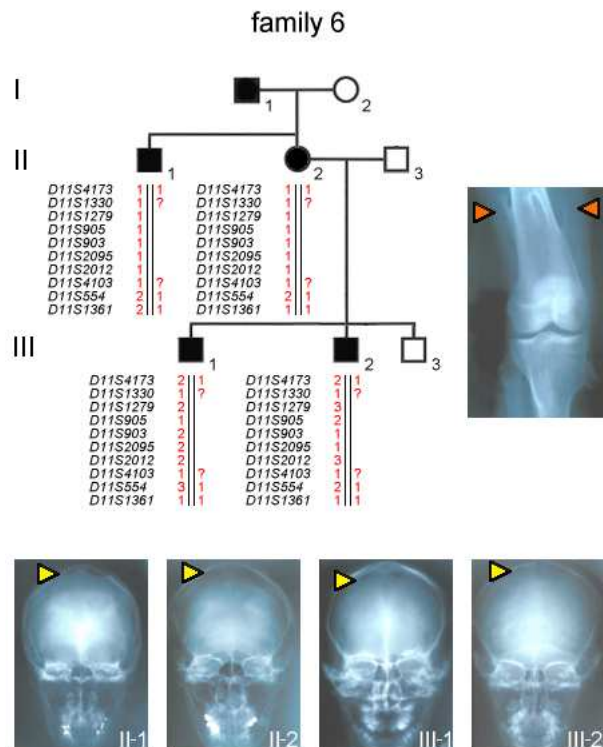


Figure 3.12: A heterozygous deletion of 11p11–p12 spanning *ALX4* and *EXT2* segregating in a family with enlarged parietal foramina and multiple exostoses of the long bones — a new case of P11pDS. Deletion mapping and haplotyping are based on data for several short tandem repeat loci in individuals II-1, II-2, III-1, and III-2. The deleted segment extends, at least, from *D11S1279* telomerically to *D11S2012* centromerically — inferred from non-inheritance of the maternal alleles in III-1 and III-2. Markers *D11S1330* and *D11S4103* are uninformative, whereas *D11S4173* and *D11S554* showed heterozygosity in at least one affected individual and hence flank the deletion. Classical bilateral enlarged parietal foramina are visible in the skull radiographs of the four examined deletion carriers (*yellow arrowheads*). Exostotic outgrowths of the long bones were also consistently seen — *red arrowheads* point to the protuberances adjacent to the epiphysis of the femur in a relative who is also a documented deletion carrier (but was unavailable for analysis and is not included in the current pedigree). (Radiographs courtesy of Dr Michael Owen.) Demarcation of the deletion boundaries and deduction of haplotypes assumes a continuous, stably inherited deletion and no recombination between the markers. The pedigree displays family 6 (see appendix B and section 2.2).

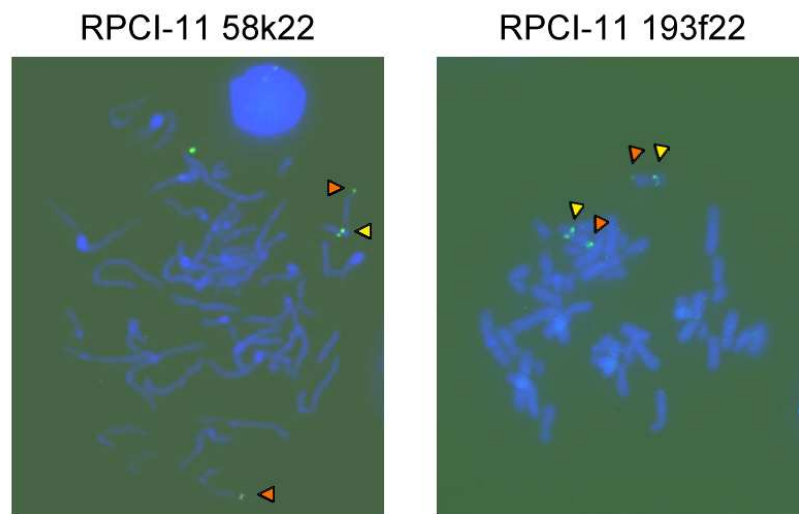


Figure 3.13: Direct confirmation of the 11p11–p12 deletion in family 6 and finer delimitation of its centromeric breakpoint by FISH analysis. Two mapped and sequenced BAC clones were used to probe metaphases of individual II-1 (see Figure 3.12): RPCI-11 58k22 maps adjacent to *D11S2012* on the centromeric side, while RPCI-11 193f22 maps further proximally, containing *D11S4103*. Hemizyosity is seen for the former whereas the latter is represented on both chromosomes (*yellow arrowheads*); a probe specific for the chromosome 11q telomere was applied as control (*red arrowheads*). The spot at the top in the left image is non-specific noise. The results were consistently observed in several metaphases. Both images were digitally enhanced.

of the remaining 17 families (see appendix B and section 2.2) were analysed by screening techniques, but eventually sequencing of the coding parts of the two *MSX2* exons was performed in at least one affected individual per family, with the exception of families 19 and 20. Two new heterozygous mutations were identified, both predicted to cause frameshifts either upstream of or within the homeobox (417_418delCA, resulting in H139fsX243; and 505_508dupATTG, resulting in A170fsX245).

3.3.1.1 Family 7 — 417_418delCA (H139fsX243) and Enlarged Parietal Foramina

In this unpublished nuclear family referred by Dr Tom Letteboer (Figure 3.14), the proband, II-1, was diagnosed with PFM/cranium bifidum and familial incidence of the defect was suggested when a wide posterior fontanelle was noticed in his son, III-1, at birth. A DHPLC shift in exon 2 was followed by sequencing, performed in the sample from II-1, which indicated a heterozygous 2 bp deletion, 417_418delCA, immediately upstream of the homeobox. The deletion homoduplex was isolated by cloning and sequencing confirmed the deduced change which was also assayed by allele-specific hybridisation. Although a frameshift is predicted at triplet 139, the new reading frame remains open for a considerable distance — H139fsX243.

3.3.1.2 Family 8 — 505_508dupATTG (A170fsX245) and Parietal Foramina with Clavicular Hypoplasia

Family 8 (Figure 3.15), which has not been described before and was referred by Dr Sixto Garcia-Miñaur, stood out for featuring the distinctive combination of PFM with hypoplasia of the clavicles — PFMCCD. The syndrome has been regarded as distinct from either classical PFM or CCD, and is listed as a separate OMIM entry under number 168550. A diagnosis of PFMCCD was established in the proband, III-1, manifesting bilaterally enlarged foramina and short, laterally hypoplastic, narrow clavicles (skull and chest radiographs at the age of 32 years). His father,

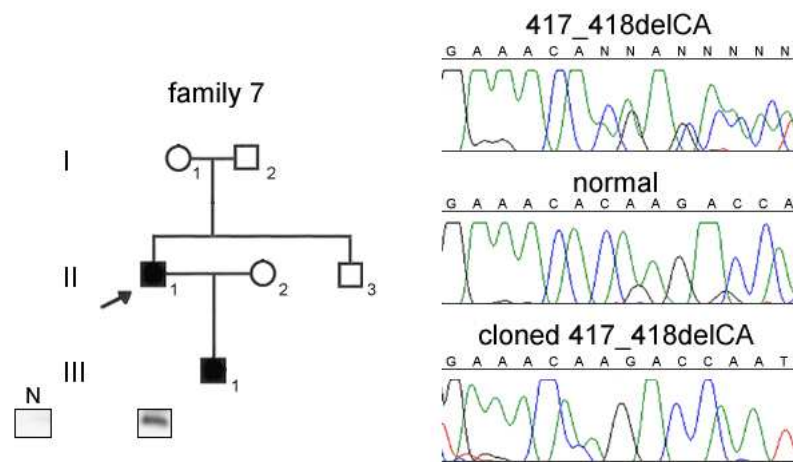


Figure 3.14: The heterozygous 417_418delCA mutation, causing a frameshift at codon 139 (H139fsX243), identified in a nuclear family with enlarged parietal foramina. The sequence overlap of the deletion and normal alleles in the sample from II-1 was resolved by cloning and the change was confirmed by hybridisation: the second exon of *MSX2* was amplified from the proband and a normal DNA sample (N), the products were blotted, and probed with an allele-specific oligonucleotide. The pedigree depicts family 7 (see appendix B and section 2.2).

II-1, showed small foramina and broad, coarse clavicles with tapering ends (skull and chest radiographs at the age of 63 years). The elder son of the proband, IV-1, presented with a similar combination of defects (skull radiograph at the age of $3\frac{1}{2}$ years; chest radiograph at the age of 5 years) and his younger brother had persistent, wide anterior and posterior fontanelles in infancy (not shown). The paternal grandmother was reputed to have sloping shoulders but was not available for examination. DHPLC analysis pointed to a change in exon 2 and subsequent sequencing, carried out in the sample from III-1, demonstrated a heterozygous 4 bp duplication, 505_508dupATTG, almost in the middle of the homeobox. The mutant homoduplex was isolated by cloning and sequencing verified the deduced alteration; in addition, a diagnostic restriction digest showed co-segregation of the variant with the defects. The change causes a frameshift at triplet 170 – A170fsX245 – but a termination codon in the new reading frame is not reached nearby. The molecular analysis of this family has been published (Garcia-Miñaur et al. 2003).

3.3.2 Could *MSX2* Mutations Be Associated with Dental Defects?

Apart from the skull defects, dental problems had been noticed in individuals II-1 and I-1 in family 11 who both carry the heterozygous 475_480delCGCAAG mutation (Wilkie et al. 2000). Since the original publication, the pedigree was enriched (see appendix B and section 2.2) and it became apparent that a history of advanced caries or a requirement for artificial teeth was a common finding in several other family members. The dental phenotype of homozygous null mice for *Msx2*, namely enamel deficiency due to degeneration of ameloblasts (Satokata et al. 2000), the expression pattern of *Msx2* during tooth development (MacKenzie et al. 1992), and the tooth agenesis in humans caused by mutations in the related homeobox gene *MSX1* (Vastardis et al. 1996; van den Boogaard et al. 2000) made *MSX2* a valid candidate for this facet of the phenotype as well. Additional members were typed by means of allele-specific hybridisation and the mutation was traced to the mater-

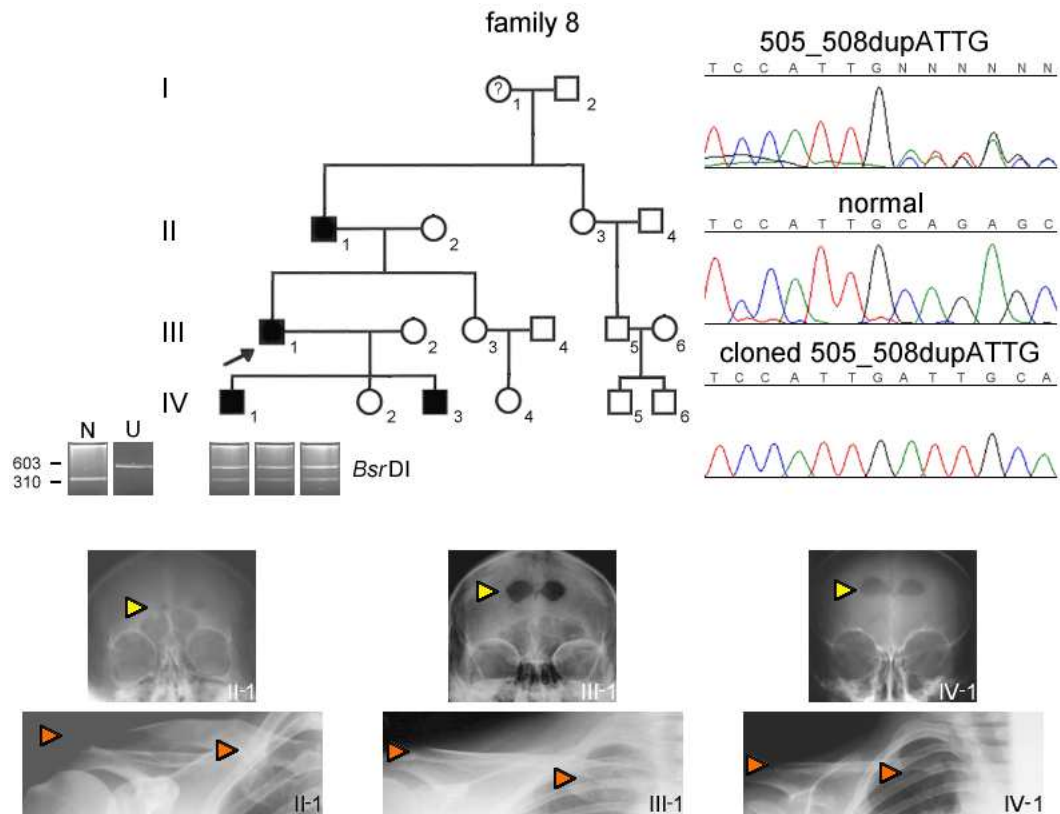


Figure 3.15: The heterozygous 505_508dupATTG mutation, resulting in a frameshift at codon 170, segregating in a three-generation family with the combination of enlarged parietal foramina and clavicular hypoplasia. The change was inferred by sequencing (overlap of the duplication and normal alleles in the trace from individual III-1), resolved by cloning, and confirmed by restriction digest: the second exon of *MSX2* was amplified and cut with *BsrDI*. Normally, a unique cleavage site divides the 496 bp product in two fragments of 154 and 342 bp. This is eliminated by the duplication and the full-size product persists. N, normal control; U, uncut PCR product. Skull radiographs, courtesy of Dr Sixto Garcia-Miñaur, demonstrated bilateral enlarged parietal foramina in II-1, III-1, and IV-1 (yellow arrowheads); the severity correlates negatively with age. Chest radiographs revealed tapering clavicles in all three individuals — red arrowheads mark their lateral and medial ends. The pedigree represents family 8 (see appendix B).

nal grandfather. However, it showed discordance with the dental defects (data not shown).

3.4 Other Investigations

In the remaining families of the panel (12–20; see appendix B), screening and/or sequencing of the coding regions of *ALX4* and *MSX2* did not reveal abnormal variants. Although inherently a less promising subset – clinically more heterogeneous, with usually unclear patterns of inheritance, and with pedigrees of limited size –, either point mutations outside of the coding parts of the two genes or small deletions could not, in principle, be excluded. Detection of heterozygosity at polymorphic sites within or around the *ALX4* exons (see section 4.2.1) ruled out full-gene deletion events in families 12–14, 16, 17, 19, and 20 (data not shown). For *MSX2*, detection of intragenic heterozygosity (see section 4.3.1; also typing data for the intragenic locus *MSX2-CA*) made full-gene deletion events unlikely in families 12–14 (data not shown). To address the possibility of exonic deletions in *ALX4*, comparative Southern blot analysis was performed, employing the non-homeobox exons 1 and 4. Hybridisation of an *EcoRI* digest of genomic DNA with a mixture of exon 1- and exon 4-specific probes – detecting distinct bands of approximately 5 and 7.5 kb respectively (Figure 3.3) – did not reveal an abnormally high or low exon 1 : exon 4 ratio in affected individuals of families 12–18; families 19–20, obtained at a later stage, were not analysed. In the case of *MSX2*, a paralogous intronless copy at 17q23 (Ensembl v. 14.31.1 — see appendix F), apparently a processed pseudogene, provided a control for gene dosage analysis (an exon 1-specific probe detects two bands: one around 6.5 kb, representing the functional copy, and another around 10.5 kb derived from the pseudogene). However, insufficient amounts of material hindered its application in families 15–17 and 19–20; in family 18 the gene : pseudogene ratio appeared normal.

3.5 Summary

With hindsight, the mutational, structural, and expression data for mouse *Alx4* made the human orthologue a natural choice for the second PFM locus (Table 1.2). Original genetic mapping of *Strong's luxoid* on chromosome 2 was imprecise, placing the locus within an interval where conservation of synteny with human chromosome 11p breaks down, and this had initially misled the analysis (Vogt and Leder 1996; Peichel et al. 1998). As a matter of fact, two of the other three potential transcription units identified within the minimal critical region remain poorly characterised so far while the product of the gene coding for a putative human ACS, *PHACS*, lacks this enzymatic activity (Koch et al. 2001). The proposed physical map around *EXT2* and *ALX4* is in complete agreement with the local organisation which has emerged from the assemblies of human genomic sequences (Ensembl v. 14.31.1 and UCSC genome browser April 2003 freeze — see appendix F): the two genes lie tail-to-tail, approximately 10 kb away, separated by *D11S2095* while *D11S2012* maps proximal to *ALX4*; and the *ALX4* exons are unequally distributed with the first one located around 20 kb away from the cluster of exons 2–4. Strictly speaking, *ALX4* resides immediately outside of the originally proposed minimal critical interval (Wuyts et al. 1999), yet the placement of the centromeric boundary at *D11S2095* was based on heterozygosity at this locus in a single patient with P11pDS (McGaughan et al. 1995); a more parsimonious delimitation would have set the proximal boundary further proximally at *D11S1361/D11S554*. Interestingly, this discrepancy is likely to reflect either a position effect (Milot et al. 1996) on *ALX4* expression or the presence of a crucial regulatory element at the 3' of the gene.

At the same time, two other research groups converged independently on *ALX4*; they had, also, relied on the same set of draft genomic sequences from the region. An initial report that human *ALX4* maps adjacent to *EXT2* (Wu et al. 2000) and could be the favoured candidate gene for the PFM locus on chromosome

11p was, however, accompanied by an erroneous cDNA sequence containing three single-nucleotide deletions that alter part of the reading frame (accession number: AF294629) — exonic segments of draft genomic sequence were assembled into a putative cDNA without experimental confirmation. A second publication (Wuyts et al. 2000a) also outlined the genomic organisation of *ALX4* (accession numbers: AF308822–AF308825) and, importantly, described two heterozygous mutations in two families segregating isolated PFM, corroborating the present findings. The single nucleotide deletion 504delT causes a frameshift (D169fsX180) whereas the 815G→C substitution alters the 59th residue of the homeodomain (R272P).

Out of the twenty families investigated, five distinct heterozygous mutations in *ALX4* were ascertained in six kindreds, including pedigrees 1 and 2 where linkage to proximal 11p was originally detected; in total, mutations were identified in twenty-nine individuals. Excluding the P11pDS family 6, these comprise three intragenic nonsense substitutions and one missense change associated with non-syndromic PFM and/or cranium bifidum (Figure 3.16). Apart from the two families with *MSX2* mutations, the residual families may harbour changes in non-coding parts of *ALX4*, such as the recently characterised promoter region (Wuyts and Van Hul 2002) or could point to further genetic heterogeneity. Although the three nonsense changes (418C→T, translating as Q140X; 736C→T, translating as Q246X; and 620C→T, translating as S207X) are predicted to eliminate, completely or partially, the homeodomain, transcript degradation is a more likely outcome. As all mutations map upstream of the terminal exon, within exons 1 and 2, their presence may trigger the mechanism of nonsense-mediated decay (Hentze and Kulozik 1999). Either way, by being unambiguously loss-of-function events they suggest that *ALX4* haploinsufficiency can cause PFM/skull ossification defects in humans.

The missense mutation 653G→A, encoding R218Q, is of special interest from several points of view. It was found twice, in families 1 and 5, but different haplotypes for the polymorphisms 104G→C, 304C→T, and 1464C→T (see section

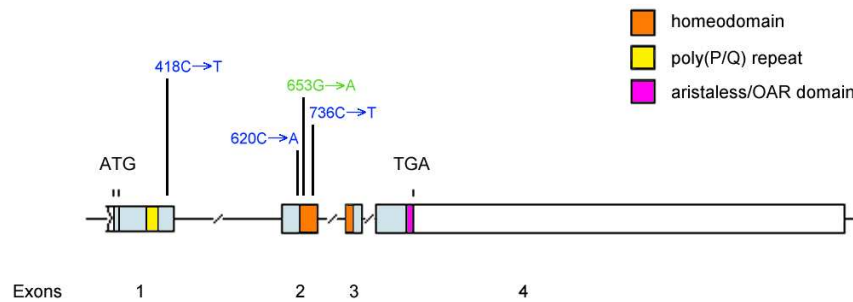


Figure 3.16: Summary of the intragenic point mutations in *ALX4* associated with PFM. The nature and location of the changes is shown on the genomic structure of the gene in the context of the major protein domains (see Figure 3.2). The three nonsense substitutions (*in blue*) map within the homeobox, immediately upstream of it, and in exon 1. The missense change 653G→A (*in green*), coding for R218Q and encountered in two unrelated families, alters the fifth highly conserved arginine residue of the homeodomain.

4.2.1) point to independent mutation events (data not shown). The arginine 218 residue, occupying the fifth position in the *ALX4* homeodomain, is almost invariant in homeoproteins (Bürglin 1994). This strong selective pressure correlates with its role in DNA recognition. The N-terminal arm of the homeodomain is flexible in solution, but upon DNA binding it insinuates into the minor groove and stabilises the entire structure (Figure 9.3). In particular, arginine 5 establishes direct contacts with the atoms of the bases (Gehring et al. 1994; Wilson et al. 1995) and marks the only discernible mutation hotspot outside the homeodomain helices (D’Elia et al. 2001). Furthermore, the equivalent change, R206Q, underlies *Alx4^{lst}* in the mouse and has been shown to abolish DNA binding and transcriptional activation in vitro while not interfering with the wild-type protein — interpreted as pure loss of function without dominant-negative effects (Qu et al. 1998). Human and murine *Alx4* homeodomains are identical and the human mutation is likely to represent, at least in certain aspects, a loss-of-function allele; nevertheless, lack of knowledge about

the *in vivo* targets of *Alx4* cannot exclude subtle dominant-negative effects and renders this suggestion provisional.

Quite apart, now that the major and consistent phenotypic features of P11pDS – long bone exostoses and PFM – have been molecularly dissected, the syndrome can join the short list of the true autosomal contiguous gene deletion syndromes (Strachan and Read 1999). The third occasional finding in P11pDS, mental retardation, awaits finer mapping. To this end, placement of the deletion in family 6, where learning disability is not a feature, in context may provide additional information.

On review, the skull phenotype associated with *ALX4* haploinsufficiency ranges from severe under-ossification in infancy – cranium bifidum – to non-penetrance, but most mutation carriers exhibit classical bilateral PFM which tend to decrease in size with age. In agreement with a previous clinical suggestion (Little et al. 1990), at least *ALX4*-caused cranium bifidum and PFM are developmental variants of the same entity, as exemplified by family 5 (Figure 3.10). A meningeal defect was the only non-osseous abnormality consistently observed. In families 5 and 3, a characteristic space occupied by cerebrospinal fluid in the posterior cranial fossa at the level of the cerebellum is associated with *ALX4* mutations; lack of brain imaging data in other cases implies that this feature may be under-diagnosed in patients with PFM — indeed, it has been documented in a P11pDS case previously (Wuyts et al. 1999). This may represent a defect in and/or abnormal insertion of the cerebellar tentorium.

Taxonomically, human *ALX4* is placed in a sub-branch of the aristaless-related family of paired-like homeoproteins (Galliot et al. 1999; Banerjee-Basu and Baxeavanis 2001): the vertebrate *Alx4*, *Cart1* and *Alx3* proteins (Meijlink et al. 1999), with the recent addition of a sea urchin homologue, *Alx1* (Ettensohn et al. 2003), define this small but distinct, deuterostome-specific group — the *Alx* group. Rat *Cart1* was cloned from a chondrosarcoma cell line (Zhao et al. 1993) and was found to be

expressed in the frontonasal process, the branchial arches, the lateral plate mesoderm and the limb buds, the somitic mesoderm; and later in the mesonephros, the lung buds, the tendons, and, characteristically, in prechondrogenic condensations and cartilaginous elements (Zhao et al. 1993; Zhao et al. 1994); the human orthologue has also been described (Gordon et al. 1996). A proportion of mice homozygous for a targeted disruption of *Cart1* may die perinatally with meroanencephaly and acrania, caused by defective closure of the neural tube (Zhao et al. 1996). Hamster *Alx3* was isolated from an insulinoma library (Rudnick et al. 1994) and mouse and human orthologues have also been reported (ten Berge et al. 1998; Wimmer et al. 2002). During mouse development *Alx3* transcripts are seen in the frontonasal process, the branchial arches, as well as in the lateral plate mesoderm; and in the limb buds, the tail, the body wall, and the genital tubercle (ten Berge et al. 1998). Contrary to the null alleles for *Alx4* and *Cart1*, homozygosity for a targeted disruption of *Alx3* in the mouse is not associated with any abnormalities (Beverdam et al. 2001). All members of the Alx group are structurally strongly related, featuring highly similar homeodomains, conserved C-terminal regions and an aristaless/OAR domain at the very end; they show significant overlap in terms of expression patterns especially during skull and limb development (Beverdam and Meijlink 2001); and demonstrate heterodimerisation potential upon DNA binding in vitro (Qu et al. 1999). Human ALX4 conforms to the structural blueprint, also possessing a rather extensive simple-sequence poly(P/Q) repeat at the N-terminus. The ALX4 homeodomain can recognise the generic core element TAAT (Tucker and Wisdom 1999) but its physiological targets remain elusive. Recent data suggest that the aristaless/OAR domain may function as a built-in attenuator for DNA binding (Brouwer et al. 2003), whereas the poly(P/Q) tract could be a protein-protein interaction module (Boras and Hamel 2002).

Evidently, *ALX4* function during human skull development is dosage-sensitive and this is also true for the mouse — the *Alx4^{lst}* allele in heterozygosity

causes a small calvarial fissure (Forsthoefel 1962). Yet, the cardinal abnormality in heterozygous mutant mice is preaxial polydactyly (Forsthoefel 1962; Qu et al. 1997b) which, on focused clinical evaluation, is absent in human mutation carriers. Hence, the impact of *Alx4* haploinsufficiency is species-specific, affecting mostly limb bud patterning in the mouse and skull ossification in humans.

The list of *MSX2* mutations in PFM (Wilkie et al. 2000; Wuyts et al. 2000b) is extended by two new entries. Both the heterozygous two-nucleotide deletion 417_418delCA and the four-nucleotide duplication 505_508dupATTG are predicted to cause frameshifts, translating as H139fsX243 and A170fsX245 respectively. If mutant proteins are synthesised, they are unlikely to be functional as either the helices II and III or the entire homeodomain are missing; additionally, the potential products in the new frame, which is common for the two mutations, share no similarity with other proteins. If transcription and translation take place, haploinsufficiency of *MSX2* is the most likely outcome, although a dominant negative effect cannot, in principle, be excluded. Alternatively, the mutant transcript and/or protein may be unstable, also leading to haploinsufficiency. The identification of an *MSX2* mutation in family 8 elucidates the molecular basis of PFMCCD and indicates that clavicular development is also sensitive to *MSX2* dosage. This is the third family with PFMCCD to be published (Eckstein and Hoare 1963; Hall 1982; Golabi et al. 1984). On the basis of true allelism with isolated PFM and given the mildness of the clavicular defects as well as the absence of other features of CCD, the designation parietal foramina with clavicular hypoplasia (PFMCH), as suggested by Golabi et al. (1984), may be a more apt heading for this syndrome.

Chapter 4

Do *ALX4* or *MSX2* Mutations Cause Craniosynostosis?

4.1 Introduction

Genetic haploinsufficiency in a biological system reflects exquisite physiological sensitivity for protein concentration and/or activity. This limited tolerance may, in turn, uncover an evolutionary constraint: protein amount and/or activity are marginally above a deficiency threshold because an excess could also be disruptive. Substantiated or presumed functional haploinsufficiency is a recurrent theme in the mutational repertoire of transcription factors involved in skeletal development (Hermanns and Lee 2001) and homeoproteins in general (D'Elia et al. 2001). On the contrary, gain-of-function, dominant-positive mutations in transcription factors that could either augment existing normal properties or confer new capabilities are anticipated to be very rare events.

Even so, both scenarios are exemplified by the mutational spectrum of the *MSX2* gene. A large deletion encompassing *MSX2* argues for true haploinsufficiency in PFM in humans (Wilkie et al. 2000). On the other hand, a unique missense mutation within the homeodomain – P148H – has been shown to segregate with a private craniosynostotic condition, the variable Boston-type craniosynostosis (Warman et al. 1993; Jabs et al. 1993), catalogued in OMIM under 604757. This variant

displays enhanced DNA binding affinity but shows no sign of altered specificity in vitro (Ma et al. 1996). Early suture closure was reproduced in mice carrying either a mutant or a wild-type transgene driven by a broadly active promoter (Liu et al. 1995); moreover, focused overexpression using a wild-type transgene under part of the native promoter resulted in obliteration of the sagittal suture — invasion of the sutural space and overlap of the parietal bones (Liu et al. 1999). Thus, dominant-negative interference is effectively ruled out in favour of a genuine dominant-positive effect.

Craniosynostosis, the premature fusion of the cranial sutures, occurring either as an isolated finding or in a syndromic context, can be viewed, to a certain extent, as a contrasting manifestation to skull ossification defects: one or more sutures fail to form at the lines of apposition of the calvarial bones, resulting in skull deformations of variable presentation and severity (Muenke and Wilkie 2001). Localised, gain-of-function, heterozygous mutations in three of the four fibroblast growth factor receptor genes (see section 1.4.2), *FGFR1–3*, and haploinsufficiency of the *TWIST1* gene, encoding a basic helix-loop-helix transcription factor (see section 1.3.2.2), cause the major craniosynostosis syndromes and account for 15–20% of total prevalence (Muenke and Wilkie 2001; Wilkie and Morriss-Kay 2001). Mutations in *FGFR2* cause the Apert and Crouzon syndromes (OMIM 101200 and 123500 respectively); Pfeiffer syndrome is due to mutations in either *FGFR2* or *FGFR1* (OMIM 101600); specific substitutions in *FGFR3* define Muenke syndrome (OMIM 602849) and Crouzon syndrome with acanthosis nigricans (also under OMIM 123500); and various loss-of-function mutations in *TWIST1* are associated with SCS (OMIM 101400). As mentioned (see section 1.3.2.2), PFM may co-exist with craniosynostosis in the broad phenotypic spectrum of SCS (Friedman et al. 1977; Thompson et al. 1984; Young and Swift 1985; Wilkie et al. 1995b).

It is likely that further genetic heterogeneity in craniosynostosis, especially in non-syndromic or atypical cases, awaits dissection. Three lines of evidence made

ALX4 a valid candidate: the established paradigm of *MSX2*; the infrequent but documented association of skull defects with craniosynostosis in SCS; and the presence of coronal synostosis in a case of P11pDS, where *ALX4* is deleted (Lorenz et al. 1990; Bartsch et al. 1996). This prompted a screen for sequence variants in a panel of selected craniosynostosis samples; at the same time *MSX2* was scrutinised for additional mutations.

4.2 Screening of *ALX4* in a Panel of Craniosynostosis Samples

A set of 211 samples from individuals with a diagnosis of either non-syndromic or, less frequently, syndromic craniosynostosis where the most common mutations in the *FGFR2*, *FGFR3*, *TWIST1*, and *FGFR1* genes had been excluded was compiled for screening. This filtration step ruled out point mutations in exon 7 of *FGFR1* (essentially the P252R substitution), exons 7 and 10 of *FGFR3* (essentially the P250R and A391E substitutions), exons 8 and 10 of *FGFR2* (the mutational hotspots of the gene) as well as point mutations in exon 1 and heterozygous deletions of *TWIST1* (Elanko et al. 2001; Kan et al. 2002). The coding regions of *ALX4* were analysed by DHPLC and, following short-listing, by sequencing; controls for the most common normal variants (which had been encountered during early investigations of the gene in the PFM families) aided selection. This approach revealed two variants of potential interest whilst consolidating the list of polymorphisms in the coding region of *ALX4*.

4.2.1 Polymorphisms

Thirteen single nucleotide variants that map within and around the coding regions of *ALX4* were detected (Table 4.1). Two frequent substitutions in exon 1 encode protein variants — R35T and P102S. Although the contribution of a variant towards the craniosynostotic phenotype cannot be fully assessed in this type of study, it

Table 4.1: Sequence Polymorphisms in *ALX4*

Nucleotide Change	Protein Variation	Frequency Estimate ^a	dbSNP Reference
63C→T	—	rare	—
69G→C	—	very rare	—
104G→C	R35T	0.48 (0.37–0.58)	rs3824915
304C→T	P102S	0.43 (0.32–0.53)	—
594C→A	—	<0.03	—
729G→A	—	0.08 (0.04–0.16)	—
778-11G→A	—	0.06 (0.02–0.13)	—
879C→T	—	0.07 (0.03–0.14)	—
906+32C→T	—	very rare	—
1074C→T	—	0.30 (0.21–0.40)	rs3802805
1282G→A	—	very rare	—
1392G→A	—	very rare	—
1464C→T	—	0.25 (0.16–0.34)	rs4755798

Notes: Variants were identified during screening of the PFM and/or the craniosynostosis sample collections. A quantitative estimate is mentioned for the changes that were evaluated against a panel of at least 94 chromosomes from normal individuals; from this group, only 594C→A was not represented in the control set, but was found in two families and is not segregating with skull defects. All five others were encountered more than once in the craniosynostosis sample collection (no scoring in a control population was carried out) and, with the exception of 63C→T and 69G→C, reside in non-coding parts of the gene. Frequency values refer to the less common allele. The dbSNP cluster identifier (Molecular Databases at NCBI — see appendix F), if the variant has been catalogued, is also cited. Accession numbers for reference sequences: AJ279074, AJ279075, AJ279076 and AJ279077.

^a $P=0.95$ for the quantitative estimates.

seems unlikely that these changes have major pathogenic effects. Several variants are common with the rare allele frequency being above 1%, by convention defined as the threshold for polymorphisms. All rare or very rare variants appear neutral on the protein level and are not predicted to alter splicing signals.

4.2.2 Ambiguous Protein Variants

Two unique nucleotide variants, predicted to result in protein changes that cannot readily be considered neutral, were ascertained (Table 4.2).

The first one, a T→G transversion at position 605, encoding L202W, was identified in a patient with apparently sporadic craniosynostosis and a clinical dia-

Table 4.2: Equivocal *ALX4* Variants in Craniosynostosis

Nucleotide Change	Protein Variation	Comments
605T→G	L202W	Not represented in 168 normal chromosomes; residue not highly conserved
314_325delCGCAGCCGCAGC	P105_Q108del	Detected in reportedly unaffected parent

Notes: The 605T→G transversion maps just upstream of the homeodomain while the 314_325 in-frame deletion causes contraction of the poly(P/Q) tract in the N-terminus. Accession numbers for reference sequences: AJ279074, AJ279075, AJ279076 and AJ279077.

gnosis of Crouzon syndrome (Figure 4.1); further genetic analysis was precluded by the unavailability of parental samples. Leucine 202 is located at position -12 relative to the homeodomain and is not conserved in the vertebrate *Alx* proteins, even within *ALX4* orthologues themselves. However, it is replaced by methionine, glycine, or proline (Figure 3.5) and the 605T→G change was not found in 168 chromosomes from normal controls, suggesting a population frequency of less than 0.02 (at $P=0.95$).

The second variant is a 12 bp deletion that removes two consecutive proline-glutamine doublets from the N-terminal poly(P/Q) tract — namely 314_325delCGCAGCCGCAGC, resulting in P105_Q108del. The proband, affected with isolated uni-coronal synostosis, inherited the deletion from the reputedly unaffected father (Figure 4.2). Although the change was not evaluated against a panel of normal controls, there was no indication of repeat instability from the analysis of all remaining samples of the craniosynostosis panel itself. This low-complexity part of *ALX4* shows little evolutionary conservation but the human repeat has expanded when compared to the mouse, rat, and chicken equivalents (Figure 3.5).

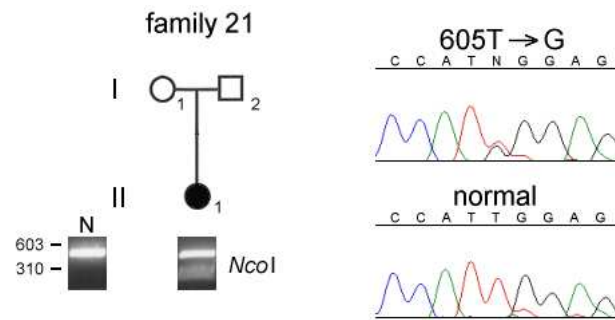


Figure 4.1: Identification of the ambiguous 605T→G substitution, encoding L202W, in an apparently sporadic case of craniosynostosis with a clinical diagnosis of Crouzon syndrome; samples from the parents were not available for analysis. The second exon of *ALX4* was amplified and cut with *NcoI*. A new site created by the variation splits the 417 bp product in two fragments of 206 and 211 bp, visible as a single fuzzy band. N, normal control. The pedigree represents family 21 (see appendix B and section 2.2).

4.3 Screening of *MSX2* in a Panel of Craniosynostosis samples

An analogous screen for *MSX2* detected no suspicious variants — only polymorphisms were identified.

4.3.1 Polymorphisms

All three polymorphisms that were encountered within and around the *MSX2* coding region are listed in Table 4.3. The protein variant T129M is not infrequent and has been reported before (Jabs et al. 1993).

4.4 Summary

This investigation of a substantial number of selected samples from craniosynostosis patients with no mutations in the established hotspots of the major loci — *FGFR1–3* and *TWIST1* — failed to identify any clearly pathogenic variation in the coding regions of *ALX4* and *MSX2*, especially within their homeodomains or the

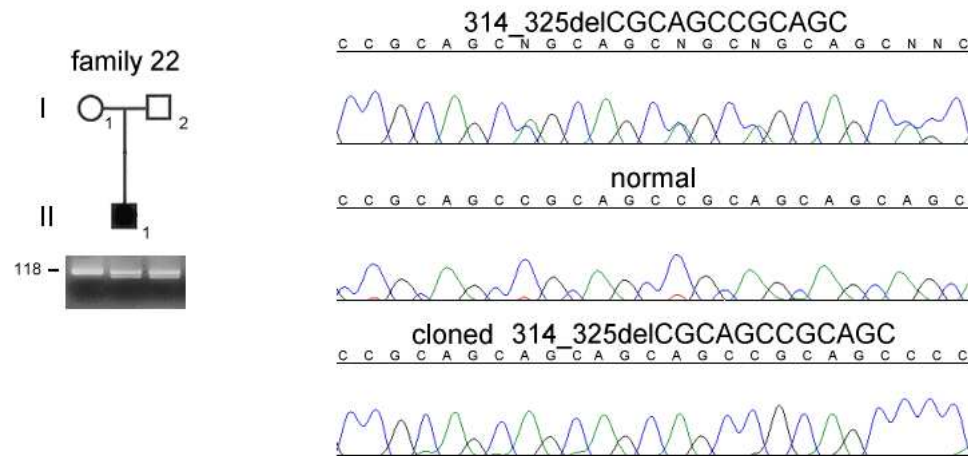


Figure 4.2: Identification of the ambiguous 12 bp deletion 314_325delICGCAGCCGCGCAGC, encoding P105_Q108del, in an apparently sporadic case of uni-coronal synostosis. The change is also present in the reputedly unaffected father. The poly(P/Q) run in the first exon of *ALX4* was amplified and resolved. The deletion homoduplex is visible as a 105 bp fragment, just below an upper band representing either the pure 117 bp-long normal amplicon or its mixture with the heteroduplex. The sequence panel illustrates, in addition to the heterozygous variant and a normal control, the cloned deletion. The pedigree depicts family 22 (see appendix B and section 2.2).

aristaless/OAR domain of *ALX4*. Nevertheless, it produced a comprehensive list of the polymorphic positions within and around the coding regions of the two genes which may facilitate future mutational studies; part of the repertoire of polymorphisms in *ALX4* and *MSX2* was uncovered independently (Verdyck et al. 2003).

MSX2 is a minor locus for craniosynostosis and its implication is of mechanistic and historical rather than of epidemiological interest. Similarly, based on this screen, it is highly unlikely that *ALX4* mutations account for a significant fraction of craniosynostosis cases. A less deterministic involvement cannot be ruled out though. Certain *ALX4* sequence variants – especially the ones encoding protein variants – may be preferentially associated with craniosynostosis, acting as

Table 4.3: Sequence Polymorphisms in *MSX2*

Nucleotide Change	Protein Variation	Frequency Estimate ^a	dbSNP Reference
-17C→G	–	0.07 (0.03–0.15)	rs4647952
379+59A→G	–	common	–
386C→T	T129M	0.06 (0.02–0.13)	rs4242182

Notes: Variants were identified during screening of the PFM and/or the craniosynostosis sample collections. A quantitative estimate is mentioned for the two changes that were evaluated against a panel of at least 86 chromosomes from normal individuals. The third one maps within the intron and was encountered frequently in the craniosynostosis sample collection. Frequency values refer to the less common allele. The dbSNP cluster identifier (Molecular Databases at NCBI — see appendix F), if the variant has been catalogued, is also cited. Accession numbers for reference sequences: L22498 and L22499.

^a $P=0.95$ for the quantitative estimates.

penetrance and/or expressivity modifiers for causative mutations. This question can be addressed with a population case-control study or by applying a transmission disequilibrium test in selected kindreds. It is hard to exclude categorically any pathogenic effect of the two ambiguous *ALX4* variants identified. The L202W change replaces a small aliphatic side chain with the bulky aromatic residue of tryptophan at the N-terminal homeodomain extension and is expected to be very rare. However, the lack of evolutionary conservation and the nature of the residues found in *ALX4* homologues indicate that this part of the protein may be structurally flexible. Whether it represents a de novo change or not is an open question — unfortunately, it proved impossible to re-establish contact with the family and obtain parental samples. Yet, re-examination of the available medical history pointed to a different etiology and mode of inheritance: both the proband and an affected sibling, also showing mild craniosynostosis, had Marfanoid connective tissue lesions whilst a consanguineous loop in the pedigree became apparent. The in-frame deletion in the poly(P/Q) repeat cannot be a fully penetrant causative change. However, the repeat is the interface for binding by the transcription factor LEF1 (Boras and

Hamel 2002) and incidences of reduced penetrance for *FGFR2* and *FGFR3* mutations are known (Robin et al. 1998; Johnson et al. 2000b).

Chapter 5

Expression Patterns of *Alx4* & *Msx2* During Skull Development

5.1 Introduction

The skull vault defects caused by *Alx4* and *Msx2* mutations in humans and mice involve, at least in the early stages, both the frontal and the parietal bones; gradually, the unossified space shrinks under circumferential bone growth (Qu et al. 1997b; Satokata et al. 2000; Wilkie et al. 2000). This could imply not just spatial and temporal co-expression of the two genes in the calvaria but a critical role in determining the growth rate of the osteogenic fronts while not being essential for terminal osteoblast differentiation.

Indeed, *Msx2* expression has been documented in the osteogenic mesenchyme at E12.5 and, subsequently, in the growing parietal bones, including the sagittal suture, between E15 and up to birth (MacKenzie et al. 1992; Jabs et al. 1993; Kim et al. 1998; Liu et al. 1999). Briefly, *Msx2* transcripts are seen in the mid-sutural mesenchyme, the underlying dura mater, and the approximating fronts of the parietal bones in the embryonic stages; the gene is down-regulated after birth as the suture matures — reviewed by Opperman (2000). In fact, its pattern of expression in the bones becomes discontinuous, suggesting restriction in individual cells (Kim et al. 1998). Limited data for *Alx4* during calvarial development indicate expres-

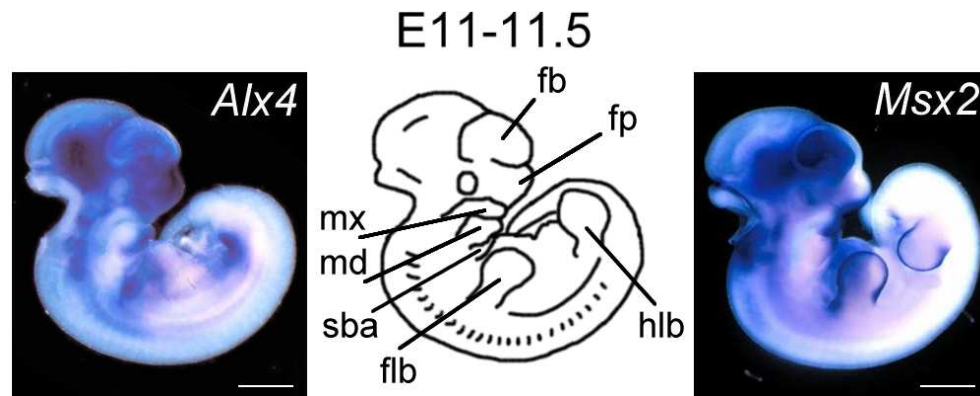


Figure 5.1: Expression patterns of *Alx4* and *Msx2* in the mouse embryo at E11–11.5. Apart from the growing limb buds, the frontonasal process, and the branchial arches, both genes are expressed in the cephalic region, outlining a domain posteriorly and dorsally to the eye. Staining in the lateral brain ventricles is an artefact, caused by probe trapping. Whole-mount in situ hybridisation using digoxigenin-labelled antisense probes, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense probes produced no significant staining (not shown). Scale bar: ~ 1 mm. fb, forebrain; flb, forelimb bud; fp, frontonasal process; hlb, hindlimb bud; md, mandibular process; mx, maxillary process; sba, second branchial arch.

sion in the frontal and parietal bones and, possibly, in the dura mater (Hudson et al. 1998).

In order to assess the expression patterns of *Alx4* and *Msx2* during skull development, a comprehensive comparative analysis by in situ hybridisation in whole-mount embryos and on sets of transverse and coronal frozen sections was undertaken. The starting point was set at E11–11.5, before the appearance of pre-cartilaginous condensations, while ending at E16.5–17, when the majority of the adult skeletal elements are recognisable (Kaufman 1992; Kaufman and Bard 1999). Three genes were employed as markers, to facilitate interpretation: *Runx2* encodes the transcription factor Cbfa1 which is indispensable for osteogenesis (see section 1.4.7) and provides an early marker for the osteoblast lineage (Komori 2002); *Spp1* directs production of osteopontin, a matrix glycoprotein characteristic of mature bone (Butler 1989); and *Col2a1*, specifying the $\alpha 1$ chain of type II procollagen, serves

as an early and abundant – though not exclusive, as a splice variant is expressed in non-cartilaginous mesenchyme (Lui et al. 1995) – marker for cartilage (Zhao et al. 1997). This approach had several goals: (1) to trace globally the expression of *Alx4* itself during a critical time window for skull development; (2) to enrich the body of expression data for *Msx2* in the calvaria, by including the coronal suture; (3) to compare and contrast directly the expression patterns of the two genes; (4) and finally, to gain insights about the subpopulation(s) where either/both *Alx4* and *Msx2* are active.

5.2 Expression at E11–11.5

At embryonic day E11–11.5, *Alx4* is characteristically expressed at the anterior aspect of the limb buds, the frontonasal process, and at the mandibular and maxillary components of the first branchial arch (Qu et al. 1997b; Qu et al. 1997a; Beverdam and Meijlink 2001). Similarly, as anticipated (Bendall and Abate-Shen 2000), *Msx2* is expressed along the margins of the limb buds (possibly representing activity in the AER and the progress zone) and, also, at the edges of the frontonasal, maxillary, and mandibular processes (Figure 5.1).

However, staining for both transcripts was seen in a domain dorsally and posteriorly to the eye, not reported before. Sectioning excluded probe trapping and revealed expression in a layer of mesenchyme, just beneath the ectoderm, that extends to the level of the mesencephalon (Figure 5.2).

5.3 Expression at E12.5–13

All five genes were analysed in serial transverse (Figure 5.3) and coronal (Figure 5.4) sections. At E12.5–13, chondrification has commenced or is just about to take place in several elements: Meckel's cartilage, the nasal capsule-prechordal cartilages, the anlage of the body of the sphenoid – fused hypophyseal cartilages –, the anlage of a

E11-11.5 transverse sections

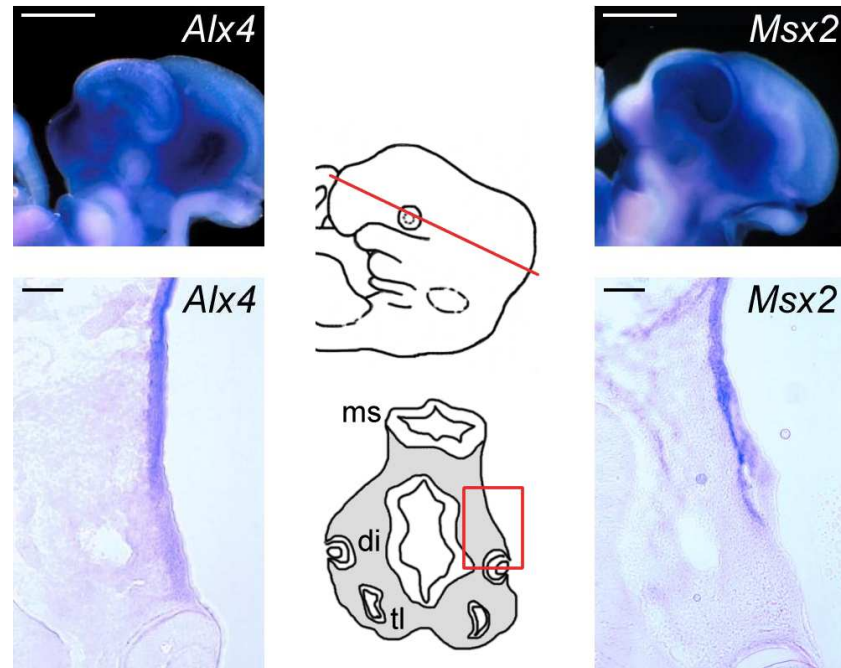


Figure 5.2: Expression patterns of *Alx4* and *Msx2* in the mouse head at E11–11.5 — transverse sections. *Upper panels* show magnifications of Figure 5.1. Transverse sections of hybridised specimens (*lower panels*) reveal expression of both genes in a layer of mesenchyme adjacent to the surface ectoderm. Whole-mount in situ hybridisation using digoxigenin-labelled antisense probes, followed by immunohistochemical detection employing NBT/BCIP, and sectioning. Control hybridisations using sense probes produced no significant staining (not shown). Scale bars: ~ 1 mm (upper panels) and 0.1 mm (lower panels). di, diencephalon; ms, mesencephalon; tl, telencephalon.

part of the orbitosphenoid, the alisphenoid cartilages, the otic capsule and the surrounding cartilaginous masses, as well as the occipital ones (coalesced parachordal cartilages and sclerotomes of the occipital somites) — all are highlighted by *Col2a1* expression. *Runx2* transcripts are present in the future nasal bone and the future mandible and maxilla, including the upper molar tooth primordium; and in part of the future orbitosphenoid bone, possibly in/around the occipital cartilages and, importantly, in the osteogenic ectomeninx surrounding the brain.

Alx4 is active not only in a subset of *Runx2*-positive structures, namely the ectomeninx, the nasal bone, the emerging symphysis menti between the two parts of the future mandible along the ventral midline, and an arc-shaped domain posteriorly to the eye of unclear destiny, but also in other regions, not associated with bone formation. These include the growing eyelid and a domain just ventrally to the eye that could correspond to a future facial muscle. The expression pattern for *Msx2* shares three regions in common with *Alx4*: the ventromedial parts of the future mandible, the ectomeninx, and the prominent arc behind the eye. In addition, *Msx2* transcripts were detected in the upper molar tooth primordium as well as in the alisphenoid, temporal and occipital cartilages. As the expression levels of both *Alx4* and *Msx2* in the thin membranous layer of the ectomeninx are relatively low, a panel of high-power comparable photomicrographs that cover both the future frontal and parietal regions is shown in Figure 5.5. The zone of continuous *Runx2* expression demarcates the ectomeninx, located laterally to the orbitosphenoid and alisphenoid/temporal cartilages. *Alx4* transcripts are confined within this layer, while *Msx2* expression spreads to the adjoining cartilage.

5.4 Expression at E14.5–15

In a similar approach, two sets of five sections in the horizontal plane and five in the coronal plane were analysed for all five genes at E14.5–15 (Figures 5.6 and 5.7). At this stage the first signs of ossification are evident as *Spp1* transcripts mark the

E12.5-13 transverse sections

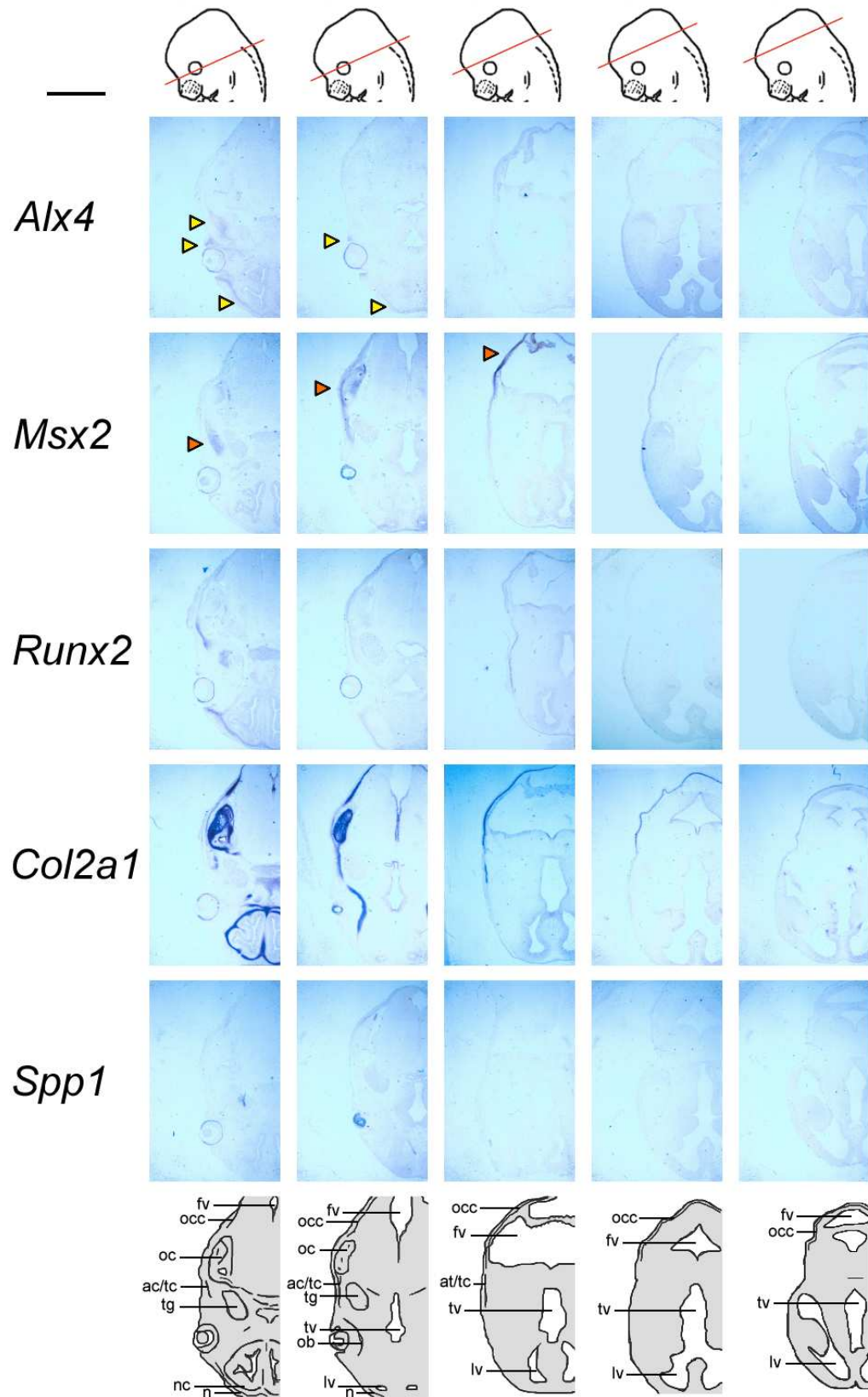
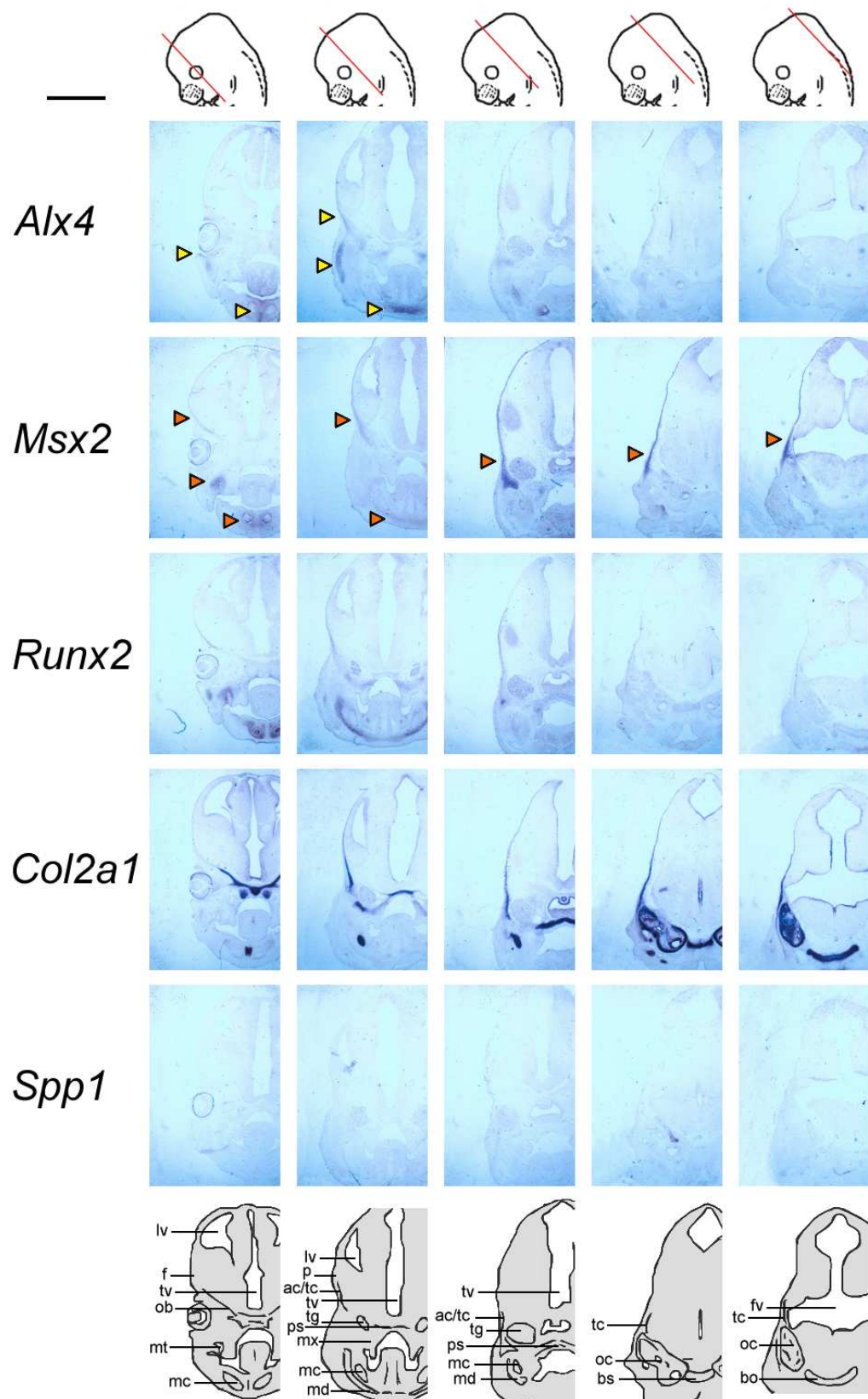


Figure 5.3: *Previous page.* Expression patterns of *Alx4* and *Msx2* in the mouse skull at E12.5–13 compared to control genes — serial transverse sections. Both *Alx4* and *Msx2* are expressed in an arc, posteriorly to the eye. Only *Alx4* transcripts are present in the growing eyelid and the future nasal bone while *Msx2* is active in the alisphenoid/temporal and occipital cartilages. Visualisation of the retina is due to natural pigmentation. *Yellow arrowheads* mark *Alx4*-expressing structures; *red arrowheads* mark *Msx2*-expressing ones. Approximate planes of sections are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 1 mm. Selected anatomical landmarks: ac, alisphenoid cartilage; fv, fourth ventricle; lv, lateral ventricle; n, nasal bone primordium; nc, nasal capsule; oc, otic capsule; occ, occipital cartilage; tc, temporal cartilage; tg, trigeminal ganglion; tv, third ventricle.

Figure 5.4: *Next page.* Expression patterns of *Alx4* and *Msx2* in the mouse skull at E12.5–13 compared to control genes — serial coronal sections. Both *Alx4* and *Msx2* are expressed in the future mandible and in the osteogenic layer that will form the frontal and parietal bones — documented in detail in Figure 5.5. Only *Alx4* transcripts are detected in the growing eyelid and, additionally, outline a domain posteriorly and ventrally to the eye that may correspond to the future zygomatic bone and/or to a facial muscle. *Msx2* is active in the alisphenoid/temporal and occipital cartilages as well as in the tooth primordium. Visualisation of the retina is due to natural pigmentation. *Yellow arrowheads* mark *Alx4*-expressing structures; *red arrowheads* mark *Msx2*-expressing ones. Approximate planes of sections are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 1 mm. Selected anatomical landmarks: ac, alisphenoid cartilage; bo, basioccipital cartilage; bs, basisphenoid cartilage; f, frontal bone primordium; fv, fourth ventricle; lv, lateral ventricle; mc, Meckel's cartilage; md, mandible primordium; mt, molar tooth; mx, maxilla primordium; ob, orbitosphenoid cartilage; oc, otic capsule; p, parietal bone primordium; tc, temporal cartilage; tg, trigeminal ganglion; tv, third ventricle.

E12.5-13 coronal sections



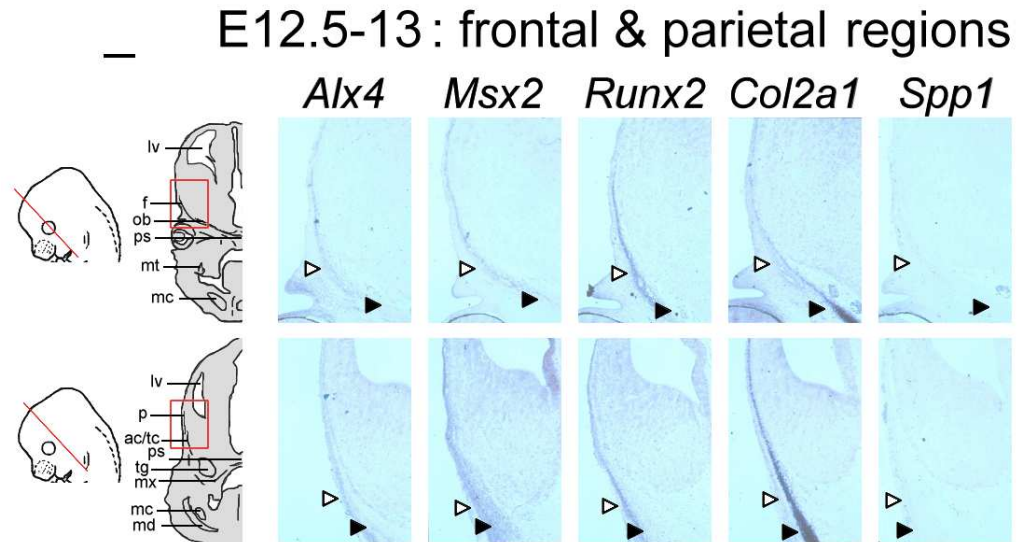


Figure 5.5: Expression patterns of *Alx4* and *Msx2* in the mouse skull at E12.5–13 compared to control genes — coronal sections, focusing on the future frontal (*upper row*) and parietal areas (*lower row*). *Alx4* and *Msx2* are expressed in the osteogenic membrane that is undergoing condensation, eventually to shape into the frontal and parietal bones as well as the dura. *Alx4* transcription is restricted to this structure while *Msx2* is also active in the adjacent alisphenoid/temporal cartilages. *White arrowheads* indicate the prospective frontal and parietal bones while *black arrowheads* point to the underlying cartilaginous elements — orbitosphenoid and alisphenoid/temporal respectively. Approximate planes of sections and areas of cover are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 0.2 mm. Selected anatomical landmarks: ac, alisphenoid cartilage; f, frontal bone primordium; lv, lateral ventricle; mc, Meckel's cartilage; md, mandible; mt, molar tooth; mx, maxilla primordium; ob, orbitosphenoid cartilage; p, parietal bone primordium; ps, presphenoid cartilage; tc, temporal cartilage; tg, trigeminal ganglion.

palatine shelves of the maxilla and the inferior parts of both the frontal and parietal bones, while another focus is seen in the medial segment of the basioccipital. *Col2a1* expression is wide: it highlights the nasal capsule, Meckel's cartilage, the anlage of a part of the orbitosphenoid, the alisphenoid cartilage, the anlagen of the presphenoid and the basisphenoid, the otic capsule and the surrounding temporal cartilages, the anlage of the basioccipital, and the cartilages of the supraoccipital/interparietal region. Few spots that are not apparently associated with cartilaginous structures, as in the maxilla, are likely to reflect weak expression in mesenchyme. *Runx2* is also widely and relatively intensely expressed, mostly in structures undergoing ossification: in the mandible, the maxilla (including the molar tooth primordium), the future turbinate bones, the frontal and the parietal bones, the future tympanic ring, and the central part of the basioccipital bone.

Both *Alx4* and *Msx2* are active in the newly-ossified inferior parts of the frontal and parietal bones as well as in the mandible and maxilla; a small shared domain of expression was also seen in the tympanic ring. As in the earlier stage, only *Msx2* is expressed in the posterior cartilaginous structures and sole *Alx4* transcripts were seen not only in the future nasal and turbinate bones but also in the growing eyelid, the extrinsic ocular muscles, and, interestingly, in the perpendicular fold of the dura mater into the brain, the falx cerebri. A closer examination of the growing frontal and parietal bones for *Alx4* and *Msx2* expression (high-power photomicrographs in Figure 5.8), revealed a discontinuous, patched pattern in the already trabeculated inferior part of the frontal bone, and either confinement to small islands or weak uniform expression in the less-developed parietal bone — probably depending on the particular domain represented in the sections. The prospective coronal suture, presenting as a gap in *Runx2* expression, displays a smooth pattern with less intense expression for both genes. As previously, *Msx2* transcripts were seen in the adjoining alisphenoid cartilage. An analogous picture emerged after focusing on the developing mandible and maxilla (Figure 5.9): *Alx4* and *Msx2* tran-

E14.5-15 transverse sections

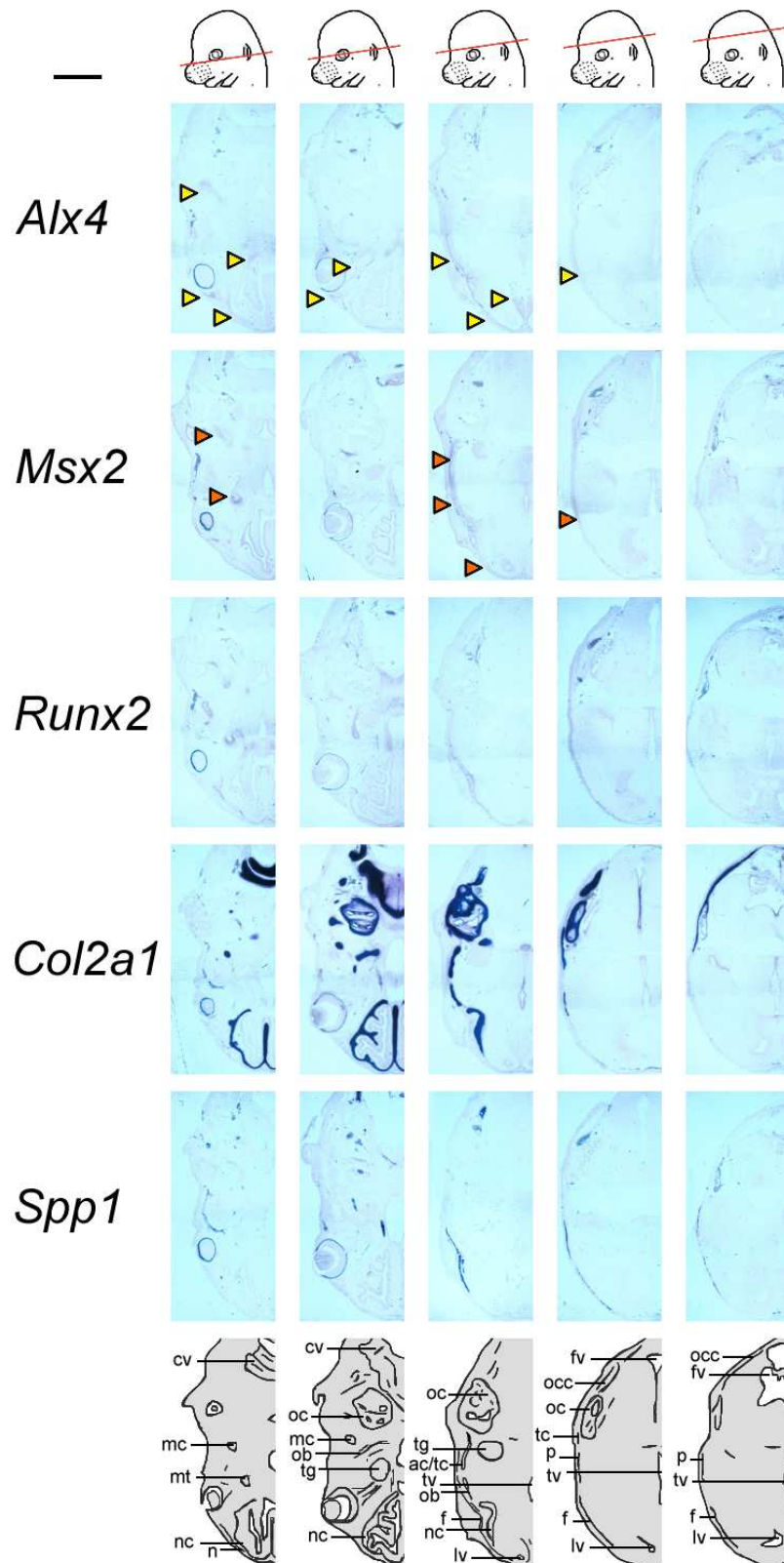
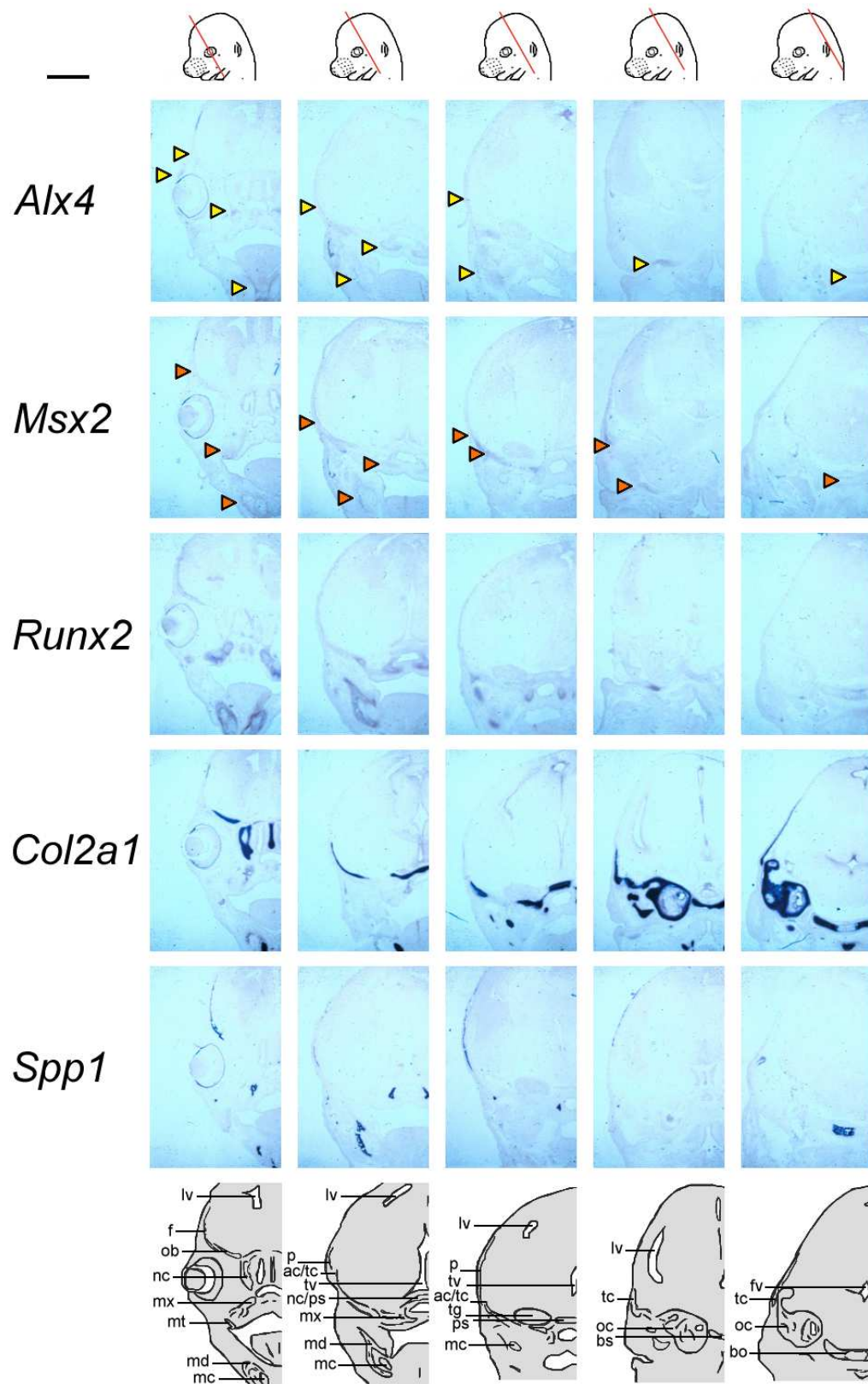


Figure 5.6: *Previous page.* Expression patterns of *Alx4* and *Msx2* in the mouse skull at E14.5–15 compared to control genes — serial transverse sections. Both *Alx4* and *Msx2* are active in the inferior part of the ossifying frontal and parietal bones — shown in detail in Figure 5.8. In addition, *Alx4* transcripts are seen in the growing eyelid, the extrinsic ocular muscles, the future nasal and turbinate bones, and the falx cerebri. *Msx2* is also expressed in the alisphenoid/temporal cartilages as well as in the tooth primordium. An arc just ventrally to the otic capsule, outlined by expression of both genes, may be part of the future tympanic ring. Visualisation of the retina is due to natural pigmentation; staining of the vessels is an artefact. *Yellow arrowheads* mark *Alx4*-expressing structures; *red arrowheads* mark *Msx2*-expressing ones. Approximate planes of sections are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 1 mm. Selected anatomical landmarks: ac, alisphenoid cartilage; cv, cervical vertebrae; f, frontal bone; fv, fourth ventricle; lv, lateral ventricle; mc, Meckel's cartilage; mt, molar tooth; n, nasal bone primordium; nc, nasal capsule; ob, orbitosphenoid cartilage; oc, otic capsule; occ, occipital cartilage; p, parietal bone; tc, temporal cartilage; tg, trigeminal ganglion; tv, third ventricle.

Figure 5.7: *Next page.* Expression patterns of *Alx4* and *Msx2* in the mouse skull at E14.5–15 compared to control genes — serial coronal sections. Both genes are active in structures undergoing ossification: the frontal and parietal bones, the mandible, the maxilla, and the basioccipital bone — shown in detail in Figures 5.8, 5.9, and 5.10. *Alx4* transcripts are also present in the eyelid and the future turbinate bones. *Msx2* is also expressed in the alisphenoid/temporal and occipital cartilages and the tooth primordium. A domain below the otic capsule, where both genes are expressed, could be the future tympanic ring. Visualisation of the retina is due to natural pigmentation. *Yellow arrowheads* mark *Alx4*-expressing structures; *red arrowheads* mark *Msx2*-expressing ones. Approximate planes of sections are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 1 mm. Selected anatomical landmarks: bo, basioccipital bone/cartilage; ac, alisphenoid cartilage; bs, basisphenoid cartilage; f, frontal bone; fv, fourth ventricle; lv, lateral ventricle; mc, Meckel's cartilage; md, mandible; mt, molar tooth; mx, maxilla; nc, nasal capsule; ob, orbitosphenoid cartilage; oc, otic capsule; p, parietal bone; tc, temporal cartilage; tg, trigeminal ganglion; tv, third ventricle.

E14.5-15 coronal sections



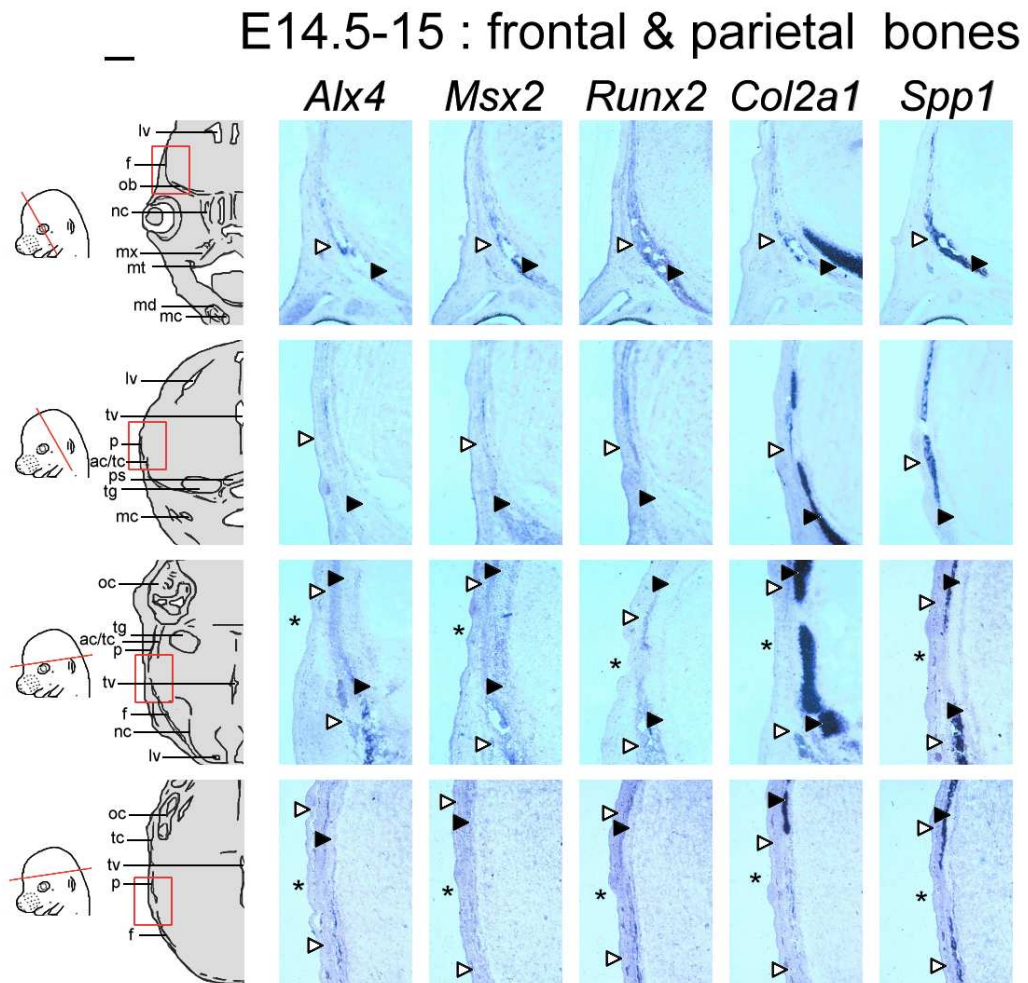


Figure 5.8: Expression patterns of *Alx4* and *Msx2* in the mouse skull at E14.5–15 compared to control genes — coronal sections, focusing on the frontal (*first row*) and parietal bones (*second row*) as well as transverse sections encompassing the future coronal suture (*third and fourth rows*). Individual cells within/around the growing osseous trabeculae express *Alx4* and *Msx2*, producing a speckled pattern which blurs into uniformity in immature regions. *Msx2* is also active in the underlying alisphenoid/temporal cartilages. *White arrowheads* indicate the frontal and parietal bones while *black arrowheads* point to the underlying cartilaginous elements — orbitosphenoid and alisphenoid/temporal respectively. The location of the future coronal suture is marked by a *star*. Approximate planes of sections and areas of cover are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 0.2 mm. Selected anatomical landmarks: ac, alisphenoid cartilage; f, frontal bone; lv, lateral ventricle; mc, Meckel's cartilage; md, mandible; mt, molar tooth; mx, maxilla; nc, nasal capsule; ob, orbitosphenoid cartilage; oc, otic capsule; p, parietal bone; tc, temporal cartilage; tg, trigeminal ganglion; tv, third ventricle.

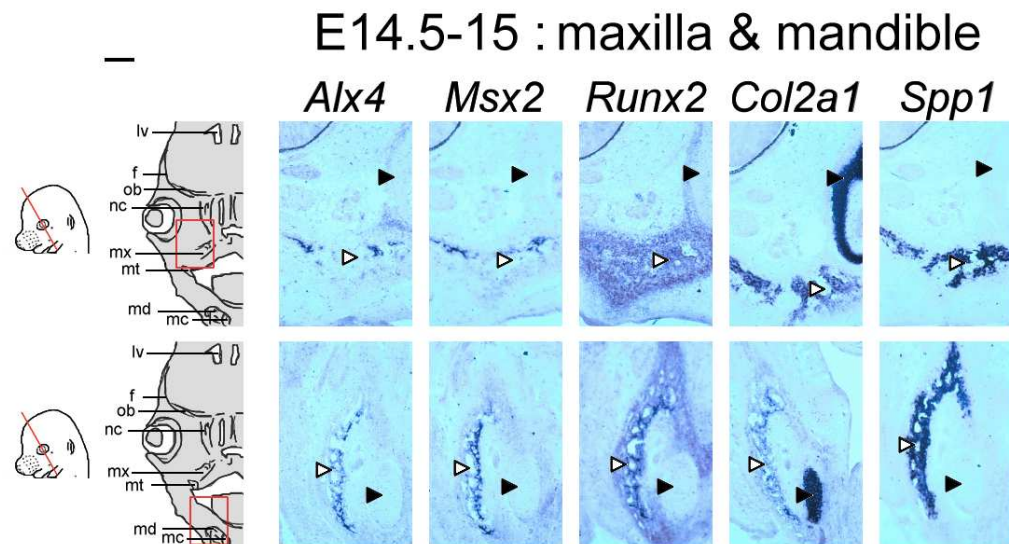


Figure 5.9: Expression patterns of *Alx4* and *Msx2* in the mouse skull at E14.5–15 compared to control genes — coronal sections, focusing on the maxilla (*upper row*) and mandible (*lower row*). *Alx4* and *Msx2* expression highlights a cell population near the edges of the developing bones. *White arrowheads* indicate the growing maxillary and mandibular trabeculae while *black arrowheads* point to the adjacent cartilaginous elements — nasal capsule and Meckel's cartilage respectively. Approximate plane of section and areas of cover are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 0.2 mm. Selected anatomical landmarks: f, frontal bone; lv, lateral ventricle; mc, Meckel's cartilage; md, mandible; mt, molar tooth; mx, maxilla; nc, nasal capsule; ob, orbitosphenoid cartilage.

E14.5-15 : basioccipital bone

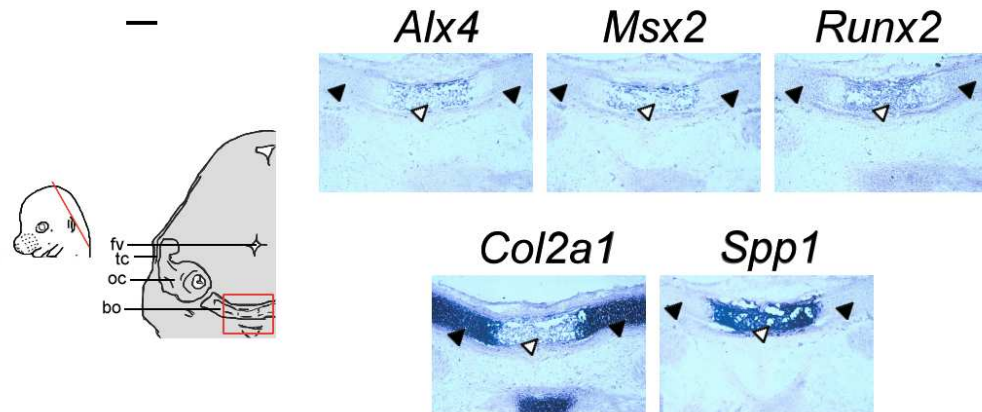


Figure 5.10: Expression patterns of *Alx4* and *Msx2* in the mouse skull at E14.5–15 compared to control genes — coronal section, focusing on the basioccipital bone. Distinct cells within/around the osseous centre, where cartilage has been resorbed, express both *Alx4* and *Msx2*. *White arrowheads* indicate the newly-ossified medial part while *black arrowheads* point to the still cartilaginous lateral segments. Approximate plane of section and area of cover are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within the set ranges from 80 to 100 μm . Scale bar: ~ 0.2 mm. Selected anatomical landmarks: bo, basioccipital bone/cartilage; fv, fourth ventricle; oc, otic capsule; tc, temporal cartilage.

scripts are less widely distributed compared to *Runx2* and, in particular, define a group of cells close to the edges of the growing bones. Relatively weak expression of both genes, also in speckled fashion, was seen in the medial part of the basioccipital, where cartilage is being resorbed and replaced by bone (Figure 5.10).

5.5 Expression at E16.5–17

Finally, at E16.5–17 (Figures 5.11 and 5.12) ossification has progressed substantially and *Spp1* expression defines several bones, including the nasal, the zygomatic, the maxilla, the mandible, part of the orbitosphenoid, the alisphenoid, the frontal, the parietal, part of the temporal, the auditory ossicles, the medial segment of the basioccipital, and the interparietal. *Runx2* shows a grossly similar pattern, with the addition of the upper and lower molar teeth primordia. However, many structures of the skull base remain cartilaginous: *Col2a1* transcripts are present in the nasal capsule, the anlage of a part of the orbitosphenoid, the anlagen of the presphenoid and basisphenoid, the condylar process of the mandible, the otic capsule; and in the anlage of a part of the temporal, the anlagen of the lateral segments of the basioccipital, and the cartilages in the supraoccipital/interparietal region. As previously, relatively weak expression was seen in few non-cartilaginous structures.

Both *Alx4* and *Msx2* are down-regulated at this stage in the frontal and parietal bones as well as in the maxilla and mandible (Figures 5.13 and 5.14); new sites of combined expression were seen in the interparietal bone and, notably, in the developing hair follicles.

5.6 Summary

Taken together, the data for *Alx4* could support a multiphasic model of expression during skull development in mesenchymal cells of diverse origin. The location of the stripe at the E11–11.5 head defined by *Alx4* (and *Msx2*) transcripts suggests partial derivation from mesoderm and likely participation in an epithelial-

E16.5-17 transverse sections

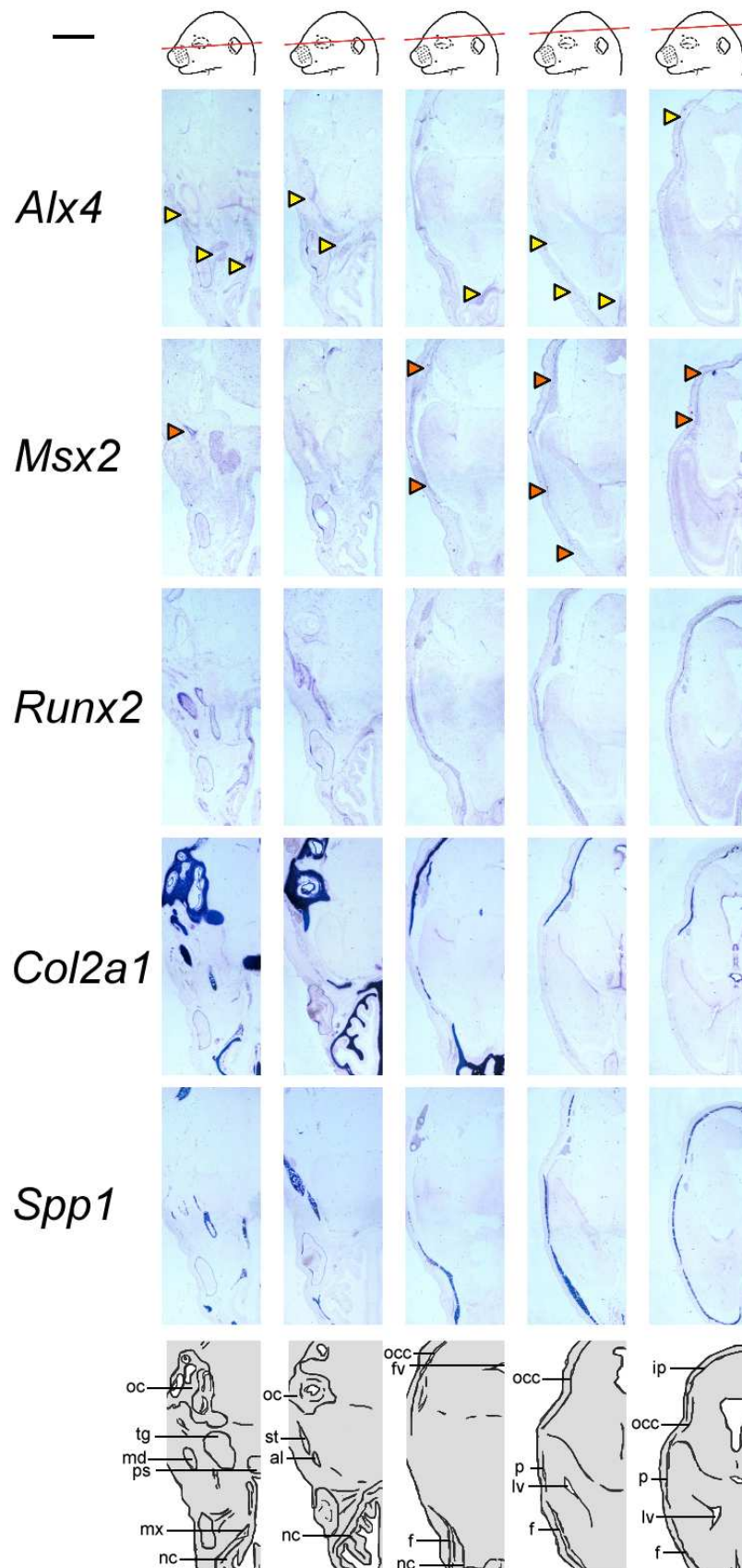
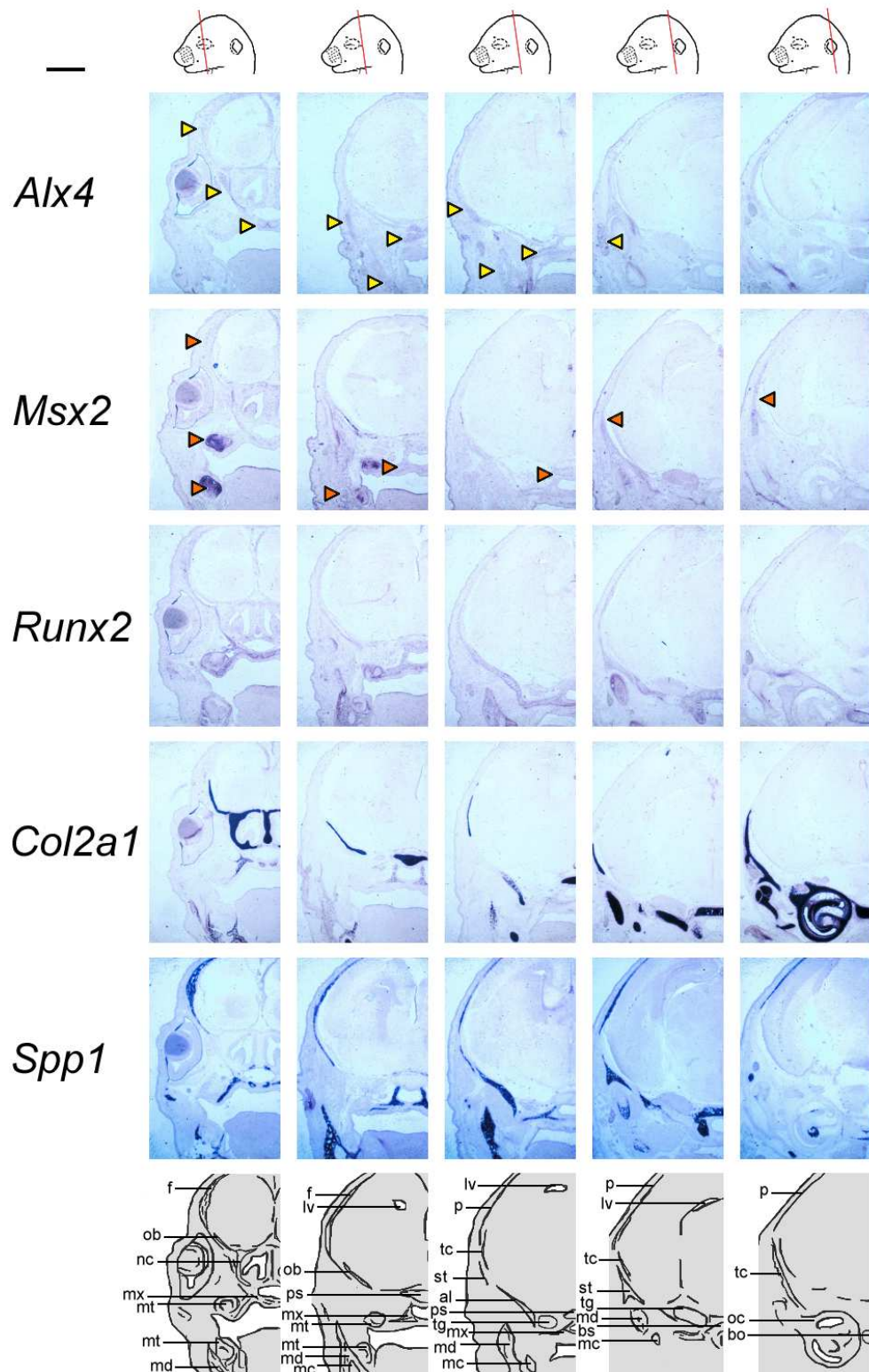


Figure 5.11: *Previous page.* Expression patterns of *Alx4* and *Msx2* in the mouse skull at E16.5–17 compared to control genes — serial transverse sections. Both genes show reduced expression levels in the frontal and parietal bones (details in Figure 5.13). New sites of activity are the interparietal bone and the hair follicles. *Alx4* is weakly expressed in the mandible and maxilla, the zygomatic process, the extrinsic ocular muscles, and the falx cerebri. *Msx2* is solely expressed in the alisphenoid/temporal and occipital cartilages; a domain laterally to the trigeminal ganglion could correspond to the auditory ossicles. Visualisation of the retina is due to natural pigmentation; staining of the vessels is an artefact. *Yellow arrowheads* mark *Alx4*-expressing structures; *red arrowheads* mark *Msx2*-expressing ones. Approximate planes of sections are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 1 mm. Selected anatomical landmarks: al, alisphenoid bone; f, frontal bone; fv, fourth ventricle; ip, interparietal bone; lv, lateral ventricle; md, mandible; mx, maxilla; nc, nasal capsule; oc, otic capsule; occ, occipital cartilage; p, parietal bone; ps, presphenoid cartilage; st, squamous temporal bone; tg, trigeminal ganglion.

Figure 5.12: *Next page.* Expression patterns of *Alx4* and *Msx2* in the mouse skull at E16.5–17 compared to control genes — serial coronal sections. *Alx4* and *Msx2* expression has decreased significantly in the frontal and parietal bones as well as in the mandible and the maxilla — demonstrated in detail in Figures 5.13 and 5.14. *Alx4* transcripts are present in the future turbinate bones, the zygomatic process, and the parotid glands. *Msx2* is also expressed in the teeth primordia and the temporal cartilages. Visualisation of the retina is due to natural pigmentation. *Yellow arrowheads* mark *Alx4*-expressing structures; *red arrowheads* mark *Msx2*-expressing ones. Approximate planes of sections are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 1 mm. Selected anatomical landmarks: al, alisphenoid bone; bo, basioccipital bone/cartilage; bs, basisphenoid bone/cartilage; f, frontal bone; lv, lateral ventricle; mc, Meckel's cartilage; md, mandible; mt, molar tooth; mx, maxilla; nc, nasal capsule; ob, orbitosphenoid cartilage; oc, otic capsule; p, parietal bone; ps, presphenoid cartilage; st, squamous temporal bone; tc, temporoparietal cartilage; tg, trigeminal ganglion.



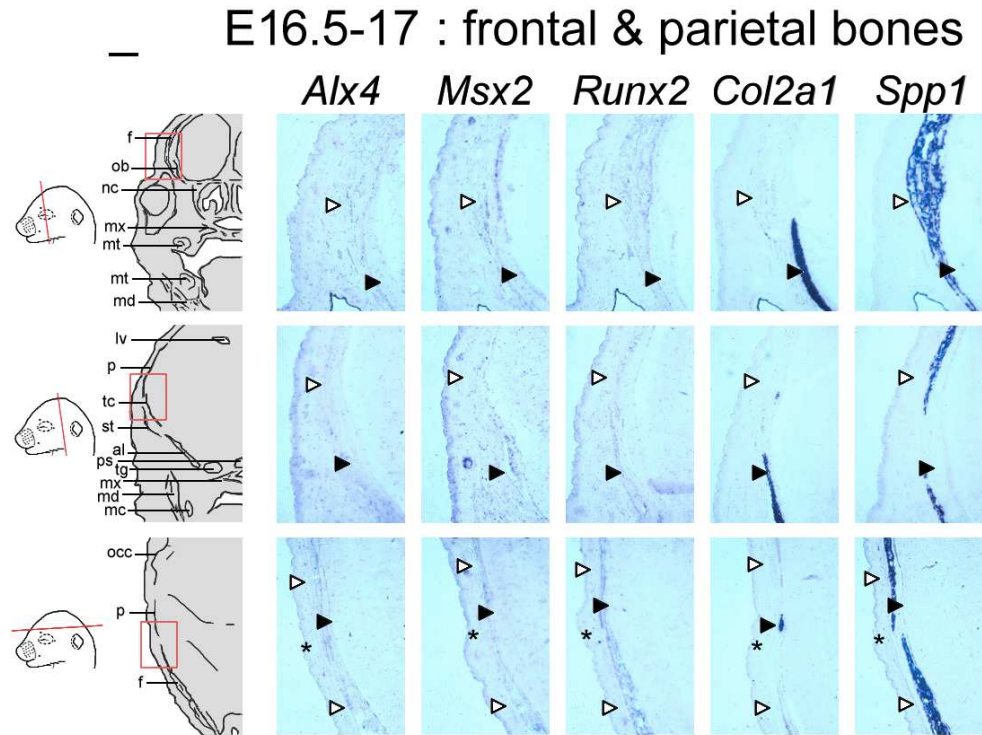


Figure 5.13: Expression patterns of *Alx4* and *Msx2* in the mouse skull at E16.5-17 compared to control genes — coronal sections, focusing on the frontal (*first row*) and parietal bones (*second row*) as well as transverse sections encompassing the future coronal suture (*third row*). *Alx4* and *Msx2* transcription has declined. Their expression is just above background in cells within the frontal bone, being slightly more intense in the parietal bone (transverse section) and the coronal suture. *White arrowheads* indicate the frontal and parietal bones while *black arrowheads* point to the underlying cartilaginous elements — orbitosphenoid and alisphenoid/temporal respectively. The location of the future coronal suture is marked by a *star*. Approximate planes of sections and areas of cover are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 0.2 mm. Selected anatomical landmarks: al, alisphenoid bone; bo, basioccipital bone/cartilage; bs, basisphenoid bone/cartilage; f, frontal bone; lv, lateral ventricle; mc, Meckel's cartilage; md, mandible; mt, molar tooth; mx, maxilla; nc, nasal capsule; ob, orbitosphenoid cartilage; p, parietal bone; ps, presphenoid cartilage; st, squamous temporal bone; tc, temporal cartilage; tg, trigeminal ganglion.

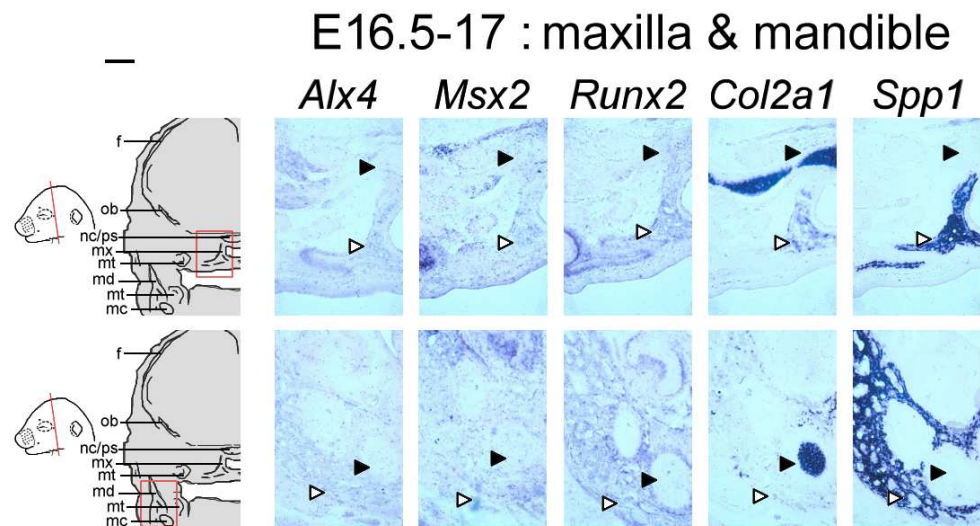


Figure 5.14: Expression patterns of *Alx4* and *Msx2* in the mouse skull at E16.5–17 compared to control genes — coronal sections, focusing on the maxilla (*upper row*) and mandible (*lower row*). *Alx4* and *Msx2* are down-regulated. Very weak expression persists in cells within the mandible and within/close to the edges of the maxilla. *White arrowheads* indicate the growing maxillary and mandibular trabeculae while *black arrowheads* point to the adjacent cartilaginous elements — nasal capsule/presphenoid cartilage and Meckel's cartilage respectively. Approximate plane of section and areas of cover are indicated in the schemes, derived from Kaufman (1992). In situ hybridisation using digoxigenin-labelled antisense probes on frozen sections, followed by immunohistochemical detection employing NBT/BCIP. Control hybridisations using sense *Alx4* and *Msx2* probes produced no significant staining (not shown). Each section is 20 μm thick and the average distance within each set ranges from 80 to 100 μm . Scale bar: ~ 0.2 mm. Selected anatomical landmarks: f, frontal bone; mc, Meckel's cartilage; md, mandible; mt, molar tooth; mx, maxilla; nc, nasal capsule; ob, orbitosphenoid cartilage; ps, presphenoid cartilage.

mesenchymal interplay during head expansion — as for the limb buds and the craniofacial processes at this stage. In principle, other cells may start expressing *Alx4* soon afterwards deeper in the mesenchyme, but it would not be unreasonable to envisage that this cell population could contribute to the primitive ectomeninx, condensing around the neural tube; partial spatial continuity of expression with a domain posteriorly to the eye at the E12.5–13 head could support this assumption. The ectomeninx, where both *Alx4* and *Msx2* are specifically expressed, gives rise to the calvarial bones as well as the dura mater and has a definitive contribution from the neural crest; *Runx2* expression implies that it contains cells already committed to the osteoblast fate. Later, when the first depositions of osteoid are evident, the gradual restriction of *Alx4* (and *Msx2*) expression in spots within and around the growing dermal – and, mostly, neural crest-derived – bones from E14.5–15 onwards (exemplified by the thicker mandible) would be compatible with exclusion from the increasing number of osteoblasts and confinement in undifferentiated pre-osteoblasts or their precursors. Notably, an indistinguishable pattern is observed in the medial part of the basioccipital bone when endochondral ossification commences; however, this segment is clearly of mesodermal (cephalic or somitic) origin. When compared, the expression patterns for both *Alx4* and *Msx2* share several developing skull elements in common, including the frontal and parietal bones as well as the coronal suture, but also show significant differences. Only *Msx2* is expressed in cartilaginous structures; on the other hand, *Alx4* transcripts are detected in non-osseous tissues, as in the ocular muscles, the eyelid, and the dura mater.

Chapter 6

Potential Interactions between *Alx4* & *Msx2*: the Genetic Level

6.1 Introduction

The phenotypic overlap of *Alx4* and *Msx2* mutations in humans and mice and, importantly, the coincidental expression of the two genes during calvarial development, described in chapter 5, raise a plausible question: do they interact in regulation of cell processes such as precursor proliferation, differentiation, or survival? *Alx4* and *Msx2* may interact hierarchically in linear pathway(s), by influencing the expression of each other; they could participate in signalling loops between cell groups; or, they may control synergistically the expression of target genes. Alternatively, they could act in parallel pathways, involving either common or distinct downstream genes, that somehow converge and regulate cell behaviour.

Any significant interplay between *Alx4* and *Msx2* during skull development is likely to be reflected at the genetic level, presenting broadly as non-additivity of the individual phenotypes in compound mutants. The genetic approach is facilitated by two facts: firstly, the calvarial defects caused by *Alx4* and *Msx2* mutations in mice are not identical; and secondly, in heterozygosity the *Msx2* mutation is non-penetrant. Double homozygotes for the mutant alleles may show a multiplicative outcome – disproportionate deterioration of the defects –, reveal

epistatic relationships – full or partial masking of one phenotype by the other –, or exhibit combinatorial effects, exposing novel anomalies in the skull or in other systems. The *Msx* mutation in heterozygosity could act as a modifier when combined with a manifesting status in the *Alx4* locus, accentuating or altering the existing abnormalities; or, as previously, the association could uncover new anomalies. Naturally, such analysis presupposes comprehensive phenotypic accounts of the single mutants for *Alx4* and *Msx2*.

Four *Alx4* mutant alleles are available: *Alx4^{lst}*, harbouring a R206Q substitution (Qu et al. 1998), equivalent to the human mutation R218Q — see sections 3.2.4.1 and 3.2.4.5; *Alx4^{lst-J}*, caused by an intrahomebox 16 bp deletion (Qu et al. 1998; Takahashi et al. 1998); *Alx4^{tm1Rwi}*, a targeted disruption (Qu et al. 1997b); and *Alx4^{lst-Alb}*, a large induced deletion spanning the *Alx4* locus (Qu et al. 1998). However, sufficiently detailed morphological assessments have been published only for animals carrying the classical Strong's luxoid allele *Alx4^{lst}* (Strong and Hardy 1956; Strong 1961; Forsthoefel 1962; Forsthoefel 1963; Forsthoefel et al. 1966) and the engineered mutation *Alx4^{tm1Rwi}* (Qu et al. 1997b). On the original so-called DP background – a mixture of the CBA, N, JK (overall 50%), and C57 (50%) strains – *Alx4^{lst/+}* produces hindlimb preaxial polydactyly and a small sutural interfrontal, bone; the penetrance decreases slightly on the C57 background. On the same background, *Alx4^{lst/lst}* homozygotes show multiple abnormalities, but with variable expressivity. Development is globally retarded and the spectrum of the skeletal patterning anomalies and growth defects includes: preaxial polydactyly of the autopod in all four limbs usually with appearance of posterior-like, triphalangeal digits with extra metatarsals and metacarpals, reduction and partial duplication of the anterior element of the zeugopod (i.e. the radius or tibia), absence of the deltoid crest of the humerus, missing condyles of the femur, hypoplasia of the pubis, skull vault defects, occasional coronal synostosis, compression of the anterior skull base, and an overall distorted skull shape, featuring an abnormally low

length : width ratio. Partial hemimelia strains the remaining long bone, resulting in enlargement and bending of ulna and fibula — presenting as 'luxation'. As previously, the penetrance of the polydactyly as well as of the radial and tibial duplications drops on transfer to the C57 strain. The proportions of the brain parts are modified, the cerebral hemispheres protrude through the skull opening, and a lobule of the cerebellar hemispheres is missing. Furthermore, the eyelids are open and there is temporary dorsal alopecia. The male and female external genitalia are abnormally shaped and the umbilicus is misplaced due to a generalised defect in the posterior part of the abdominal body wall; in fact, the weak body wall may tear apart during birth, explaining the under-representation of homozygotes in litters. Nevertheless, the gonads are functional in both sexes and the pronounced infertility is due to copulation difficulties. Consistent with the effect of genetic background, selection experiments underscored the sensitivity of both the heterozygous and homozygous phenotypes to modifiers (Forsthoefel 1968). Heterozygotes for the *Alx4^{tm1Rwi}* allele on the 129/SvEvTac background appear normal, but when out-crossed to C57BL/6J, hindlimb polydactyly emerges. *Alx4^{tm1Rwi/tm1Rwi}* animals are rarely born alive, as the severe ventral body wall defect causes herniation either in utero or perinatally. The pattern of preaxial polydactyly of all four limbs is comparable to that caused by the *Alx4^{lst}* allele, and a wide midline gap is present in the skull vault at birth which eventually heals spontaneously. Shortening of the radius or tibia is not apparent, although other features might have been unnoticed. In the original studies, Forsthoefel (1963) attributed the calvarial defects in the homozygous mutant to the pressures exerted by the expanding brain which cannot be accommodated in the reduced anterior fossa, pushing the bone plates apart. Current evidence does not support this view. PFM in heterozygous human carriers, the normal anterior skull base of *Alx4^{tm1Rwi/tm1Rwi}* animals, as well as the continuing bone growth that fills the gap suggest delayed and/or deficient ossification as the primary cause — and not just mechanical distortion.

Animals heterozygous for the targeted disruption of *Msx2*, *Msx2^{tm1 Rilm}*, are reportedly normal. In *Msx2^{tm1 Rilm/tm1 Rilm}* homozygotes growth is globally delayed and pleiotropic defects of the skeleton and the skin appendages (Satokata et al. 2000; Ma et al. 2003) are notable. The skull shows a midline ellipsoid hiatus at the interface of the frontal and parietal bones and hypoplasia of the interparietal and supraoccipital bones; in addition, the long bones of the axial and appendicular skeleton are reduced in size because of defective proliferation at the epiphyseal growth plates. In the brain, a lobule of the vermis of the cerebellum is absent and certain cell layers fail to form; later in life, the animals demonstrate seizure-like episodes. Teeth develop, but the incisors are brittle and misaligned whereas the molars degenerate, making the animals incapable of chewing solid food; the defect is traced to ameloblast death and poor accumulation of enamel. Early hair loss is followed by out-of-phase cycles of growth and regeneration, presenting as pelage patchiness; moreover, the hair is structurally abnormal. Mammary gland development is arrested at the sprout stage and no branching of the epithelial invagination occurs. The background influence – analysis in mixed BALB/c, mixed C57BL/6J, and inbred 129/Sv strains – on the expressivity of the *Msx2^{tm1 Rilm}* allele appears negligible.

Genetic interaction studies have been performed for *Msx2* and *Msx1* (Satokata et al. 2000) and for all possible combinations of the *Alx* group members: *Alx4* and *Cart1* (Qu et al. 1999), *Alx4* and *Alx3* (Beverdam et al. 2001), *Cart1* and *Alx3* (Beverdam 2001). However, no genetic analysis has been reported on any *Alx* and *Msx* double mutant. In the following sections, the morphological findings on the *Alx4* ; *Msx2* compound mutant mice are described and certain inferences are pointed out. A complementary idea was also investigated: whether common protein variants in *ALX4* or *MSX2* could act as reciprocal penetrance modifiers for PFM in humans.

Table 6.1: Offspring from the $Alx4^{+/-}; Msx2^{+/-} \times Alx4^{+/-}; Msx2^{+/-}$ Cross

Genotype	Number per Stage of Typing						Total	Ratio(%)
	E16.5–17.5	P0	P1	P2	P4	P6		
$Alx4^{+/+}; Msx2^{+/+}$	2	6	–	5	–	1	14	11.5
$Alx4^{-/-}; Msx2^{-/-}$	1	2	–	–	–	–	3	2.5
$Alx4^{-/-}; Msx2^{+/+}$	1	3	–	2	–	1	7	5.7
$Alx4^{+/+}; Msx2^{-/-}$	–	1	–	1	–	–	2	1.6
$Alx4^{+/-}; Msx2^{-/-}$	5	3	–	4	–	3	15	12.3
$Alx4^{-/-}; Msx2^{+/-}$	3	6	–	2	–	–	11	9.0
$Alx4^{+/-}; Msx2^{+/+}$	6	8	1	4	–	–	19	15.6
$Alx4^{+/+}; Msx2^{+/-}$	1	12	1	2	1	2	19	15.6
$Alx4^{+/-}; Msx2^{+/-}$	3	14	1	12	1	1	32	26.2
Total	22	55	3	32	2	8	122	100

Notes: Overall, 122 late embryos and neonates were unambiguously characterised. All nine possible genotypic classes are present, but single $Msx2^{-/-}$ homozygotes and double $Alx4^{-/-}; Msx2^{-/-}$ homozygotes appear to be under-represented.

6.2 The $Alx4^{+/-}; Msx2^{+/-} \times Alx4^{+/-}; Msx2^{+/-}$ Cross

The $Alx4^{tm1Rwi}$ allele (Qu et al. 1997b), hereafter $Alx4^{-}$, was obtained in a 129 background while the $Msx2^{tm1Rilm}$ allele (Satokata et al. 2000), hereafter $Msx2^{-}$, was made available in a mixed background, approximately 80% : 20% C57BL/6 : BALB/c. Heterozygotes for either mutation were mated with corresponding local wild-type 129 and C57BL/6 strains, and the animals used in subsequent experimental crosses had been generated through, on average, five matings with pure strains; for the $Msx2^{+/-}$ line, this had brought the C57BL/6 background representation to over 99%. A colony of double heterozygotes was established by crossing the single heterozygotes, employing male $Alx4^{+/-}$ and female $Msx2^{+/-}$ animals and vice versa. The two genes are not linked – $Alx4$ maps on chromosome 2; $Msx2$ on chromosome 13 – (Mouse Genome Informatics at the Jackson Laboratory — see appendix F) and double heterozygotes were produced with the anticipated frequency

Table 6.2: χ^2 Test on the $Alx4^{+/-}; Msx2^{+/-} \times Alx4^{+/-}; Msx2^{+/-}$ Cross

Genotype	Observed Number	Estimated Number	χ^2 values
$Alx4^{+/+}; Msx2^{+/+}$	12	6.25	5.29
$Alx4^{-/-}; Msx2^{-/-}$	2	6.25	4.08
$Alx4^{-/-}; Msx2^{+/+}$	6	6.25	
$Alx4^{+/+}; Msx2^{-/-}$	2	6.25	
$Alx4^{+/-}; Msx2^{-/-}$	10	12.5	0.50
$Alx4^{-/-}; Msx2^{+/-}$	8	12.5	1.62
$Alx4^{+/-}; Msx2^{+/+}$	13	12.5	0.02
$Alx4^{+/+}; Msx2^{+/-}$	18	12.5	2.42
$Alx4^{+/-}; Msx2^{+/-}$	29	25	0.64
Total	100	100	14.57

Notes: χ^2 test, for compliance with Mendelian dihybrid genotypic ratios, on the set of 100 newborn offspring obtained from the $Alx4^{+/-}; Msx2^{+/-} \times Alx4^{+/-}; Msx2^{+/-}$ cross. A cumulative χ^2 value of 14.57, corresponding to a probability value of just under 0.025, confirms the deviation seen on inspection of Table 6.1. Disproportionally few $Msx2^{-/-}$ homozygotes and double $Alx4^{-/-}; Msx2^{-/-}$ homozygotes, as well as an excess of wild-type animals contribute significantly to this distortion. Data for the late embryonic stages were excluded in order to focus on animals that survive up to birth. The three rarest classes, comprising the single and double null homozygotes, are merged for analysis.

of around 0.25. The animals showed no gross morphological anomalies and were fertile.

First generation double heterozygotes were crossed and all possible combinations of genotypes were obtained (Table 6.1) over several litters, although a discrepancy (significant at $P = 0.025$) with Mendelian dihybrid ratios was observed (Table 6.2). Only two $Alx4^{-/-}; Msx2^{-/-}$ double mutant homozygotes and two $Alx4^{+/+}; Msx2^{-/-}$ single mutant homozygotes were found among 100 newborns; on the other hand, wild-type animals were over-represented. These figures could indicate partial embryonic and/or perinatal lethality for the mutant genotypes in this mixed, practically 50% : 50% 129 : C57BL/6, background. However, figures

for the more frequent $Alx4^{+/-}$; $Msx2^{-/-}$ combination do not exclude a deviation within sampling error for all $Msx2^{-/-}$ homozygotes.

Newborn mice carrying the $Msx2^{-/-}$ genotype were smaller and growth was apparently retarded, whereas the ones bearing the $Alx4^{-/-}$ genotype had typical polydactyly in all four limbs and often showed bilateral encephalocele-like bulging in the anterior frontal region. The two $Alx4^{-/-}$; $Msx2^{-/-}$ double homozygotes were already dead when collected. Both were strikingly small, with a rather narrow trunk but without apparent abdominal herniation. Lethality during late gestation and/or at birth seems likely; consistently, the genotype was encountered once in a set of 22 embryos sampled between E16.5–E17.5 (Table 6.1).

6.3 Skull Defects in $Alx4$; $Msx2$ Compound Mutants

At least two skulls from each genotypic class were examined after dual bone/cartilage staining with Alizarin Red and Alcian Blue respectively at P0. Genotyping was in accordance with two diagnostic features at this stage: hindlimb and forelimb polydactyly for the $Alx4^{-/-}$ status, and extreme delay in the supraoccipital ossification centres for the $Msx2^{-/-}$ status (Figure 6.2).

The skull base appeared unaffected even in the double homozygous mutant (Figure 6.3), and the facial elements seemed, also, grossly normal in all combinations of mutations (Figure 6.2). Skull vault defects of variable severity were evident, culminating in the double homozygous mutant (Figure 6.1). They are illustrated in Figure 6.4 and their interpretation will focus on three points: (1) the nature and extent of the calvarial defects in the double $Alx4^{-/-}$; $Msx2^{-/-}$ homozygote; (2) the influence of $Alx4^{+/-}$ heterozygosity on the presentation of $Msx2$ -caused vault defects; (3) and reciprocally, the influence of $Msx2^{+/-}$ heterozygosity on the presentation of $Alx4$ -associated vault defects.

The skull vault phenotypes associated with $Alx4^{-/-}$ and $Msx2^{-/-}$ homozygosity are distinct. The former is a long midline defect, involving both the frontal

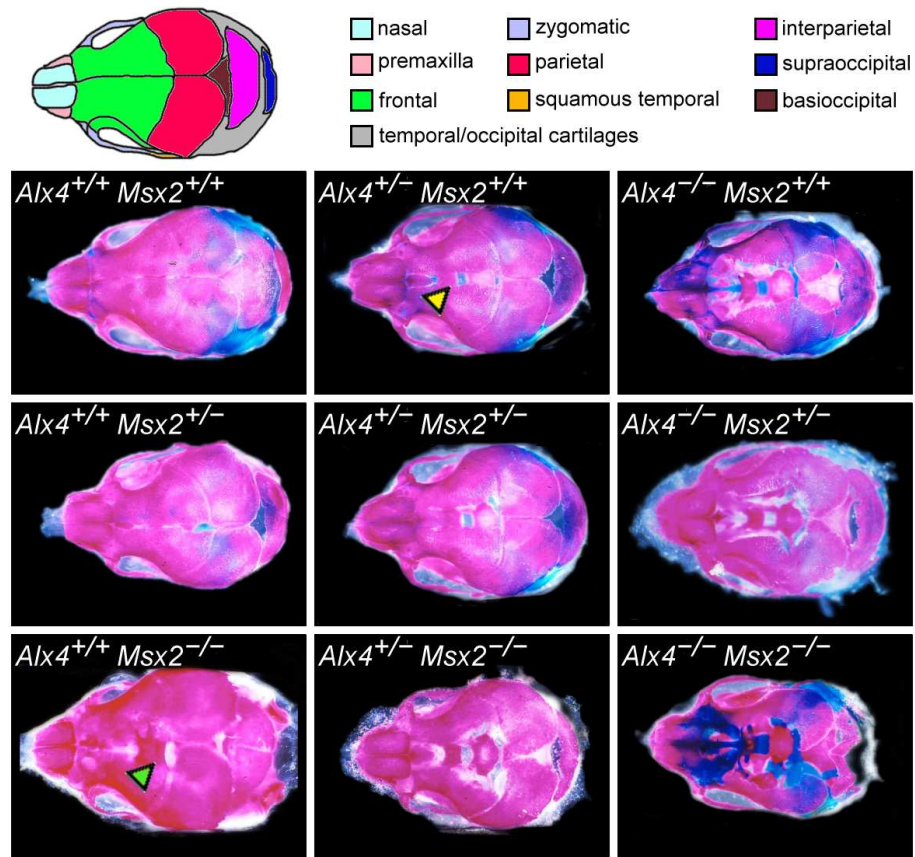


Figure 6.1: Representative skull phenotypes of newborn mice at P0, carrying all nine combinations of wild-type and mutant *Alx4* and *Msx2* alleles — dorsal views. At this stage in the wild-type the paired frontal and parietal bones have approached, shaping the metopic and sagittal sutures, and only the triangular posterior fontanelle remains open. Ablation of one *Alx4* allele causes a narrow midline gap (*yellow arrowhead*) in the frontal region, often accompanied by an intrasutural bone. On the contrary, the skull vault of the *Msx2*^{+/-} animal is normal. In the double heterozygote, *Alx4*^{+/-} ; *Msx2*^{+/-}, the defect is, on average, aggravated. The *Alx4*^{-/-} homozygote presents with a substantial defect, spanning both the frontal and the parietal bones and extending anteriorly to the line of articulation with the nasal bones. The *Msx2*^{-/-} homozygote displays a wide opening in front of the sagittal suture (*green arrowhead*), reminiscent of a persistent anterior fontanelle. The *Alx4*^{-/-} ; *Msx2*^{+/-} compound genotype results in a manifestation grossly resembling that of *Alx4*^{-/-} but with increased involvement of the posterior frontal region. The reciprocal combination, *Alx4*^{+/-} ; *Msx2*^{-/-}, is associated with a mixed, slightly worse presentation compared to *Msx2*^{-/-} homozygote. Finally, in the double null homozygote, *Alx4*^{-/-} ; *Msx2*^{-/-}, only the infero-lateral parts of the frontal and parietal bones have ossified, outlining a severe vault defect; additionally, the coronal suture is not visible. Dissected skulls, double-stained with Alizarin Red (bone) and Alcian Blue (cartilage); elements of the skull base appear, out of focus, though the openings. Variability in cartilage staining is a technical artefact and does not correlate with the extent of chondrification. The *Alx4*^{+/-} ; *Msx2*^{-/-} skull is of advanced biological age, and homozygosity for the mutant *Alx4* allele is associated with reduced overall size. A schematic representation of the vaults only, omitting the basal structures to facilitate interpretation, is shown in Figure 6.4

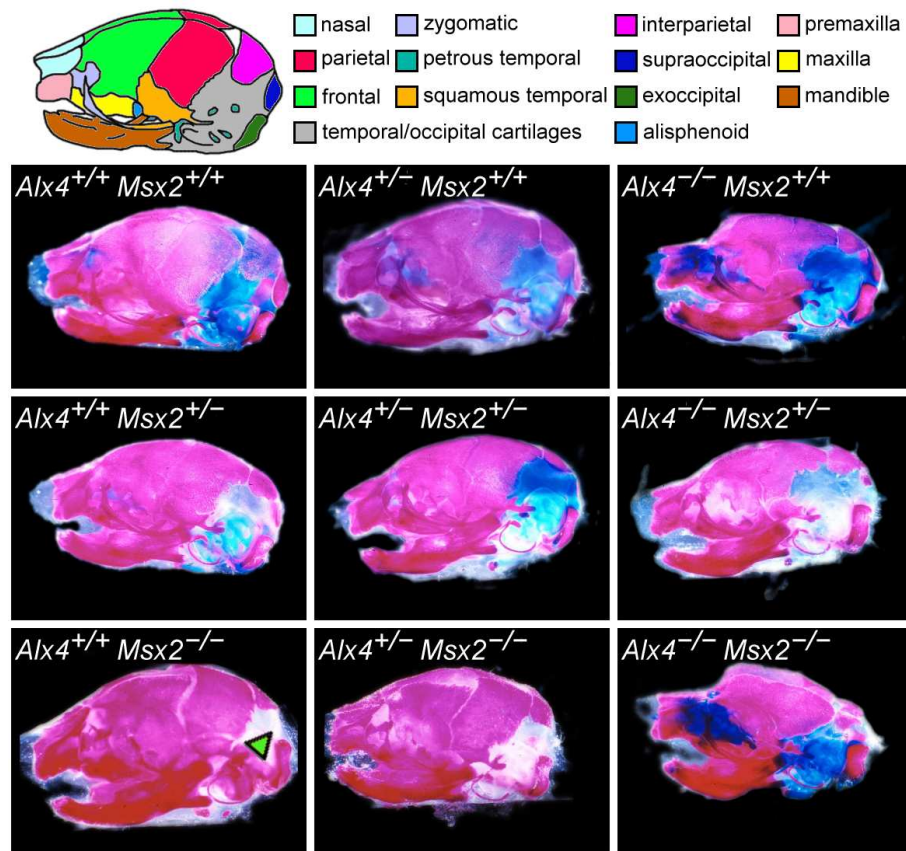


Figure 6.2: Representative skull phenotypes of newborn mice at P0, carrying all nine combinations of wild-type and mutant *Alx4* and *Msx2* alleles — lateral views. In the mutants with severe vault defects the contour of the skull is distorted, but the only additional abnormality is the absence of the supraoccipital bone (*green arrow-head*) in animals that carry the *Msx2*^{-/-} genotype. The premaxilla and mandible, the zygomatic bone, and the temporal bone are unremarkable. Dissected skulls, double-stained with Alizarin Red (bone) and Alcian Blue (cartilage). Variability in cartilage staining is a technical artefact and does not correlate with the extent of chondrification. The *Alx4*^{+/+} ; *Msx2*^{-/-} skull is of advanced biological age and homozygosity for the mutant *Alx4* allele is associated with reduced overall size.

and parietal bones, bound anteriorly by the nasal bones while ending posteriorly at the interparietal bone; moreover, its width increases anteriorly. The latter is an oval or ellipsoid gap in the posterior frontal region, just in front of the sagittal suture, diminishing anteriorly. The *Alx4*^{-/-} ; *Msx2*^{-/-} skull showed consistently, in

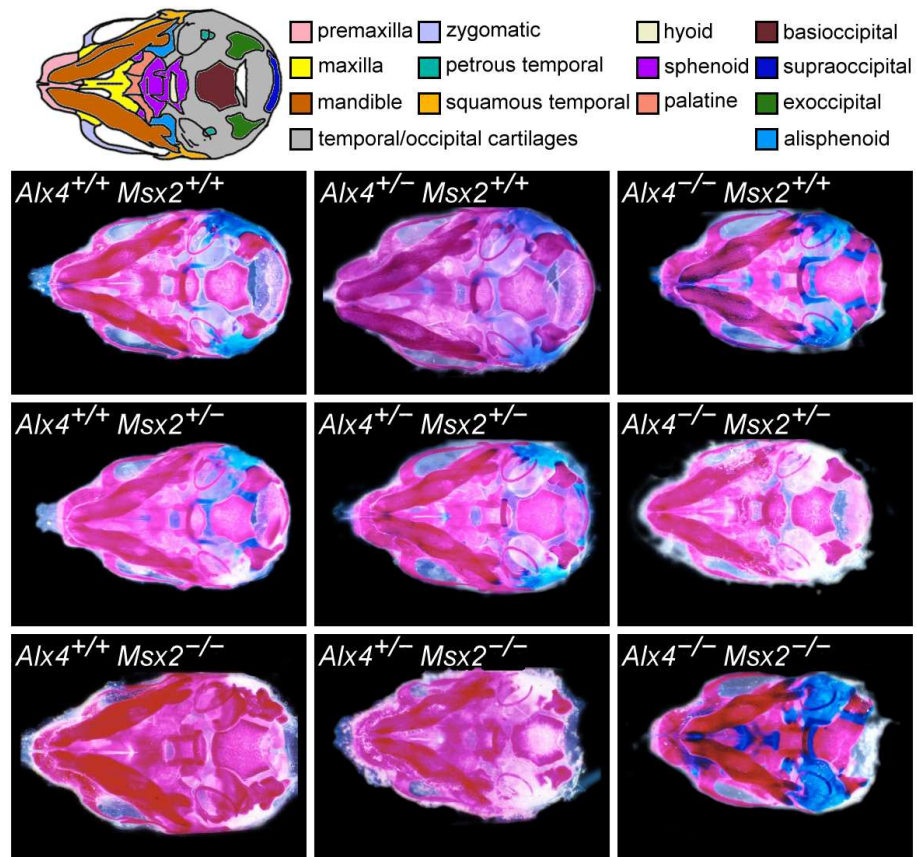


Figure 6.3: Representative skull phenotypes of newborn mice at P0, carrying all nine combinations of wild-type and mutant *Alx4* and *Msx2* alleles — ventral views. No abnormalities are noticeable in the skull base. The exoccipital bones, the basioccipital bone, the basisphenoid and presphenoid bones, as well as the mandible appear normal and of proportional size. A notch in the basioccipital of the $Alx4^{-/-} ; Msx2^{-/-}$ specimen is due to damage; the $Alx4^{-/-} ; Msx2^{+/-}$ specimen is tilted, concealing the premaxilla. Dissected skulls, double-stained with Alizarin Red (bone) and Alcian Blue (cartilage). Variability in cartilage staining is a technical artefact and does not correlate with the extent of chondrification. The $Alx4^{+/+} ; Msx2^{-/-}$ skull is of advanced biological age and homozygosity for the mutant *Alx4* allele is associated with reduced overall size.

both specimens obtained, a more severe but roughly additive defect. The overall size is not disproportionate for the combination and, interestingly, the characteristic widenings for the constituent manifestations in both the anterior and posterior frontal region are present — as if the two phenotypes have merged. Ossification has

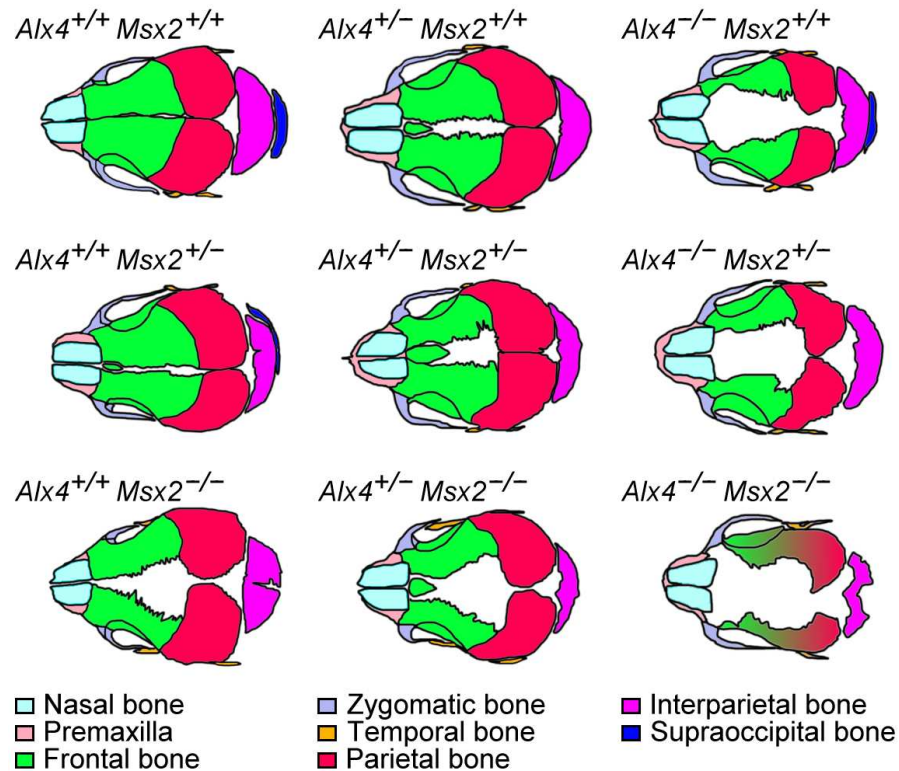


Figure 6.4: Schematic depiction of representative skull vault phenotypes of newborn mice at P0, carrying all nine combinations of wild-type and mutant *Alx4* and *Msx2* alleles. A detailed description of the defects is provided in Figure 6.1. In heterozygosity, only the *Alx4* mutation is penetrant, and in homozygosity the defects associated with *Alx4* and *Msx2* mutations differ. *Alx4*^{-/-} skulls show a large midline gap: anteriorly, it starts from the fronto-nasal boundary and stretches posteriorly to the interparietal bone; the medial edges of the parietal bones are either free or have just met each other. In *Msx2*^{-/-} skulls the discontinuity involves the frontal region only and is diminishing towards the anterior part. The defect in the double homozygote, *Alx4*^{-/-} ; *Msx2*^{-/-}, is the most severe; however, the frontal and parietal bones are still represented. The coronal suture is obliterated bilaterally. The schemes were drawn directly from the images in Figure 6.1.

taken place and the bone plates, especially the parietals, have grown to a certain extent but the line of apposition between them is obliterated, indicating synostosis of the coronal suture. Other intramembranous elements, such as the interparietal bone and the nasal bones, are not affected.

Alx4^{+/-} heterozygotes display a narrow fissure between the frontal bones which anteriorly surrounds a small intrasutural bone. The *Alx4*^{+/-} ; *Msx2*^{-/-} combination exhibited a somewhat exacerbated defect of the *Msx2* type with the characteristic interfrontal bone. The presentation is compatible with an additive effect, but an interaction is not completely ruled out: after subtracting its contribution from the compound phenotype, the *Alx4*^{+/-} genotype could act as a modifier, as the remaining defect is slightly more severe in comparison to the *Msx2*^{-/-} homozygote.

Msx2^{+/-} heterozygotes are completely normal and, hence, any modifying effect of the genotype should be readily visible. Indeed, in the double heterozygote, *Alx4*^{+/-} ; *Msx2*^{+/-}, the observed defect was, on average, wider and more triangular-shaped when compared to the even fissure of the *Alx4*^{+/-} skull. The *Alx4*^{-/-} ; *Msx2*^{+/-} skull corroborates this trend: the *Alx4*^{-/-}-caused defect was found altered, enlarged posteriorly.

However, all assessments were based on representative skulls for each genotype, and morphological variability, though limited, was observed. Not surprisingly, given the documented sensitivity of *Alx4* alleles to modifiers, the defects associated with the *Alx4*^{+/-} ; *Msx2*^{+/+} and *Alx4*^{+/-} ; *Msx2*^{+/-} genotypes were, mostly, of variable severity. Although all animals studied were derived from the intercross (F2) of double heterozygotes (F1; see section 6.2) and, consequently, shared a 50% : 50% 129 : C57BL/6 background on average, random assortment of chromosomes and one round of recombination had occurred, ensuing on patchy, not strictly comparable genomes. To investigate the possibility of confounding background effects, several *Alx4*^{+/-} and *Msx2*^{+/-} animals were generated on a mixed but unfragmented 50% : 50% 129 : C57BL/6 background, after outcrossing the stock, single mutant strains. When examined alongside their F2 equivalents comparable fluctuation was apparent, indicating that the variability is inherent in *Alx4* heterozygosity (Antonopoulou 2002).

6.4 Other Skeletal Abnormalities in *Alx4* ; *Msx2* Compound Mutants

Apart from skull defects, mice of the *Alx4*^{-/-} genotype exhibit preaxial polydactyly in both the hindlimbs and forelimbs and hypoplasia of the pelvic girdle, in particular strong reduction of the pubis at P0. The entire skeleton of *Msx2*^{-/-} mice is relatively smaller and, as mentioned in section 6.3, the supraoccipital bone is almost absent at P0.

As expected, these characteristic skeletal abnormalities were seen combined in the *Alx4*^{-/-} ; *Msx2*^{-/-} double homozygote. In addition, unforeseen anomalies were evident in the thoracic region (Figure 6.5): the thoracic cage is narrow, laterally compressed; the clavicles are misaligned relative to the sternum, somewhat shorter, and their medial ends are free; a defect of the anterior part of the sternum, where the future manubrium is to be formed, contributes to this failure of attachment; and the xiphoid process, at the posterior edge of the sternum, is split. Other important structural aspects are, nevertheless, normal: the usual number of ribs (thirteen) and sternebrae (six) are present, and no costal fusions or bifurcations were seen.

6.5 Cardiac Abnormalities in the Double Null Homozygote

In one of the *Alx4*^{-/-} ; *Msx2*^{-/-} double homozygotes the thoracic viscera were excised and examined (Antonopoulou 2002) — Figure 6.6. Externally, both the lungs and the heart are smaller; furthermore, the lungs have not been inflated and appeared collapsed. Sectioning revealed a conspicuously malformed heart. A preliminary evaluation would score the lumina of the ventricles as almost completely eliminated, the outflow tract as misplaced, and the atrioventricular valves as incomplete.

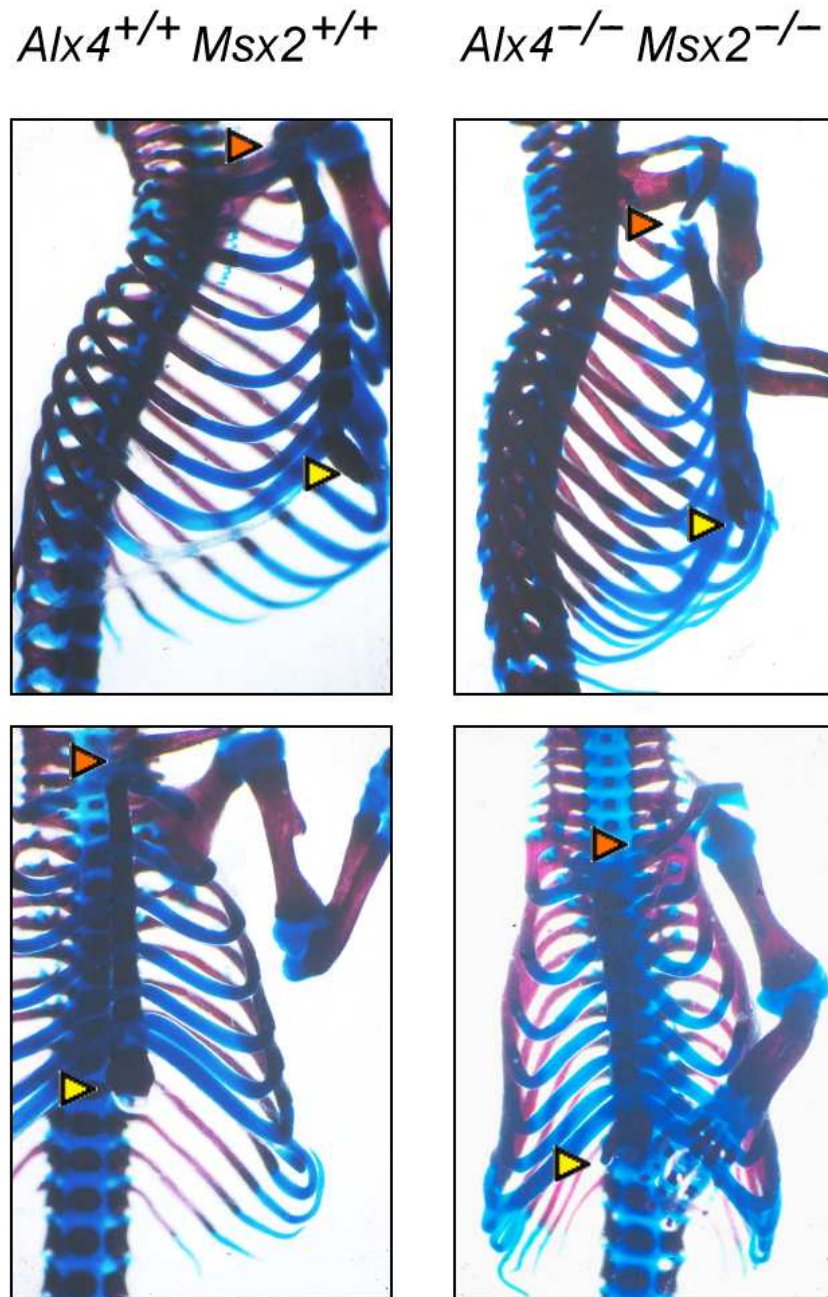


Figure 6.5: Malformed thoracic cage in the $Alx4^{-/-} ; Msx2^{-/-}$ compound homozygote in comparison to a wild-type animal at P0 — lateral (*upper row*) and ventral (*lower row*) views. The thoracic cage is disproportionately narrow in the mutant and the clavicles are misaligned and hypoplastic. They are not attached to the sternum (*red arrowheads*), as its very anterior segment – corresponding to part of the future manubrium – is missing. The xiphoid process appears forked (*yellow arrowheads*). However, there is a normal number of sternabrae and ribs. Partially dissected thoracic cages, double-stained with Alizarin Red (bone) and Alcian Blue (cartilage). The abnormalities were observed in both mutant specimens available from this stage.

$Alx4^{+/+} Msx2^{+/+}$ $Alx4^{-/-} Msx2^{-/-}$

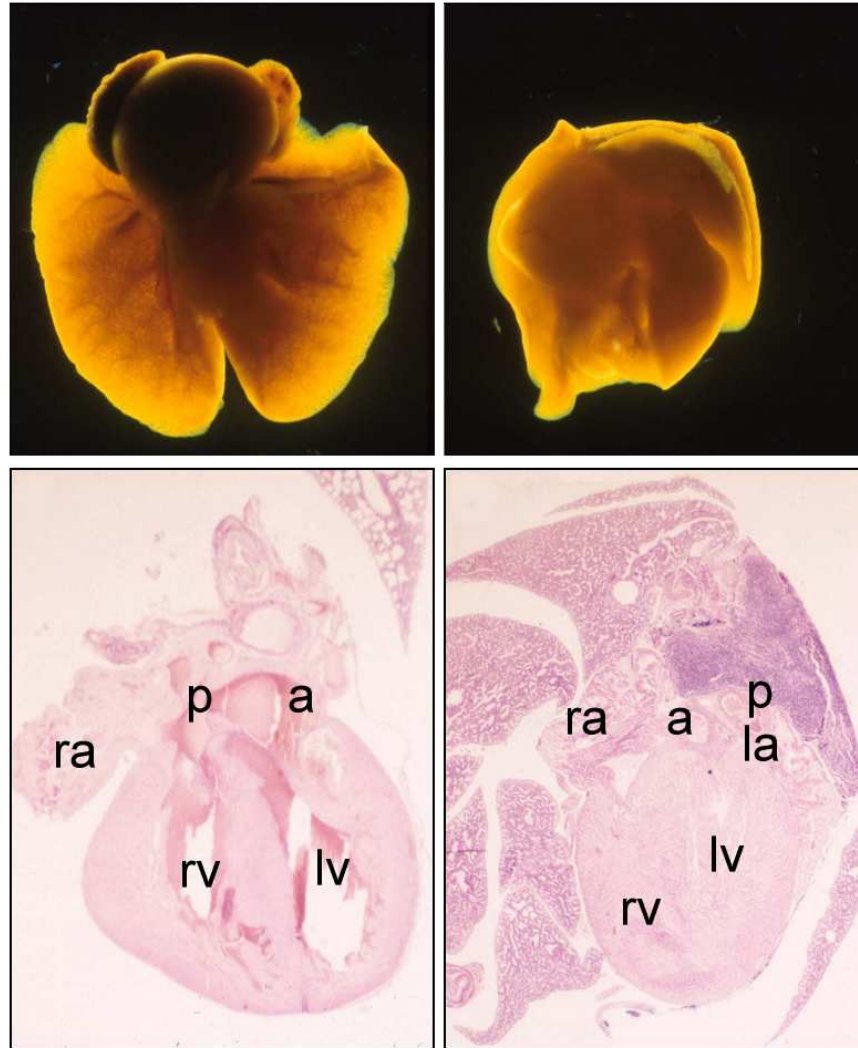


Figure 6.6: Major heart anomalies in the $Alx4^{-/-} ; Msx2^{-/-}$ compound homozygote in comparison to a wild-type animal at P0 — gross morphology (*upper row*) and appearance after sectioning (*lower row*). The heart of the mutant is overall smaller, surrounded by the lungs that failed to expand. The ventricles appear collapsed – their lumina are totally effaced – and the outflow tract shifted. Photographs kindly provided by Dr I. Antonopoulou (Antonopoulou 2002). a, aortic trunk; la, left atrium; lv, left ventricle; p, pulmonary trunk; ra, right atrium; rv, right ventricle.

6.6 Could *ALX4* Variants Influence the Penetrance of *MSX2* Mutations and Vice Versa?

From analysis of single nucleotide polymorphisms, two very frequent protein variants are predicted for human *ALX4*, R35T and P102S, and one not uncommon for human *MSX2*, T129M — see sections 4.2.1 and 4.3.1 respectively. Incidents of non-penetrance of PFM in human pedigrees segregating *ALX4* or *MSX2* mutations cannot be always accounted by the advanced age of the individuals and the concomitant extreme narrowing of the defects; they may point to distinct contributions by modifiers elsewhere in the genome or reflect stochastic effects, intrinsically associated with gene dosage imbalances. If specific modifiers exist, variants in the second, unmutated PFM locus are good candidates.

In family 2, where the Q140X mutation was identified in *ALX4* (see section 3.2.4.2 and appendix B), a 43-year-old carrier, II-4, is radiologically unaffected, and a 5-year-old child, IV-5, showed rather borderline defects. On the contrary, the mutation is penetrant in the 72-year-old carrier, I-2. The coding region of *MSX2* was analysed in the former individuals and no variants were detected; the same was true for a manifesting family member, IV-4.

Reciprocally, in family 9, segregating a deletion around *MSX2* (Wilkie et al. 2000) (see appendix B), one case of non-penetrance in a 59-year-old carrier, II-2, was encountered. The coding region of *ALX4* was analysed alongside a manifesting carrier, III-1, and no protein variants were found.

6.7 Summary

Purely genetic data may not allow definitive conclusions with regard to biological interactions to be drawn but offer helpful insights. Although genetic interactions between *Alx4* and *Msx2* are detected, a model of a simple, linear, hierarchical cascade that places the one factor immediately downstream of the other appears un-

likely. The double null homozygote, $Alx4^{-/-}$; $Msx2^{-/-}$, shows skull vault defects that cannot be described as epistatic, are not disproportionately severe, and no novel skull anomalies are apparent. The $Msx2^{-/-}$ -associated defect is less prominent compared to the one caused by $Alx4^{-/-}$, indicating that $Msx2$ is unlikely to be upstream – and a major positive regulator – of $Alx4$. An epistatic relationship with $Alx4$ upstream of $Msx2$ is, also, hardly compatible with the phenotypes: had $Alx4$ been upstream, exerting a substantial positive influence on $Msx2$, no significant effect would have been observed on ablation of $Msx2$ in $Alx4^{-/-}$ animals. On the other hand, $Msx2^{+/-}$ heterozygosity clearly modulates the presentation of the defects seen on both $Alx4^{+/-}$ and $Alx4^{-/-}$ skulls; in fact, the effect may be considered as increased penetrance for the $Msx2^{-}$ mutation once at least one $Alx4$ copy has been removed from the system. The impact of $Alx4^{+/-}$ heterozygosity on $Msx2$, if any, is subtle. Collectively, the genetic data for the mouse model support cumulative and possibly co-operative actions of $Alx4$ and $Msx2$ on downstream targets during calvarial development. Interestingly, with the exception of coronal synostosis, skull patterning is not disrupted in the $Alx4^{-/-}$; $Msx2^{-/-}$ animal and terminal maturation of bone is not compromised — implying that even in the double mutant the main problem remains essentially a growth delay. In humans, a preliminary investigation found no evidence that $ALX4$ or $MSX2$ variants could act reciprocally as penetrance modifiers in PFM.

If the $Alx4^{-/-}$; $Msx2^{-/-}$ embryos survive to term, they die soon after birth presumably from respiratory failure. The collapsed lungs suggest that no air has been inhaled. Further histological analysis (Antonopoulou 2002) revealed unexpanded alveoli, but some of the larger bronchioles were found very dilated and lymphatic infiltration of the trachea was noticed. It is unclear if the primary cause of death is the structurally abnormal thoracic cage that does not permit expansion, the malformed heart that is unable to cope with the postnatal pattern of circulation and pump blood into the pulmonary trunk, or other anomalies that were not ini-

tially considered, i.e. weakness of the diaphragm and/or the intercostal muscles. The thoracic cage appears distorted but with a normal number of ribs and sternbrae. The forked xiphoid process probably arises from delayed fusion of the paired sternal bars alongside the ventral midline and the anterior defect, involving the medial edges of the clavicles, reflects delayed and/or deficient ossification in local centres — distinct from the ones in sternbrae. The tissue origins of the sternum are not clear: somitic, and/or lateral plate mesoderm are likely to contribute. It is intriguing that the clavicular defect contrasts with the one seen in PFMCH in humans – caused by a mutation in *MSX2* (see section 3.3.1.2) – which affects predominantly the lateral segment. The malformed heart discloses functional redundancy of *Alx4* and *Msx2* during cardiac development as well. The atria are hardly visible and at the time of death no blood is filling the ventricles; further histological analysis (Antonopoulou 2002) recorded absence of the membranous part of the interventricular septum and an undivided proximal part of the outflow tract, but deduced that the architecture of the aortic arches is normal. These manifestations could be broadly viewed as failure of atrioventricular partitioning. This process requires induction of the endocardial cushions, consisting of a peculiar tissue that has undergone an epithelial-mesenchymal transition. No investigation of *Alx4* expression in the developing heart has been published, but *Msx2* is known to be expressed in the atrioventricular endocardial cushion and other cell populations at the atrioventricular interface (Abdelwahid et al. 2001).

Chapter 7

Potential Interactions between *Alx4* & *Msx2*: the Expression Level

7.1 Introduction

Inevitably, the genetic approach of chapter 6 ended with inferences about *Alx4* and *Msx2* relationships. Although the participation of the two genes in a linear pathway seems unlikely, any further investigation has to rely on expression data in mutants — whether *Msx2* is required for *Alx4* transcription during skull development and vice versa.

In a different but related system, the limb bud, collateral data from expression studies in the *Alx4*^{-/-} mutant suggest that *Msx2* is not immediately downstream of *Alx4*. An initial allusion to a grossly normal *Msx2* expression pattern in the *Alx4*^{-/-} limb bud (Qu et al. 1997b) was verified by a second independent study (Dunn et al. 1997). Despite the abnormal morphology (anterior prominence in the *Alx4*^{-/-} limb bud), the expression level and spatial distribution of *Msx2* transcripts between E10.5–13.5 appeared unaltered by in situ hybridisation. On the other hand, *Alx4* expression has not been assessed at all in the *Msx2*^{-/-} background so far.

However, in the context of the skull any analogous study would not be technically straightforward. The fact that both genes are not highly expressed in the early mesenchyme condensations or the newly-ossified bones (see chapter 5)

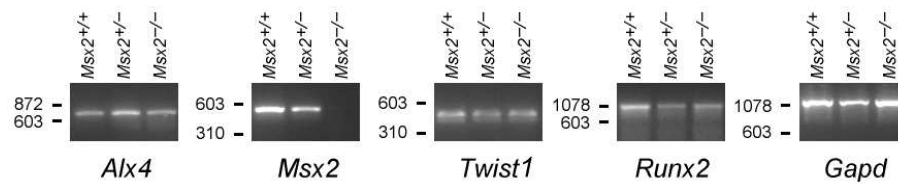


Figure 7.1: Representative RT-PCR results for expression of *Alx4*, *Twist1*, and *Runx2* in the head of *Msx2*^{+/+}, *Msx2*^{+/-}, and *Msx2*^{-/-} embryos at E11.5–12. *Msx2* expression is abolished in the null homozygote, but *Alx4* expression persists. *Twist1* shows no difference and the fluctuation observed for *Runx2* was not reproducible. The housekeeping gene *Gapd* serves as a rough quantitative control. RT-PCR on comparable amounts of total RNA using primer pairs that map in different exons and variable number of cycles, as described in section 2.11. Product sizes: *Alx4*, 672 bp; *Msx2*, 534 bp; *Twist1*, 449 bp; *Runx2*, 795 bp; and *Gapd*, 983 bp. No amplification from genomic DNA was detectable (not shown).

may challenge the sensitivity of any approach based on in situ hybridisation. In addition, if RNA had to be isolated and extracted, the size and tissue heterogeneity of the head beyond E11–12 would have necessitated reliable microdissection of specific parts. As a compromise, expression levels were analysed by semi-quantitative RT-PCR in whole heads of mutants at E11.5–12. At this stage, *Alx4* and *Msx2* are intensely expressed in mesenchymal cell populations and their patterns display substantial overlap (Bendall and Abate-Shen 2000; Beverdam and Meijlink 2001), including the medial and lateral nasal processes, the distal edges of the maxillary and mandibular processes, and a domain posteriorly and dorsally to the eye (Figure 5.1). Besides *Alx4* and *Msx2*, two other genes crucially implicated in skull development were considered. *Twist1* is active at this stage in the mesenchyme of the frontonasal process and the branchial arches (Wolf et al. 1991; Füchtbauer 1995); *Runx2* is also expressed, albeit weakly (Ducy et al. 1997; Otto et al. 1997).

7.2 Expression of *Alx4* in the *Msx2*^{-/-} Background

Equalised total RNA preparations from heads of comparable *Msx2*^{+/+}, *Msx2*^{+/-}, and *Msx2*^{-/-} E11.5–12 embryos, in a practically C57BL/6 background, were

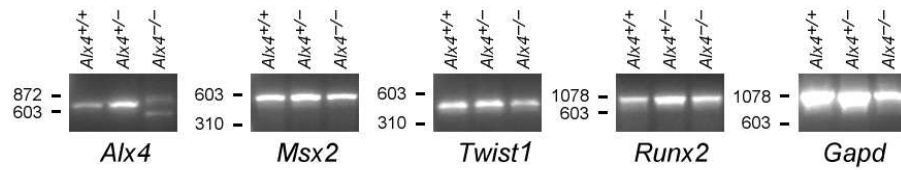


Figure 7.2: Representative RT-PCR results for expression of *Msx2*, *Twist1*, and *Runx2* in the head of *Alx4*^{+/+}, *Alx4*^{+/-}, and *Alx4*^{-/-} embryos at E11.5–12. The *Alx4*-specific primer pair produces two weak bands in the null homozygote: the lower represents an exon skipping event that bypasses the disrupted exon 3 (data not shown); the upper is PCR noise, perhaps derived from another member of the *Alx* group. *Msx2* expression persists and no significant differences are seen for *Twist1* and *Runx2*. The housekeeping gene *Gapd* serves as a rough quantitative control. RT-PCR on comparable amounts of total RNA using primer pairs that map in different exons and variable number of cycles, as described in section 2.11. Product sizes: *Alx4*, 672 bp; *Msx2*, 534 bp; *Twist1*, 449 bp; *Runx2*, 795 bp; and *Gapd*, 983 bp. No amplification from genomic DNA was detectable (not shown).

reverse-transcribed and subsequently used in transcript-specific PCR reactions for *Alx4*, *Msx2*, *Twist1*, *Runx2*, and *Gapd* (Figure 7.1). *Gapd*, encoding the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase and considered a housekeeping gene, provided a rough quantitative control. *Alx4* expression appeared consistently unaffected, even in the *Msx2*^{-/-} background, and the same was true for *Twist1*. The reduction seen for *Runx2* was not a reproducible finding — probably related to the high number of cycles required for amplification.

7.3 Expression of *Msx2* in the *Alx4*^{-/-} Background

A converse analysis, using heads of *Alx4*^{+/+}, *Alx4*^{+/-}, and *Alx4*^{-/-} E11.5–12 embryos in a pure 129 background, failed to detect any effect on transcription of *Msx2*, as well as on *Twist1* and *Runx2* (Figure 7.2).

7.4 Summary

The expression data in the mutants make a hierarchical model for *Alx4* and *Msx2* interactions extremely unlikely although the interpretation extrapolates from a slightly different context. At the E11.5-12 head, *Alx4* and *Msx2* are co-expressed in outer domains of mesenchyme that interact with the adjoining epithelia during head shaping. Later, during condensation and osteogenesis in deeper mesenchymal layers, they regulate locally the cell behaviours. It is assumed that any interdependence in the latter context would be more-or-less mirrored in the former. A complementary study by in situ hybridisation on sections of *Msx2*^{-/-} and *Alx4*^{-/-} skulls at E14–16 detected normal expression levels of *Alx4* and *Msx2* respectively (Antonopoulou 2002).

Chapter 8

Potential Interactions between *Alx4* & *Msx2*: the Protein Level

8.1 Introduction

Members of the Msx and Alx groups bind to DNA sequences containing the generic homeodomain recognition site TAAT in vitro (Gehring et al. 1994). In particular, Msx proteins exhibit a preference for the CTAATTG element (Catron et al. 1993; Catron et al. 1996; Semenza et al. 1995), whereas Alx dimers tend to assemble synergistically on TAATYNRATTA palindromes (Wilson et al. 1993; Wilson et al. 1995; Tucker and Wisdom 1999). Msx proteins are potent transcriptional repressors and this activity (Semenza et al. 1995; Catron et al. 1995; Catron et al. 1996; Newberry et al. 1997b) maps chiefly to the N-terminal part of the homeodomain and its conserved extension (Zhang et al. 1996a; Catron et al. 1996; Newberry et al. 1997b); on the other hand, Alx proteins present as modest activators — although their behaviour may be ambivalent, depending on the experimental context (Gordon et al. 1996; Hudson et al. 1998; Qu et al. 1999; Boras and Hamel 2002; Brouwer et al. 2003).

Nevertheless, their functional repertoire is not limited to direct recognition of cognate sites on DNA; several of their actions may be exerted through protein-protein interactions. This is especially true for the Msx group. The repressive po-

tential is distinctively independent of DNA binding (Catron et al. 1995; Semenza et al. 1995; Catron et al. 1996; Newberry et al. 1997b) and an increasing number of partners and contacts has been reported. A compilation of potential interactants includes components of the basal transcription apparatus such as Tbp in TFIID (Zhang et al. 1996a; Shetty et al. 1999) and Rap74 in TFIIF (Newberry et al. 1997b); sequence-specific transcription factors such as Sp1 (Shetty et al. 1999), Cebpa (Zhou et al. 2000a), G22p1 and Xrcc5 – collectively known as the Ku nuclear antigen complex – (Willis et al. 2002), and several homeoproteins of the Dlx, Pax, and LIM types (Zhang et al. 1997; Newberry et al. 1998; Bendall et al. 1998; Bendall et al. 1999); the co-activators Crebbp and Ep300 (Shetty et al. 1999; Mehra-Chaudhary et al. 2001), but also the histone deacetylase Hdac1 (Mehra-Chaudhary et al. 2001); and the less well-characterised proteins Mint (a histone deacetylase-associated repressor), Miz1 (a possible activator), and Maged1 (a potential regulator of both Msx and Dlx proteins) (Wu et al. 1997; Newberry et al. 1999; Masuda et al. 2001). Under the current model, put forward by Bendall and Abate-Shen (2000), at least four mechanisms could underlie the functional diversity of Msx proteins: (1) conventional DNA binding, (2) inhibitory contacts with the basal transcriptional apparatus, (3) interactions with sequence-specific transcription factors while bound on DNA and (4) antagonism with activators by sequestration in heterodimers. The latter attribute – complex formation at the expense of DNA binding – involves other homeodomain partners and the association is mediated by the homeodomains themselves on either side (Bendall et al. 1998; Bendall et al. 1999; Zhang et al. 1997).

Establishment of homo- and heterodimers on suitable DNA sites is a typical feature of paired-related homeoproteins (Wilson et al. 1993) and the Alx proteins are not an exception (Tucker and Wisdom 1999). Apart from group members, other proteins of the wider paired-related class may participate in formation of co-operative dimers (Tucker and Wisdom 1999; Qu et al. 1999). It is envisaged that these interactions could be part of a code that imparts specificity, dictated by the

nature of the DNA elements and the local availability of partners (Wilson et al. 1993). Additionally, at least for Alx4, there is evidence for non-homeodomain partners. The high-mobility-group-box transcription factor Lef1 contacts Alx4 via the poly(P/Q) repeat at the N-terminus (Boras and Hamel 2002) and an association with members of the retinoblastoma family of negative cell cycle regulators seems likely (Hudson et al. 1998; Wiggan et al. 1998); even the C-terminal aristaless/OAR domain may be involved in intermolecular regulatory interactions (Brouwer et al. 2003).

The attractive idea that Alx and Msx proteins could converge to common target promoters by heterodimer formation has been investigated, with a negative outcome *in vitro* (Tucker and Wisdom 1999): when recombinant Alx4 and Msx1 homeodomains were incubated with an element that supports dual binding only pure Alx4-DNA and Msx1-DNA complexes were detected. Yet, the possibility that the Msx and Alx proteins could either interact through regions outside their homeodomains or associate in solution, in the absence of DNA, remained open. Arguably, for Msx2 and Alx4 in particular, a simple model of mutual neutralisation by heterodimerisation is highly unlikely given the haploinsufficiency of their loss-of-function mutations; however, participation in a multi-protein assembly is a scenario that could be considered.

8.2 Pull-down Assay Using GST-MSX2 and ALX4

The interaction potential of human full-length MSX2 and ALX4 was assessed by the GST pull-down assay. The initial design featured a GST-ALX4 fusion, expressed in bacterial cells, and radiolabelled MSX2, generated by coupled *in vitro* transcription-translation. Despite its satisfactory yield, the GST-ALX4 protein was refractory to purification and the approach was inverted.

Partially purified GST-MSX2 (MW: ~ 48 kDa), immobilised on glutathione beads, was incubated with radiolabelled ALX4 (MW: ~ 50 kDa) under conditions

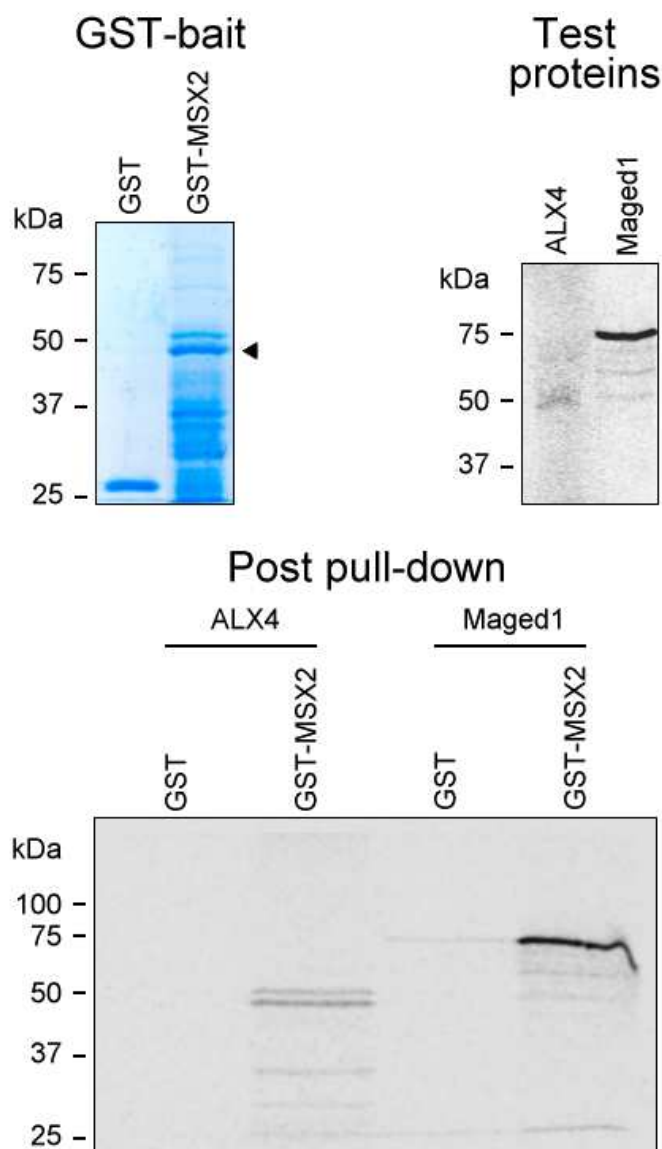


Figure 8.1: Representative GST pull-down experiment using bacterially-expressed GST-MSX2 and [^{35}S]-labelled ALX4 or Maged1 proteins, produced by *in vitro* transcription-translation. The mouse Maged1, a documented partner for Msx2, serves as a positive interaction control. The GST-MSX2 fusion (~ 48 kDa) was partially purified alongside GST (29 kDa) on glutathione beads; 2.5 times the amounts shown in the SDS-PAGE gel – *upper left panel* – were used as inputs. The yields of [^{35}S]-labelled ALX4 (~ 50 kDa) and Maged1 (~ 75 kDa) were not comparable; to compensate, 3 and 1 times the quantities shown in the SDS-PAGE gel autoradiograph – *upper right panel* – were used respectively as inputs. Following incubation and washing, all combinations were assessed for protein trapping — *lower panel*. The background produced by GST is negligible, while both ALX4 and Maged1 demonstrate binding potential for GST-MSX2. GST-MSX2 preparations were of variable purity; the specific band is the one just below the 50 kDa mark (*black arrowhead*). Approx. $0.5\text{--}1\ \mu\text{g}$ of fusion was used. ALX4 is seen as a doublet due to use of an alternative initiation codon. Relaxed conditions were applied in the experiment (see section 2.12.4).

that suppress non-specific binding. Pure GST (MW: ~ 29 kDa), to check for specificity, and a known interactant for murine *Msx2*, *Maged1* (Masuda et al. 2001) (MW: ~ 75 kDa), providing a positive control, were also included in the experiment. As neither ALX4 nor *Maged1* trapping by GST-MSX2 was detected (data not shown) under stringent conditions, the parameters were gradually relaxed. A positive result was seen for both ALX4 and *Maged1* (Figure 8.1) when rather loose conditions were applied under prolonged incubation; nevertheless, the background noise produced by GST was very low.

Given the tendency of homeodomains to bind each other, the specificity of this finding was further tested by evaluating the divergent human Hox homeoprotein HOXD13 (Dolle et al. 1991) (MW: ~ 38 kDa) against GST-MSX2 (Figure 8.2). As ALX4, HOXD13 demonstrated binding potential for GST-MSX2 under the same, relaxed, conditions.

8.3 Summary

Although the contact between ALX4 and GST-MSX2 is not an experimental artefact – as inferred by the GST control – it is unlikely to reflect an interaction of biological significance. It is more likely attributable to the inclination of homeodomains to combine under favourable conditions. The two sets of binding experiments cannot, strictly speaking, discriminate between specific and non-specific, possibly homeodomain-mediated, complex formation. However, when considered in combination with other evidence, the results lend little support to a physiological ALX4-MSX2 association. Firstly, binding disappears under increased stringency. Secondly, the GST-MSX2 - HOXD13 interaction underscores the tendency of homeodomains for non-specific mutual binding (the HOXD13 protein is expressed in the very posterior and distal parts of the trunk and the appendages and there is no evidence to suggest a functional link with MSX2 (Dolle et al. 1991)). And thirdly, complex formation should have an antagonistic effect on DNA binding by either protein — but this was not been observed when the homeodomains of *Alx4* and, the almost identical to *Msx2*, *Msx1* were used (Tucker and Wisdom 1999).

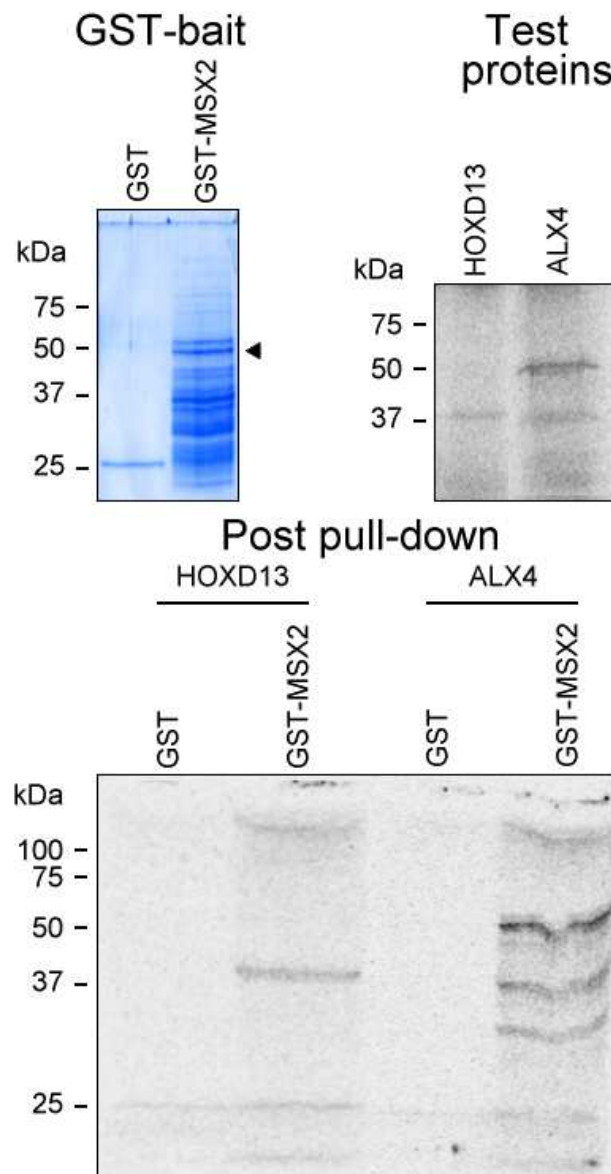


Figure 8.2: Representative GST pull-down experiment using bacterially-expressed GST-MSX2 and [^{35}S]-labelled ALX4 or HOXD13 proteins, produced by in vitro transcription-translation. The GST-MSX2 fusion (~ 48 kDa) was partially purified alongside GST (29 kDa) on glutathione beads; 2 times the amounts shown in the SDS-PAGE gel – *upper left panel* – were used as inputs. The yields of [^{35}S]-labelled ALX4 (~ 50 kDa) and HOXD13 (~ 38 kDa) were low; 2.5 and 2.6 times the quantities shown in the SDS-PAGE gel autoradiograph – *upper right panel* – were used respectively as inputs. Following incubation and washing all combinations were assessed for protein trapping — *lower panel*. The background produced by GST is negligible and both ALX4 and HOXD13 demonstrate binding potential for GST-MSX2. GST-MSX2 preparations were of variable purity; the specific band is the one just below the 50 kDa mark (*black arrowhead*). Approx. $0.5\text{--}1\ \mu\text{g}$ of fusion protein was used. ALX4 is consistently seen as a doublet due to use of an alternative initiation codon. Relaxed conditions were applied in the experiment (see section 2.12.4).

Chapter 9

General Discussion

9.1 Introduction

Starting from human genetics and exploiting the window of opportunity offered by the P11pDS, *ALX4* was isolated and characterised as the second PFM locus, harbouring mutations in a number of families. Their properties indicate that functional haploinsufficiency is the predominant mechanism behind the human phenotype. Two new *MSX2* mutations were also ascertained and one of them reveals allelism between PFMCCD, more appropriately termed PFMCH, and isolated, *MSX2*-caused PFM. Moving towards functional analysis, the expression of *Alx4* during skull development was comprehensively recorded alongside with *Msx2* and an initial analysis of the *Alx4*-*Msx2* link in the skull vault, facilitated by the availability of mutant mice, was attempted from several viewpoints. A parsimonious interpretation of these results suggests that the two genes act non-hierarchically, in parallel pathways, and have critical roles at the osteoblast precursor-mature osteoblast interface. This section sets out to elaborate on the genetics of *ALX4* and *MSX2* and, by drawing on selected pieces of information, present certain clues about the specific functions of the two genes in calvarial development. It ends with few suggestions regarding avenues of future research.

9.2 Genetics of *ALX4* and *MSX2*

9.2.1 Mutations of *ALX4* and *MSX2*: Manifestations, Phenotype-Genotype Correlations, and Genetic Mechanisms

As a result of local work (Wilkie et al. 2000; Mavrogiannis et al. 2001; Garcia-Miñaur et al. 2003) (chapter 3) and published investigations of others (Jabs et al. 1993; Wuyts et al. 2000b; Wuyts et al. 2000a), several individuals with intragenic mutations in either *ALX4* or *MSX2* have been documented. Available mutational data and clinical descriptions (Tables 9.1 and 9.2) allow for a brief review of the phenotypic spectrum and for tentative phenotype-genotype correlations to be drawn.

In both genes the mutations per se appear conventional. A significant proportion of point mutations are C→T (or G→A) transitions that occur in a CG sequence context, indicative of methylation-dependent de-amination of cytosine; in certain deletion or duplication events small repeats can be recognised in the vicinity (Cooper and Krawczak 1993). The most obvious question is whether there is any difference in the severity of the skull defects caused by *ALX4* and *MSX2* mutations. The analysis of Figure 9.1, taking into account data for both intragenic mutations and large deletions, gives a negative answer: overall, in the available datasets, no significant difference is seen. However, cranium bifidum has not been observed in association with *MSX2* mutations so far (Table 9.2); on the other hand, several cases of non-penetrance of *ALX4* mutations counterbalance this trend.

Apart from the skull defects and the occasional neurological symptoms, morphological abnormalities of the tentorium cerebelli are a consistent finding on MRI scans (Figure 3.11) of several *ALX4* mutation carriers – families 3 and 5 with the mutations R218Q and Q246X respectively and the P11pDS case of Wuyts et al. (1999) – and this facet of the phenotype may have been under-reported. Extrapolating from the other major dural infolding, the falx cerebri (see chapter 5), *Alx4* is likely to be expressed in the tentorium cerebelli. The embryonic tentorium is

Table 9.1: Phenotypic Spectrum of Intragenic *ALX4* Mutations

Nucleotide Change	Protein Change	Phenotype	References
385_394del	C129fsX177	PFM	I. Taylor, D. T. Bonthron, A. O. M. Wilkie; unpublished
418C→T	Q140X	PFM/cranium bifidum	Kutilek et al. (1997) Mavrogiannis et al. (2001)
504del	D169fsX180	PFM	Wuyts et al. (2000a) Wuyts et al. (2000b)
620C→A	S207X	PFM, dental defects, broad and short thumbs	L. A. Mavrogiannis, F. J. Ramos, A. O. M. Wilkie; unpublished
653G→A	R218Q	PFM/cranium bifidum, meningeal anomalies, neonatal convulsions	Salamanca et al. (1994) Mavrogiannis et al. (2001)
736C→T	Q246X	PFM/cranium bifidum, meningeal anomalies	Mavrogiannis et al. (2001)
815G→C	R272P	PFM	Rasore-Quartino et al. (1985) Wuyts et al. (2000a)

Notes: With the exception of 653G→A, found in two unrelated families, all other mutations are represented by a single kindred. Two missense mutations, mapping within the homeodomain have been identified so far: the R218Q substitution alters the fifth position of the homeodomain while the R272P change affects the penultimate, fifty-ninth residue of the motif. Apart from the skull defects, all other features either show concordance with the mutations in at least two individuals per pedigree or have been reported as additional manifestations in PFM families. Incomplete penetrance and variable expressivity, even for the skull defects, complicate the individual clinical presentations.

the first dural process to emerge, developing from the primitive mesenchymal condensation in the mesencephalic flexure at the metencephalic-mesencephalic boundary, around the fifth week. By the seventh–eighth week not only this initial, ventro-medial primordium has fully formed, but differentiation has spread antero- and postero-laterally and, to some extent, dorsally over the metencephalon, showing some correlation with ossification at this stage (Klintworth 1967; O’Rahilly and Müller 1986). Through degeneration of the ventro-medial part and complex move-

Table 9.2: Phenotypic Spectrum of Intragenic *MSX2* Mutations

Nucleotide Change	Protein Change	Phenotype	References
[265G→T; 266C→A]	A89X	PFM ^a	Schmidt-Wittkamp and Christians (1970)
345del	W115X	PFM, aplasia cutis congenita, meningeal, venous and cortical abnormalities	Wuyts et al. (2000b) Preis et al. (1995) Wuyts et al. (2000b)
417_418del	H139fsX243	PFM	L. A. Mavrogiannis, T. Letteboer, A. O. M. Wilkie; unpublished
443C→A	P148H	Boston-type craniosynostosis	Warman et al. (1993) Jabs et al. (1993)
456_465dup	A156fsX247	PFM, meningeal and ventricular anomalies	I. Taylor and A. O. M. Wilkie, unpublished
461T→C	L154P	PFM	Wuyts et al. (2000b)
475_480del	R159_K160del	PFM	Wilkie et al. (2000)
505_508dup	A170fsX245	PFMCH	Garcia-Miñaur et al. (2003)
515G→A	R172H	PFM, headaches	Wilkie et al. (2000) Wuyts et al. (2000b)

Notes: With the exception of 515G→A, found in two families, all other mutations are represented by a single kindred. Three missense mutations, mapping within the homeodomain have been identified so far. The craniosynostosis-causing P148H substitution alters the seventh position of the homeodomain whereas the L154P and R172H changes in PFM affect the thirteenth and thirty-first residues of the motif respectively. Two families are notable for syndromic associations — skull defects with clavicular hypoplasia (PFMCH); and scalp defects, manifesting as aplasia cutis. Incomplete penetrance and variable expressivity complicate the individual clinical presentations.

^aOriginal clinical description in German; the English summary alludes to other 'signs of degeneration' in certain members of this kindred.

ments of the lateral sheets, the definitive tentorium is shaped. Thus, the *ALX4*-associated tentorial abnormalities can be interpreted as deficient and/or delayed differentiation of the meningeal lining on the dorsal side of the future cerebellum, leaving a gap which is flooded with cerebrospinal fluid. The minor abnormalities in the network of cranial venous sinuses (Reddy et al. 2000) are probably of the same origin as the dural layer is vascularised soon after differentiation. Another potentially interesting feature is thumb broadening, scored on detailed clinical assess-

ment of family 4 and showing concordance with the underlying nonsense mutation 620C→A. It may represent very subtle anterior polydactyly, the prominent feature of *Alx4*^{-/+} mice (Strong and Hardy 1956; Strong 1961; Forsthoefel 1962; Forsthoefel 1963; Qu et al. 1997b). On re-assessment, both *Alx4*^{lst/+} (Forsthoefel 1962) and *Alx4*^{tm1Rwi/+} heterozygotes (see chapter 6) show a narrow calvarial gap, mostly in the frontal region.

Focusing on *ALX4* mutations, there is no statistically significant difference on the severity of the calvarial phenotype between all intragenic mutations and P11pDS-associated deletions (data not shown), verifying formally the notion that haploinsufficiency is the principal genetic mechanism involved. However, the R218Q variant, found in families 1 and 5, is associated with relatively wider defects, after adjusting for age, in comparison to the phenotypes of all other *ALX4* mutations (Figure 9.2). As mentioned in section 3.5, this change of a critical base-contacting arginine residue in the N-terminal arm of the homeodomain (Figure 9.3), which also underlies the murine *Alx4*^{lst} allele, appeared not to interfere with DNA binding of the wild-type protein in vitro (Qu et al. 1998). Alterations at this position are known to cause a drastic reduction in DNA-binding affinity (Ades and Sauer 1995) and with hindsight it seems that dominant-negative effects may contribute to its phenotypic outcomes in humans. In turn, this finding implies that homodimerisation of *Alx4* in solution could be physiologically important during calvarial development. This notion is not without precedence: Furukawa et al. (2002) demonstrated DNA-independent homodimerisation of *Cart1* and vulnerability of wild-type trans-activation to dominant-negative interference from variants that lack DNA-binding ability. The other missense mutation of *ALX4*, R272P, changes the penultimate position of the homeodomain (Wuyts et al. 2000a). This position is rather variable but the proline residue is rarely, if ever, encountered (Bürglin 1994) — it could, perhaps, perturb the adjacent recognition helix. Paucity of data hindered an equivalent phenotype-genotype analysis.

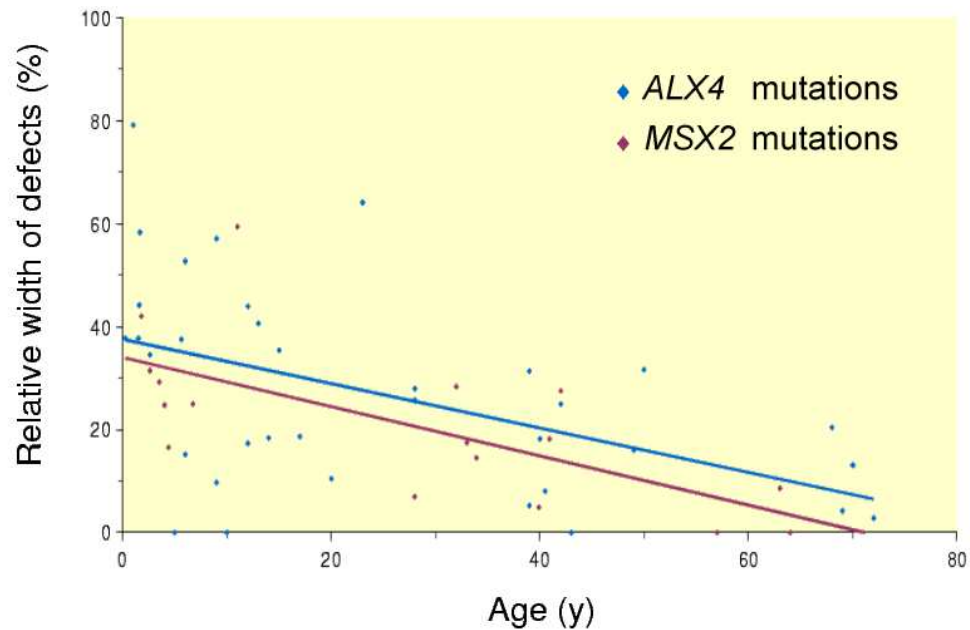


Figure 9.1: Comparison of the skull vault defects associated with *ALX4* and *MSX2* mutations. The cumulative width of the openings or of the central gap is expressed as a fraction of the maximum skull width. The age effect on the severity of the phenotype is, overall, evident but there is significant variability in either case — coefficients of correlation for the linear regression models: 0.24 for the *ALX4* class and 0.47 for the *MSX2* class. The slopes of the two lines, reflecting the average ossification rates during postnatal life, are essentially identical (-0.43 ± 0.28 for the *ALX4* class, and -0.48 ± 0.28 for the *MSX2* class ($P=0.95$)) and there is no statistically significant difference in the y-intercept points (37.6 ± 9.1 for the *ALX4* class, and 34.1 ± 9.8 for the *MSX2* class). After adjusting for these trends, a two-tailed t-test on the basis of equal sample variances returns a P value of 0.49, supporting the null hypothesis of no difference between the two groups. Age and relative width data, either calculated on available antero-posterior skull radiographs or extracted from the literature, refer to 35 individuals with *ALX4* mutations and 15 individuals with *MSX2* mutations of all types. Duplicate measurements, when available, were averaged. References for published cases: Schmidt-Wittkamp and Christians (1970); Rasore-Quartino et al. (1985); Salamanca et al. (1994); Preis et al. (1995); Bartsch et al. (1996); Potocki and Shaffer (1996); Kutilek et al. (1997); Davies et al. (1997); Wilkie et al. (2000); Wuyts et al. (2000b); Wuyts et al. (2000a); Mavrogiannis et al. (2001); Yamamoto et al. (2001); Garcia-Miñaur et al. (2003).

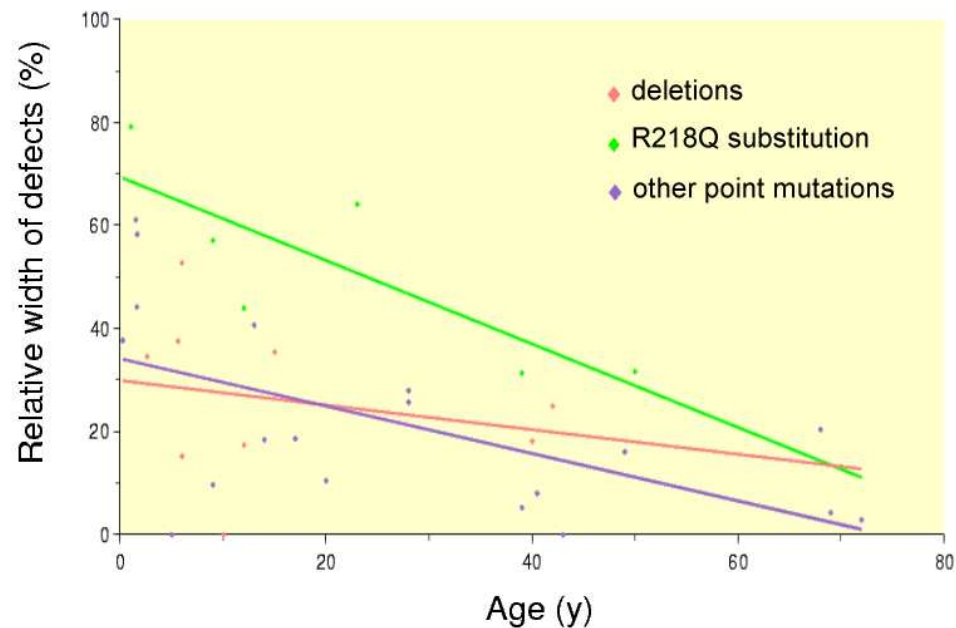


Figure 9.2: Comparison of the skull vault defects associated with the R218Q substitution, with all other intragenic mutations of *ALX4*, and with complete deletions of the gene. Analysis as in Figure 9.1. There is no significant difference in the slopes of the lines (-0.81 ± 0.46 for the R218Q group, -0.46 ± 0.32 for the group encompassing all other point mutations, and -0.24 ± 0.90 for the deletion group ($P=0.95$)), but when the y-intercept points are considered the R218Q group stands out (69.4 ± 17.3 for the R218Q group, 34.1 ± 11.7 for the group encompassing all other point mutations, and 29.9 ± 19.0 for the deletion group). Following age-adjustment, pairwise two-tailed t-tests on the basis of unequal sample variances return: $P < 0.001$ for the R218Q – deletion comparison, rejecting the null hypothesis of no difference; a marginal $P = 0.088$ for the R218Q – other-point-mutations comparison; and $P = 0.229$ for the deletion – other-point-mutations comparison, confirming that the defects are indistinguishable. Upon merging of the non-R218Q point mutation and deletion classes, a comparison against the R218Q group results in $P = 0.008$. Age and relative width data, either calculated on available antero-posterior skull radiographs or extracted from the literature represent 7 individuals with the R218Q substitution, 19 individuals with all other intragenic mutations, and 9 deletion carriers. Duplicate measurements, when available, were averaged. References for published cases: Rasore-Quartino et al. (1985); Salamanca et al. (1994); Bartsch et al. (1996); Potocki and Shaffer (1996); Kutilek et al. (1997); Davies et al. (1997); Wilkie et al. (2000); Wuyts et al. (2000a); Mavrogiannis et al. (2001); Yamamoto et al. (2001).

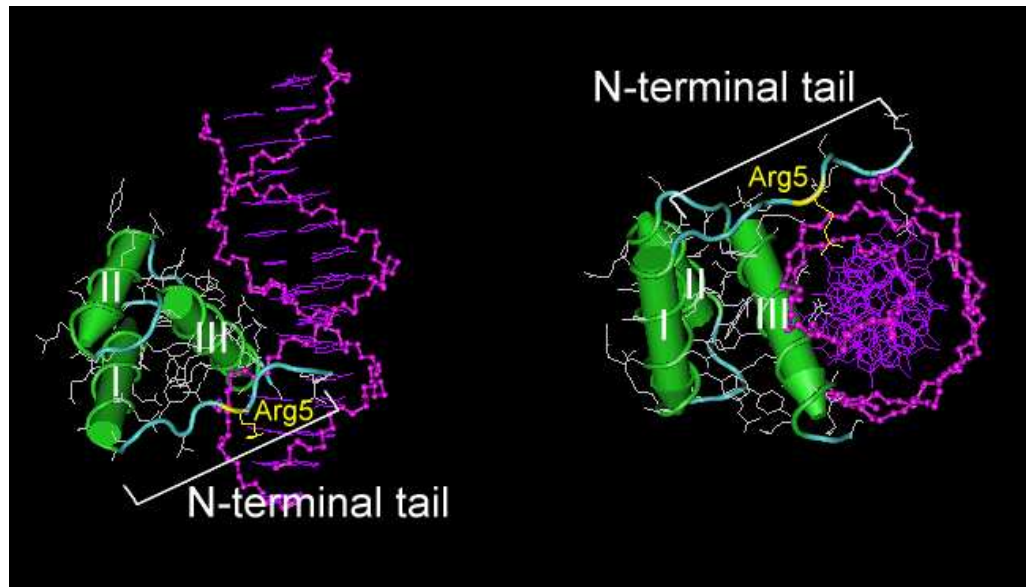


Figure 9.3: Crystal structure of a modified *Drosophila* paired homeodomain, engineered to resemble a member of the paired-like subclass (serine residue at position 50 replaced by glutamine), on a DNA duplex harbouring the core sequence TAAT. Views perpendicular to (*left*) and along (*right*) the long axis of the DNA. Of the three α helices (*green strands and cylinders*; arrows point to the C-terminus), the third slots into the major groove of the DNA (backbone *in magenta*; bases *in purple*) and makes specific contacts with the bases. The non-helical part (*cyan strands*) includes the N-terminal tail that is also involved in recognition through interactions in the minor groove. The side chain of the almost universally invariable arginine residue at position 5 is highlighted *in yellow*; it contacts, directly or indirectly, both bases (T:A) at the first position of the TAAT motif. All other side chains are shown *in grey*. The full structure refers to a co-operative homodimer bound on a palindromic DNA element (Wilson et al. 1995); for clarity only one homeodomain is depicted. Structure file id: 4645 (Molecular databases at NCBI — see appendix F).

Two of the PFM families with *MSX2* mutations exhibit other features in addition to skull defects. The deletion of nucleotide 345, resulting in W115X, segregates with PFM, overlying aplasia cutis, as well meningeal, venous, and cortical abnormalities of the brain (Preis et al. 1995; Wuyts et al. 2000b). In particular, the morphology of the tentorium cerebelli is comparable to the *ALX4*-caused defect. The 505_508 duplication, causing a frameshift, uncovers the sensitivity of the clavicle to *MSX2* dosage, presenting as PFMCH in family 8 (Figure 3.15). The mam-

malian clavicle is believed to be the sole exoskeletal remnant in the pectoral girdle (Hall 2001), once braced by an arch of dermal elements and connected to the skull in early fishes. Therefore, a uniform intramembranous pattern of ossification would appear plausible. However, the murine clavicle is a unique amalgamation, developing from two centres (Huang et al. 1997): the medial part ossifies endochondrally while the lateral part ossifies intramembranously — the two segments being separated by a growth plate. The tapered lateral ends of the clavicles in family 8 indicate that correct dosage of *MSX2* is important for intramembranous ossification. More subtle modelling defects of the central and medial parts were also observed and indicate either a concomitant defect of endochondral ossification, or that in humans the dichotomy in the developmental origins of the medial and lateral parts of the clavicle is not clear cut. *Msx2*^{-/-} mice show deficient endochondral ossification but curiously the clavicles are allegedly unremarkable (Satokata et al. 2000). Unlike to *Alx4*^{-/+} mice, *Msx2*^{-/+} heterozygotes are completely normal (Satokata et al. 2000).

Simple haploinsufficiency is the pathophysiological mechanism behind the *MSX2* mutations in PFM. A complete *MSX2* deletion is phenotypically indistinguishable from the intragenic mutations, and at least for the changes R172H and R159_K160del, predicted to destabilise the homeodomain (Li et al. 1997), loss of function has been demonstrated (Wilkie et al. 2000). In addition, there is no evidence to suggest homodimerisation of Msx proteins (Catron et al. 1993). The substitution L154P may disturb the hydrophobic core of the homeodomain between helices I and II (Bürglin 1994). The craniosynostosis mutation P148H maps within the N-terminal arm and introduces a histidine residue at a minor groove-contacting position (Gehring et al. 1994) where it is naturally only rarely encountered (Bürglin 1994).

Given the manifestations of homozygous null mice, it is tempting to ask whether *ALX4* or *MSX2* mutations could underlie other clinical entities in humans. An investigation of both genes in the dominantly-inherited Adams-Oliver syn-

drome (OMIM 100300) (Table 1.1) was unfruitful (Verdyck et al. 2003). Of the rare recessive congenital conditions in which calvarial defects are part of a constellation of abnormalities, the Al-Awadi/Raas-Rothschild syndrome (OMIM 276820) and the pseudoaminopterin syndrome (OMIM 600325) (Table 1.1) were considered, but no PFM or mild skull defects have ever been reported in parents of affected children.

Finally, insights regarding the likely phenotypic outcomes of *ALX4* and *MSX2* gain-of-function events could be obtained from trisomies of proximal 11p and distal 5q respectively. Duplications of the 5q31–qter segment are associated with growth and mental retardation and craniofacial abnormalities, but no craniosynostosis is mentioned in the literature (Rodewald et al. 1980). Similarly, craniosynostosis appears not to be characteristic of duplications that encompass the 11p11–p12 region (Strobel et al. 1980; Aalfs et al. 1997). A proximal 11p duplication that originated from a parental intrachromosomal insertion (Strobel et al. 1980), which also produced a deletion with the P11pDS signature features (Hittner et al. 1979) (and thus indisputably involved *ALX4*), was associated with cleft lip and palate, mandibular hypoplasia, and developmental delay.

9.2.2 Further Genetic Heterogeneity in Enlarged Parietal Foramina

Until recently, the only exploitable hint with regard to another locus for skull defects in humans was provided by the extremely rare ‘delayed membranous cranial ossification’ syndrome, catalogued in OMIM under 155980 (Table 1.1). All the flat bones of the skull are absent at birth although ossification subsequently ensues and by early adulthood no gaps remain (Gonzalez-del Angel et al. 1992; Cargile et al. 2000). In one of the two reported cases, a translocation, t(2;3)(p15;q12), was found to segregate with the phenotype and the breakpoints were grossly mapped (Cargile et al. 2000); despite the greatly improved sequence coverage of the critical regions in the meanwhile, no follow-up study has been published.

Chen et al. (2003) excluded both *ALX4* and *MSX2* in a large Chinese pedigree with isolated classical PFM and obtained evidence of linkage to the 4q21–q23 region. This, however, assumed non-penetrance in several individuals who had inherited the high-risk haplotype but were clinically unaffected. The minimal critical interval for the putative third PFM locus appears to be flanked by the markers *D4S2964* and *D4S2961*. Within this substantial, approximately 22 Mb segment (Ensembl v. 16.33.1 — see appendix F), three candidate genes were screened – *BMPR1B*, encoding a type I Bmp receptor; *SPP1*, encoding osteopontin; and *IBSP*, encoding integrin-binding sialoprotein – but no mutations were found.

9.2.3 Provision of Diagnostic Testing

There is scope, albeit limited, for diagnostic testing of *ALX4* and *MSX2* in individuals and families with skull defects. PFM tend to be narrow in adults but the defects can be pronounced in infants and children; a definitive molecular diagnosis may be beneficial in these cases, especially if surgical intervention is planned. Judging from the familial cases investigated so far (Wilkie et al. 2000; Wuyts et al. 2000b; Wuyts et al. 2000a; Mavrogiannis et al. 2001; Garcia-Miñaur et al. 2003), the mutation detection rate can be very high, in the range of 80%–90%, if there is inheritance of classical PFM in an autosomal dominant fashion. Furthermore, testing might be useful in differential diagnosis of atypical manifestations, even for exclusion purposes. Such investigations could, potentially, identify variants offering novel functional insights of research interest.

A cytogenetic analysis, to exclude visible rearrangements at chromosomes 5q and 11p is indicated prior to any molecular analysis. Given the small size of the genes and the random mutational spectra, direct sequencing of the coding regions of *ALX4* and *MSX2*, complemented by a contemporary dosage analysis assay (like the multiplex ligation-dependent probe amplification) can be an effective and cost-

efficient strategy. The lists of common polymorphisms in the two genes (Tables 4.1 and 4.3) would be a useful resource in that respect.

9.2.4 Mutations in Other Members of the *Alx* and *Msx* Families

No mutations in the human *CART1* and *ALX3* have been identified, but targeted disruptions of both genes have been engineered and analysed in mice. *Alx3*^{-/-} animals appear completely normal (Beverdam et al. 2001), indicating that both copies of the gene are dispensable as long as *Alx4* and *Cart1* are functional. *Cart1*^{-/+} mice are normal but *Cart1*^{-/-} mice exhibit, with markedly variable penetrance (depending on the genetic background), acrania and meroanencephaly, preaxial polydactyly, palatal clefts, and minor abnormalities of the axial and appendicular skeleton (Zhao et al. 1996; Beverdam 2001). When observed, the primary neural tube closure defect in the forebrain-midbrain boundary is caused by a deficiency of mesenchyme in the forebrain region and is, interestingly, rescuable by administration of folic acid during pregnancy.

As discussed in section 1.4.5, heterozygous loss-of-function mutations of *MSX1* in humans are not associated with skull defects but with dental defects, cleft lip/palate, and nail abnormalities (Vastardis et al. 1996; van den Boogaard et al. 2000; Jumlongras et al. 2001; Jezewski et al. 2003). *Msx1*^{-/-} mice reproduce the palatal cleft and tooth defects and also display an opening between the frontal bones (Satokata and Maas 1994).

9.3 Further Delineation of Proximal 11p Deletion Syndrome — Evidence for a Specific Mental Retardation Locus on Proximal 11p

A by-product of this work is the narrowing of the critical interval for the mental retardation locus in P11pDS. After the identification of *ALX4*, two new case reports of the syndrome were published (Yamamoto et al. 2001; Hall et al. 2001) — cases

8 and 9 in Figures 1.7 and 9.4. Neither, however, was substantially informative in refining a rough provisional mapping for the mental retardation locus, based on cases 3 and 4 (Shaffer et al. 1993; Bartsch et al. 1996): a gene could reside either in the *D11S905–D11S1393* or in the *D11S1361–D11S1344* interval (Figures 1.7 and 9.4). Mental retardation is a frequent finding in gross deletions; the larger the deleted region the higher the probability that it will encompass one or several genes essential for normal brain function. Thus, any pursuit of a specific mental retardation locus could run into uncertainties. However, the new P11pDS family ascertained in this study, case 10 (the underlying deletion is placed in context in Figure 9.4), supports the existence of a single gene crucially implicated in neural functions on proximal 11p. Firstly, absence of mental retardation in association with fairly large deletions has been observed in three families so far — cases 4 (Bartsch et al. 1996) and 9 (Hall et al. 2001) in addition to case 10. Secondly, once the P11pDS-WAGR deletion in case 6 (McGaughran et al. 1995) is excluded, a comparative analysis of cases 3, 4, and 10 maps the mental retardation locus in the *D11S1361–D11S1344* interval — a segment in the range of 1 Mb with relatively few known genes (Ensembl v. 16.33.1 — see appendix F). Although molecular re-assessment of cases 3 and 4 would be valuable towards an even finer delimitation, the prime candidate in the region is *SYT13*, encoding an atypical member of the large synaptotagmin family (Fukuda and Mikoshiba 2001; von Posser and Sudhof 2001). The synaptotagmins are involved in vesicular trafficking, including exocytosis, and synaptotagmin XIII is abundant in the adult brain; furthermore, homozygous null mice for another member, *Syt4*, exhibit various neurological and behavioural problems (Ferguson et al. 2000).

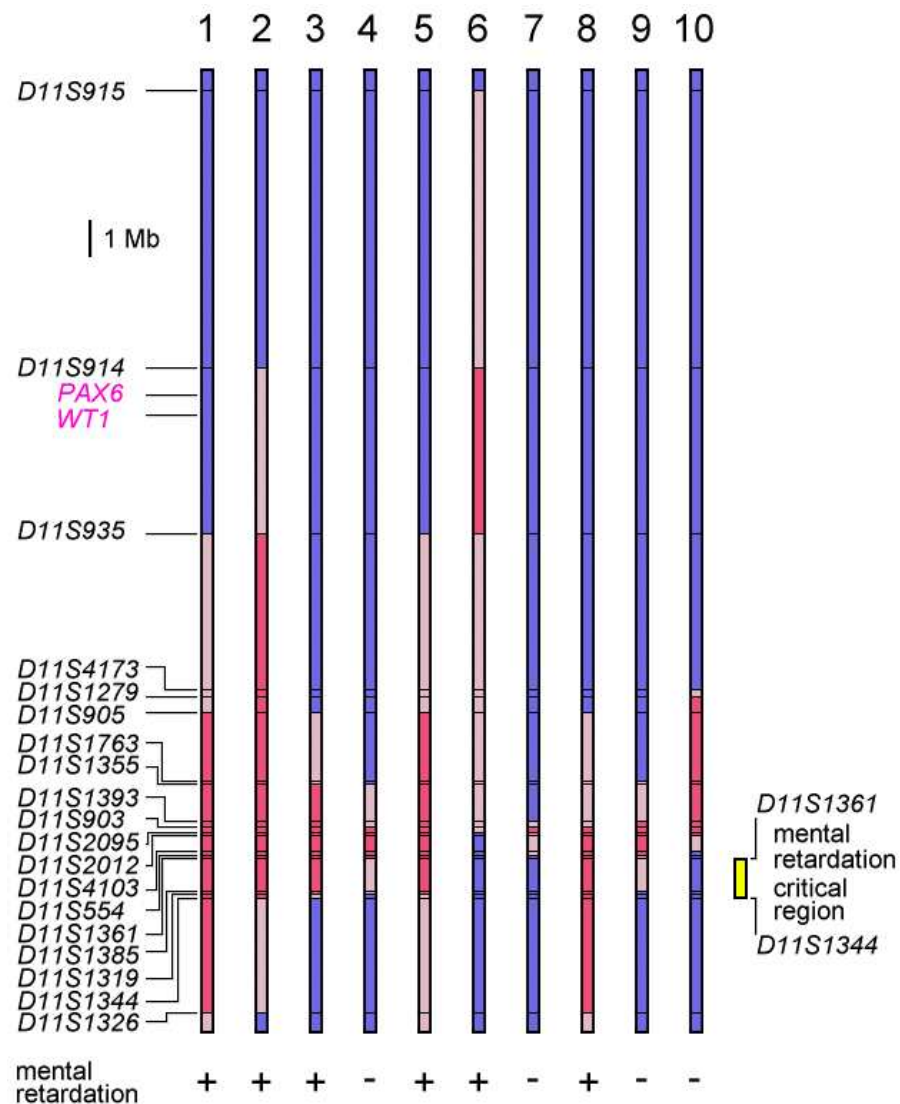


Figure 9.4: Refinement of the critical interval for the mental retardation locus in P11pDS. Mental retardation is not a feature of the new P11pDS case — case 10. Placement in context with previously published and molecularly characterised deletions, variably associated with mental retardation, narrows the interval for the relevant locus in the *D11S1361*–*D11S1344* region. Definitively deleted segments are shown *in red* with windows of uncertainty – because of limited marker coverage or non-informativeness – *in pink*; *blue* marks regions that are unambiguously present. Data from published cases were collated and integrated using the local physical map (Ensembl v. 16.33.1 — see appendix F). The *PAX6* and *WT1* genes provide a useful landmark; markers run top-to-bottom in the telomere-to-centromere direction. References: case 1 (Lorenz et al. 1990; Bartsch et al. 1996); case 2 (Bartsch et al. 1996); case 3 (Shaffer et al. 1993; Bartsch et al. 1996); case 4 (Bartsch et al. 1996); case 5 (Potocki and Shaffer 1996); case 6 (McGaughan et al. 1995; Wuyts et al. 1996); case 7 (Wuyts et al. 1999); case 8 (Yamamoto et al. 2001); case 9 (Hall et al. 2001); case 10, this study.

9.4 Roles of *Alx4* and *Msx2* in Skull Development

9.4.1 Insights from Mutant Phenotypes

The contraction, over time, of the calvarial gaps caused by *Alx4* and *Msx2* mutations in humans and mice and the fact that overt differentiation can be reached even in the double null mutant *Alx4*^{-/-} ; *Msx2*^{-/-} (see chapters 3 and 6) suggest that there is no permanent defect in osteogenesis but a growth delay. On the cellular level, this retardation could reflect reduced proliferation, an increased rate of death of precursors, or impaired early differentiation that results in a subsequent developmental lag.

The positive influence of *Msx2* on proliferation of osteoblast precursors and/or pre-osteoblasts is well established (Liu et al. 1999; Dodig et al. 1999) and in accordance with the roles of the *Msx* proteins in other systems (Bendall and Abate-Shen 2000). Indeed, in *Msx2*^{-/-} mice proliferation in the developing calvarial bones is reduced (Satokata et al. 2000; Ishii et al. 2003). However, this effect becomes apparent at a rather advanced stage during development and is preceded by a differentiation defect of the ectomeninx, as judged from expression of early markers, including *Runx2* (Ishii et al. 2003). Contrary to the expectations dictated by a simple anti-differentiation model for *Msx2* (Liu et al. 1999; Dodig et al. 1999), no precocious differentiation is observed (Ishii et al. 2003). The apparent repressive influence of *Msx2* on genes encoding products of terminal differentiation on the one hand (Hoffmann et al. 1994; Towler et al. 1994; Dodig et al. 1996; Hoffmann et al. 1996; Newberry et al. 1997a; Newberry et al. 1997b) and this requirement for duly cell commitment, just after initiation of the skeletogenic condensations, on the other could be reconciled if the *Msx2* functions are stage-specific — i.e. depend on the position of the cell along the pathway of osteoblastic differentiation. It is clear though that neural crest migration and apoptosis are not perturbed in the head of *Msx2*^{-/-} mice (Ishii et al. 2003).

An equivalent analysis for *Alx4* in the calvarial mesenchyme has not been performed but the bone plates are thinner at E16.5 and expression of *Fgfr1* and *Fgfr2* appeared decreased (Antonopoulou 2002). Extrapolating from the cell defects observed in the frontonasal mesenchyme of *Cart1*^{-/-} and *Alx4*^{-/-}; *Alx3*^{-/-} mutants (see section 9.4.5), depletion of the precursor pool through aberrant apoptosis is a possibility (Zhao et al. 1996; Beverdam et al. 2001; Beverdam 2001). It remains to be seen if this holds true for the skull vault or if there is an impact on proliferation. In *Foxc1*^{-/-} mice (see section 9.4.6), both *Alx4* and *Msx2* are down-regulated but only proliferation, not apoptosis, is affected (Rice et al. 2003).

9.4.2 Insights from Normal Expression Patterns

When considered globally, the expression patterns of the genes belonging to the *Alx* and *Msx* groups display significant differences and localised overlap. *Alx4*, *Cart1*, and *Alx3* transcripts are almost universally confined to mesenchymal populations of various origins — paraxial or lateral plate mesoderm or neural crest (Zhao et al. 1994; Qu et al. 1997a; Qu et al. 1997b; Takahashi et al. 1998; Hudson et al. 1998; ten Berge et al. 1998; Beverdam and Meijlink 2001). *Msx2* and *Msx1* expression extends beyond the mesenchyme in epithelial and neural tissues (Bendall and Abate-Shen 2000).

When the scope is restricted to *Alx4* and *Msx2* expression during skeletal development another difference emerges. Co-expression in developing bones occurs irrespective of the mode of ossification or the tissue origins, but contrary to *Msx2*, *Alx4* can only be switched on in cells committed to the osteoblast lineage and not in pre-cartilaginous condensations or immature chondrocytes (see chapter 5 and Hudson et al. (1998)). *Msx2* can be active in a *Sox9*-positive environment (Semba et al. 2000; Takahashi et al. 2001) but this selectivity of *Alx4* expression has not been explored. Given that *Runx2* is active well in advance of overt differentiation it may even exert a direct influence on *Alx4*.

9.4.3 Insights from Responses to Signalling Molecules

As discussed in section 1.4.5, the responsiveness of *Msx2* to Bmp signalling in the skull vault (Kim et al. 1998) is likely to be of physiological significance. Recently, Rice et al. (2003) demonstrated that *Alx4* can also be induced by exogenous Bmp2.

9.4.4 Insights from Conserved Domains and Protein Interactions

Apart from the homeodomain, *Msx2* is devoid of any other major motif but, as mentioned in chapter 8, *Msx* proteins display very broad binding potential, being able to interact with a range of proteins from ubiquitous components of the basal transcriptional apparatus to specific transcription factors and histone deacetylases (Zhang et al. 1996a; Zhang et al. 1997; Newberry et al. 1997b; Wu et al. 1997; Newberry et al. 1998; Bendall et al. 1998; Newberry et al. 1999; Bendall et al. 1999; Shetty et al. 1999; Zhou et al. 2000a; Mehra-Chaudhary et al. 2001; Masuda et al. 2001; Willis et al. 2002). To a great extent, this potential underlies their generic suppressive influence. On the other hand, the majority of the *in vivo* DNA targets for the homeodomain remain elusive. A report that *Msx2* can complex with and repress the transcriptional activity of *Cbfa1*, awaits verification (Shirakabe et al. 2001).

Outside of the homeodomain there are two regions in the *Alx4* protein with documented interaction potential: the C-terminal aristaless/OAR domain and the N-terminal poly(P/Q) repeat. There is evidence that the former serves as a switch: intramolecular interactions with the rest of the protein, the default status, attenuate DNA binding and transactivation; intermolecular interactions could release this repressive effect (Amendt et al. 1999; Norris and Kern 2001; Brouwer et al. 2003). Naturally, identification of variants within the domain could be of great interest. The unusually long poly(P/Q) element bears some resemblance to the recognition site of the SH3 domain (Sudol 1998), a widely-distributed interaction module in proteins involved in signal transduction. It has been shown to

bind the high-mobility-group box of the transcription factor Lef1 (Boras and Hamel 2002), a known nuclear effector of Wnt signalling that complexes with β -catenin.

9.4.5 Insights from Genetic Interactions

Double and triple mutants of *Alx4*, *Cart1*, and *Alx3*, carrying various allelic combinations, have been bred (Qu et al. 1999; Beverdam et al. 2001; Beverdam 2001) and *Msx2*^{-/-}; *Msx1*^{-/-} mutant mice have also been generated (Satokata et al. 2000). In addition, the phenotype of double *Msx2*^{-/-}; *Twist1*^{-/+} mutant mice was recently described in detail (Ishii et al. 2003). Other published compound mutants are the *Alx4*^{-/-}; *Shh*^{-/-} and *Alx4*^{-/+}; *Bmp4*^{-/+} mice but no worsening or modification of the *Alx4*-associated calvarial defect was mentioned in the available phenotypic accounts (Dunn et al. 1997; te Welscher et al. 2002b).

Although the *Alx3*^{-/-} status itself does not produce any abnormalities, when combined with *Alx4* and/or *Cart1* null alleles it acts as a penetrance enhancer; in fact, the sensitivity of the *Alx4*- and *Cart1*-associated defects on the genetic background complicates the analysis of compound mutants. Nonetheless, the major novel defects in *Alx4*^{-/-}; *Alx3*^{-/-} mice are pronounced clefting in the nasal region and distal truncation of the jaws; the skull is distorted but there is no exacerbation of the calvarial defect (Beverdam et al. 2001; Beverdam 2001). Apart from the *Cart1*-specific (but variably present) neural tube closure defect, *Alx4*^{-/-}; *Cart1*^{-/-} animals manifest an equivalent facial cleft and severe preaxial polydactyly; the skull vault bones are diminished, although the primary exencephaly does not permit a straightforward phenotypic analysis (Qu et al. 1999). Triple *Alx4*^{-/+}; *Cart1*^{-/+}; *Alx3*^{-/+} heterozygotes show facial clefts and minor defects of the axial and appendicular skeleton but no calvarial defect is evident (Beverdam 2001). These lines of genetic data indicate that the *Alx4* group proteins have redundant and specific functions. In the fully redundant mode, they act incrementally to promote fusion of the anterior midline structures. Partial redundancy exists during outgrowth of the

branchial arches and patterning of the limb bud. Haploinsufficiency of *ALX4* in the skull vault in humans illustrates the specific functions. No skull defects are seen in *Alx3*^{-/-} and even in *Cart1*^{-/-} mice when the neural tube closes properly (Beverdam et al. 2001; Beverdam 2001) although both genes are active in the calvarial mesenchyme (Zhao et al. 1994; ten Berge et al. 1998).

In the *Msx2*^{-/-} ; *Msx1*^{-/-} embryos that survive until late gestation the nasal, frontal, parietal, interparietal, supraoccipital, exoccipital, and squamous temporal bones are absent and the mandible markedly reduced. This strong genetic interaction reveals redundancy at a very early stage during calvarial osteogenesis (Satokata et al. 2000). Defective cell migration or failure of condensation could underlie the abnormalities thus masking any interaction during the later stages of interest. The *Msx2*^{-/-} ; *Twist1*^{-/+} mutant exhibits a wide calvarial defect, uncovering genetic additivity (Ishii et al. 2003). Both the early differentiation defect and the reduction in proliferation caused by loss of *Msx2* (see section 9.4.1) are aggravated in the *Twist1*^{-/+} background; surprisingly, there is no indication of enhanced apoptosis.

9.4.6 Insights from Dysregulation in Other Mutants

Msx2 and *Twist1* act non-hierarchically during calvarial osteogenesis. They exert additive effects on the skull vault (see section 9.4.5) and there is no down-regulation of either gene in the reciprocal mutant; Ishii et al. (2003) alluded to physical contacts between the *Msx2* and *Twist1* proteins. This is not the case for *Alx4* and *Twist1*. Investigations of gene expression in *Twist1*^{-/-} embryos up to mid-gestation revealed diminished expression of *Alx4* in the head mesenchyme and the forelimb bud; this effect is tissue-specific and appeared to be more-or-less characteristic for all the members of the *Alx* group (Soo et al. 2002; O'Rourke et al. 2002; Loebel et al. 2002). Furthermore, Loebel et al. (2002) identified conserved elements which

could be recognised by Twist1 upstream of mouse and human *Alx4* and *Alx3* — *Alx4* could be an immediate target of Twist1.

More recently, Rice et al. (2003) studied *Msx2* and *Alx4* in *Foxc1*^{-/-} mice. *Foxc1* encodes a winged-helix transcription factor and mutant mice exhibit multiple skeletal anomalies (Kume et al. 1998) although the most conspicuous defect is seen in the skull vault: the calvarial bones are rudimentary, have not expanded superiorly, and there is a hydrocephalic presentation (see Table 1.2). Defective proliferation underlies this growth defect which turns out to be the primary cause of the phenotype — and not secondary to exencephaly. Halted appositional growth correlates with specific down-regulation of *Msx2* and *Alx4* at the osteogenic fronts and in the meninges; at the same time, Bmp-responsiveness of both genes (see section 9.4.3) is abolished.

9.4.7 Insights from Homologues

Msx2 and *Alx4* belong to ancient families of homeoproteins. *msh*- and *al*-like genes have been identified in vertebrates and invertebrates, including cnidarians (Schummer et al. 1992; Smith et al. 2000), and certain of their archetypical functions can still be recognised in mammals. However, their involvement in skeletogenesis appears to be a novelty of the deuterostome line (Tan et al. 1998; Etensohn et al. 2003).

In *Drosophila*, *msh* is essential for correct dorso-ventral patterning of the neuroectoderm, the mesoderm, the wing imaginal disc, as well as regional specification of muscle progenitors (Lord et al. 1995; D'Alessio and Frasch 1996; Isshiki et al. 1997; Nose et al. 1998; Milan et al. 2001). On the other hand, *al* controls the morphogenesis of the extremities of the appendages – the arista of the antenna, the claw organs of the legs, the tip of the wing – and the posterior notum of the body (Schneitz et al. 1993; Campbell et al. 1993).

9.5 Future Directions

A complementary experiment to test the interaction between *Alx4* and *Msx2* at the genetic level would be to bring together loss-of-function and gain-of-function mutations. Liu et al. (1999) developed transgenic mice harbouring an *Msx2* minigene driven by a 5.2 kb segment of the native promoter — *Tg(-5.2 kb Msx2)*. Although the copy number is not known, certain stable lines of transgenic hemizygotes were established that exhibit calvarial overgrowth, causing sutural invasion and bone overlap (see section 1.4.5). There is no true synostosis, but the phenotype appears distinct enough to allow for any influence of *Alx4*^{-/-} to be scored. Assuming simple additivity, the combination is likely to produce an intermediate phenotype. Generation of the double *Alx4*^{-/-} ; *Twist1*^{-/+} mutant would also be of interest. Given that *Alx4* has a specific role during calvarial osteogenesis, if it also represents a major target of *Twist1* in the skull mesenchyme (see section 9.4.6) the combination should not aggravate the *Alx4*-caused defect.

Intuitively, a direct or indirect interaction of *Alx4* and *Msx2* with components of the cell cycle machinery seems plausible. The association of *Alx4* with a member of the retinoblastoma protein family, p130, is not an artefact (Hudson et al. 1998). Homeodomains of the paired-like subclass can interact with pRb, p107 and p130 via helices I and II and this association counteracts the activation of target promoters (Wiggan et al. 1998) — an effect analogous to E2F inhibition. However, this avenue has not been explored for *Alx4*. In addition, an important generic attribute of the *Msx* proteins appears to be the ability to up-regulate cyclin D1, one of the G₁-cdk complex cyclins (Hu et al. 2001). This effect is indirect but specific for the G₁-cdk complex and could provide a mechanistic explanation for their anti-differentiation effects: it prevents cell cycle exit and, under the in vitro conditions used, it was accompanied by a subtle increase in proliferation.

Another aspect of cell behaviour that has to be regulated during condensation and differentiation is cell adhesion. There is some evidence to suggest that expression of *Msx* proteins could decrease cadherin-dependent aggregation and confer differential sorting properties in cell populations (Lincecum et al. 1998). More specifically, Boras and Hamel (2002) demonstrated that *Alx4* and *Lef1* not only interact but also bind on adjacent sites on the promoter of *Ncam1* and modulate the expression of its product, the neural cell adhesion molecule, in mesenchymal cells.

There is a growing body of data to indicate that *Alx4* and *Msx2* could be downstream targets of Wnt signals and the pathway could be of importance during calvarial osteogenesis. Firstly, the *Alx4*-*Lef1* interaction itself has been mentioned (Boras and Hamel 2002). Secondly, there is a close relationship between *Wingless* (*Wg*), *Dpp*, and *al* during appendicular development in the fly (Campbell et al. 1993). And thirdly, the Bmp and Wnt signalling pathways have been shown to converge and interact on the *Msx2* promoter (Hussein et al. 2003). In addition, there is a well-established relationship between *Alx4* expression and Shh signalling in the limb bud. Preaxial polydactyly is associated with ectopic *Shh* transcription (Chan et al. 1995; Qu et al. 1997b) and te Welscher et al. (2002a) proposed that the repressor form of *Gli3* may direct expression of *Alx4* in the anterior aspect which, in turn, helps to constrain *Shh* activity. The Shh/Ihh and Wnt signalling pathways have attracted little or no attention during skull vault development so far. Their impact on and their modulation by *Alx4* and *Msx2* may define new research vistas. As in endochondral ossification, a negative feedback circuit should operate in order to maintain the precursor population as differentiation progresses, chiefly due to enhanced Fgf signalling. The Shh/Ihh and Wnt pathways are good candidates to transduce this regulatory signal; promisingly, it has been shown that *Lef1* can inhibit the *Cbfa1*-dependent activation of *Bglap1*, encoding osteocalcin (Kahler and Westendorf 2003).

Appendix A

The Human *ALX4* cDNA Sequence

A partial cDNA sequence of the human *ALX4*, encompassing the coding region, was assembled by direct sequencing of an RT-PCR product obtained from a normal fibroblast cell line (see section 2.5). Beneath the DNA sequence, in both the forward and the reverse strands, the deduced protein sequence is shown in bold. The nucleotides at the exon boundaries are underlined and the major protein features are highlighted: the poly(P/Q) tract (*yellow*); the homeodomain (*red*); and the aristaless/OAR domain (*magenta*). Two functional initiation codons are present and the corresponding methionines are underlined. The first one is used as the reference point in numbering. Accession number: AJ404888.


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GAAGGCCGACTCGGAGAGCAACAAGGGCAAGAAGCGGCGGAACCGGACCACCTTCACCAG
661 -----+-----+-----+-----+-----+-----+ 720
CTTCCGGCTGAGCCTCTCGTTGTTCCCGTTCTTCGCCGCCTTGGCCTGGTGAAGTGGTC
K A D S E S N K G K K R R N R T T F T S

CTACCAGCTGGAGGAGCTGGAGAAGGTCTTCCAGAAGACCCACTACCCAGACGTGTATGC
721 -----+-----+-----+-----+-----+-----+ 780
GATGGTCGACCTCCTCGACCTCTTCCAGAAGGTCTTCTGGGTGATGGGTCTGCACATACG
Y Q L E E L E K V F Q K T H Y P D V Y A

GCGGGAACAGCTGGCCATGAGGACAGACCTCACTGAGGCCCGCGTGCAGGTCTGGTTCCA
781 -----+-----+-----+-----+-----+-----+ 840
CGCCCTTGTCGACCGTACTCCTGTCTGGAGTGACTCCGGGCGCACGTCCAGACCAAGGT
R E Q L A M R T D L T E A R V Q V W F Q

GAACCGAAGGGCCAAGTGGAGGAAGCGGGAGCGTTTTGGGCAGATGCAGCAGGTTCAAC
841 -----+-----+-----+-----+-----+-----+ 900
CTTGGCTTCCCGTTACCTCCTTCGCCCTCGCAAACCCGCTCTACGTGCTCCAAGCTTG
N R R A K W R K R E R F G Q M Q Q V R T

CCACTTCTCCACTGCATATGAGCTGCCCTCCTCACCCGAGCTGAGAACTACGCCAGAT
901 -----+-----+-----+-----+-----+-----+ 960
GGTGAAGAGGTGACGTATACTCGACGGGGAGGAGTGGGCTCGACTCTTGATGCGGGTCTA
H F S T A Y E L P L L T R A E N Y A Q I

TCAGAACCCGTCCTGGCTCGGCAACAACGGGGCTGCCTCACCAGTGCCAGCCTGCGTGGT
961 -----+-----+-----+-----+-----+-----+ 1020
AGTCTTGGGCAGGACCGAGCCGTTGTTGCCCCGACGGAGTGGTCACGGTCGGACGCACCA
Q N P S W L G N N G A A S P V P A C V V

CCCCTGCGACCCGGTGCCTGCCTGCATGTCCCCTCATGCCACCCCCCTGGCTCTGGGGC
1021 -----+-----+-----+-----+-----+-----+ 1080
GGGGACGCTGGGCCACGGACGGACGTACAGGGGAGTACGGGTGGGGGGACCGAGACCCCG
P C D P V P A C M S P H A H P P G S G A

CAGCAGCGTCACCGACTTCTGAGTGTGTCTGGGGCTGGCAGTCACGTGGGCCAGACGCA
1081 -----+-----+-----+-----+-----+-----+ 1140
GTCGTCGCAGTGGCTGAAGGACTCACACAGACCCCGACCGTCAGTGCACCCGGTCTGCGT
S S V T D F L S V S G A G S H V G Q T H

CATGGGCAGCCTGTTTGGAGCAGCCAGCCTCAGCCCAGGCCTCAATGGCTACGAGCTCAA
1141 -----+-----+-----+-----+-----+-----+ 1200
GTACCCGTCGGACAAACCTCGTCGGTCGGAGTCGGGTCCGGAGTTACCGATGCTCGAGTT
M G S L F G A A S L S P G L N G Y E L N

CGGCGAGCCGGACCGCAAGACCTCGAGCATCGCGGCCCTCCGCATGAAGGCCAAGGAGCA
1201 -----+-----+-----+-----+-----+-----+ 1260
GCCGCTCGGCCTGGCGTTCTGGAGCTCGTAGCGCCGGGAGGCGTACTTCCGGTTCTCTCGT
G E P D R K T S S I A A L R M K A K E H

CAGTGCGGCCATTTCTGGGCCACATGACAGGGCACCCCTGCCCCGTCCCCACCTCGGGA
1261 -----+-----+-----+-----+-----+-----+ 1320
GTCACGCCGGTAAAGGACCCGGTGTACTGTCCCGTGGGGACGGGGCAGGGGTGGAGCCCT
S A A I S W A T *

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CACCATGGGCCACGCCCATGTTTTCCAGGCCCCAGCCTCCCACTCGACTTTCCTCTTAG
1321 -----+-----+-----+-----+-----+-----+ 1380
GTGGTACCCGGTGCGGGTACAAAAGGTCCGGGGTTCGGAGGGTGAGCTGAAAGGAGAATC

GAACCTGGCCTGGGCCAGGGGCCTGACCCTCAGCACTTTCAGCCGCCCCAAGTCTGAGGC
1381 -----+-----+-----+-----+-----+-----+ 1440
CTTGACCGGACCCGGTCCCCGGACTGGGAGTCGTGAAAGTCGGCGGGGTTCAGACTCCG

CCCGTGGACTGCTGGGAGGGAGGGGGCAGCAGGCCCTGGCCCCTCCCTGGCA
1441 -----+-----+-----+-----+-----+-----+ 1493
GGGCACCTGACGACCCTCCCTCCCCGTCGTCCGGGGACCGGGGAGGGACCGT
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Appendix B

List of Families

B.1 Families with Typical Enlarged Parietal Foramina and/or Skull Ossification Defects

The research panel of the laboratory, comprising families and individuals in whom a diagnosis of classical PFM or less typical skull ossification defects has been established or considered likely, is tabulated below — detailed data for the families are provided in section 2.2. Following the family number, each person is identified either by a pedigree number (referring to the current work if the kindred is displayed; or to a previous publication if this is not the case) or by the biological relationships with other members. For mutation carriers the year of birth is also recorded. The phenotypic designations employed denote the status (affected, A; unaffected, U; intermediate, I; or unknown, ?) and, in addition, indicate the mode of ascertainment — by radiography or CT imaging, (R); by palpation during clinical examination, (E); or by personal history, (H). The unique laboratory identifier is cited for the DNA samples, isolated from blood or buccal epithelium and lymphoblastoid cell lines, if the latter have been established. The mutations identified either in the current work or reported in previous studies are summarised in the last column.

Families with PFM/Skull Ossification Defects Investigated for Mutations in *ALX4* and *MSX2*

Family	Member	Year of Birth	Status	Sample	Cell line	Mutation Identified
1	I-2	1930	A(R)	1542	1558	<i>ALX4</i> 653G → A (ALX4 R218Q)
1	II-6	1961	A(R)	1540		<i>ALX4</i> 653G → A (ALX4 R218Q)
1	II-7		U(H)	1543		–
1	III-5	1988	A(R)	1541		<i>ALX4</i> 653G → A (ALX4 R218Q)
1	III-6	1991	A(R)	1546		<i>ALX4</i> 653G → A (ALX4 R218Q)
2	III-6	1972	A(R)	1563	1601	<i>ALX4</i> 418C → T (ALX4 Q140X)
2	IV-4	1992	A(R)	1564		<i>ALX4</i> 418C → T (ALX4 Q140X)
2	IV-5	1995	I(R)	1565		<i>ALX4</i> 418C → T (ALX4 Q140X)
2	IV-6	1998	A(R)	1566		<i>ALX4</i> 418C → T (ALX4 Q140X)
2	III-5		U(E)	1562		–
2	II-3	1950	A(R)	1596		<i>ALX4</i> 418C → T (ALX4 Q140X)
2	I-2	1927	A(R)	1595		<i>ALX4</i> 418C → T (ALX4 Q140X)
2	II-4	1956	U(R)	1597		<i>ALX4</i> 418C → T (ALX4 Q140X)
2	III-8	1982	A(R)	1598		<i>ALX4</i> 418C → T (ALX4 Q140X)
2	II-1		U(R)	2064		–
2	III-4		U(R)	2063		–
2	III-7	1980	A(R)	2062		<i>ALX4</i> 418C → T (ALX4 Q140X)
3	IV-1	1994	A(R)	1615	1638	<i>ALX4</i> 736C → T (ALX4 Q246X)
3	III-3		U(E)	1616		–
3	III-4	1958	A(R)	1617		<i>ALX4</i> 736C → T (ALX4 Q246X)
3	II-1	1931	A(R)	2113		<i>ALX4</i> 736C → T (ALX4 Q246X)
3	II-5		U(H)	2106		–
3	IV-2		U(R)	2107		–
3	III-1		U(H)	2127		–
3	III-2	1960	A(H)	2154		<i>ALX4</i> 736C → T (ALX4 Q246X)
4	I-2	1934	A(R)	2840		<i>ALX4</i> 620C → A (ALX4 S207X)
4	II-3	1963	A(R)	2835		<i>ALX4</i> 620C → A (ALX4 S207X)
4	II-4		U(E)	2836		–
4	III-1	1988	A(R)	2838		<i>ALX4</i> 620C → A (ALX4 S207X)
4	III-2	1989	A(R)	2837		<i>ALX4</i> 620C → A (ALX4 S207X)
4	III-3	1993	A(R)	2839		<i>ALX4</i> 620C → A (ALX4 S207X)
5	I-2	1947	A(R)	1918	1973	<i>ALX4</i> 653G → A (ALX4 R218Q)
5	II-2	1977	A(R)	1919		<i>ALX4</i> 653G → A (ALX4 R218Q)
5	III-2	1999	A(R)	1920		<i>ALX4</i> 653G → A (ALX4 R218Q)
6	II-2	1961	A(R)	2536	2561	Deletion encompassing <i>ALX4</i> and <i>EXT2</i>
6	II-1	1959	A(R)	2535		Deletion encompassing <i>ALX4</i> and <i>EXT2</i>
6	III-1	1989	A(R)	2537		Deletion encompassing <i>ALX4</i> and <i>EXT2</i>
6	III-2	1995	A(R)	2538		Deletion encompassing <i>ALX4</i> and <i>EXT2</i>
7	II-1	1968	A(H)	2236		<i>MSX2</i> 417_418delCA (<i>MSX2</i> H139fsX243)
8	III-1	1970	A(R)	2807		<i>MSX2</i> 505_508dupATTG (<i>MSX2</i> A170fsX245)
8	IV-1	1997	A(R)	2952		<i>MSX2</i> 505_508dupATTG (<i>MSX2</i> A170fsX245)
8	II-1	1939	A(R)	2975		<i>MSX2</i> 505_508dupATTG (<i>MSX2</i> A170fsX245)
9	II-1		U(H)	1369	1606	–
9	II-2	1940	U(R)	1370		Deletion encompassing <i>MSX2</i>
9	III-1	1964	A(R)	1372		Deletion encompassing <i>MSX2</i>
9	III-2		U(H)	1373		–
9	IV-1	1991	A(R)	1374		Deletion encompassing <i>MSX2</i>
9	IV-2	1994	A(R)	1375		Deletion encompassing <i>MSX2</i>
9	II-4		U(H)	1388		–
9	II-5	1930	?	1389		Deletion encompassing <i>MSX2</i>
9	III-4	1957	A(R)	1371		Deletion encompassing <i>MSX2</i>
9	III-3	1947	A(H)	1434		Deletion encompassing <i>MSX2</i>
10	I-1		U(H)	1529		–
10	II-1	1991	A(R)	1527		<i>MSX2</i> 515G → A (<i>MSX2</i> R172H)
10	I-2	1957	I(R)	1528		<i>MSX2</i> 515G → A (<i>MSX2</i> R172H)

Family	Member	Year of Birth	Status	Sample	Cell line	Mutation Identified
11	I-1	1976	A(R)	1532		MSX2 475_480delCGCAAG (MSX2 R159_K160del)
11	II-1	1993	A(R)	1530	1552	MSX2 475_480delCGCAAG (MSX2 R159_K160del)
11	I-2		U(H)	1531		–
11	brother of I-1		?	2004		–
11	grandmother of I-1		?	2002		–
11	grandfather of I-1	1936	?	2003		MSX2 475_480delCGCAAG (MSX2 R159_K160del)
11	sister of I-1		?	2005		–
11	son of previous		?	2006		–
11	sister of I-1		?	2007		–
11	son of previous		?	2008		–
12	mother		U(H)	1526		
12	proband		A(H)	1523	1557	
12	sister		U(H)	1525		
12	father		U(H)	1524		
13	proband		A(R)	1639	1661	
13	mother		U(H)	1641		
13	father		?	1640		
14	proband		A(R)	1645	1698	
14	father		U(E)	1646		
14	mother		A(E)	1647		
15	proband		A(R)	1839		
16	maternal grandmother		?	1854		
16	mother		U(R)	1855		
16	proband		A(R)	1856		
17	father of proband		?	1916	1953	
18	proband		A(E)	2009		
18	mother		U(H)	2010		
18	father		U(H)	2011		
19	mother		U(H)	2307		
19	father		U(H)	2308		
20	paternal grandfather		U(H)	2425		
20	paternal grandmother		U(H)	2426		
20	mother		U(H)	2427		
20	proband		A(E)	2428		
20	father		A(H)	2429		

B.2 Families with Craniosynostosis

From an initial research panel of 211 samples, consisting of families and individuals with a diagnosis of craniosynostosis where the most common mutations in the *FGFR2*, *FGFR3*, *TWIST1*, and *FGFR1* loci had been excluded, ambiguous variants in *ALX4* were found in the two families listed below — for additional clinical details see section 2.2. The pedigree numbers refer to the current work and for the variant carriers the year of birth is mentioned. Individuals are classed as either affected, A; or unaffected, U. The unique laboratory identifier is cited for the DNA samples while the sequence changes are indicated in the last column.

Families with Craniosynostosis Featuring Uncommon Variants in *ALX4*

Family	Member	Year of Birth	Status	Sample	Change Identified
21	II-1	1982	A	1291	<i>ALX4</i> 605T→G (L202W)
22	I-1		U	2477	–
22	I-2	1961	U	2478	<i>ALX4</i> 314_325delCGCAGCCGCAGC (P105_Q108del)
22	II-1	2000	A	2479	<i>ALX4</i> 314_325delCGCAGCCGCAGC (P105_Q108del)

Appendix C

Catalogue of Oligonucleotides

Project-specific or highly relevant oligonucleotides, used for a variety of purposes – PCR amplification, sequencing, hybridisation – are listed below. Primer pairs for established short tandem repeat loci (i.e. markers assigned 'D' numbers) are not included but can be readily retrieved through The Genome Database — see appendix F. For the *ALX4* allele-specific oligonucleotides there are discrepancies between primer names and sequence variants; many oligonucleotides were designed when gene characterisation was still in progress and a tentative numbering system was used. All sequences are written in the 5'-to-3' direction.

List of Oligonucleotides

Designation	Sequence (5'→3')	Location	Comments
ALX4-F3	GGAATTCGCAAGGAGTGCACAGCCACAGC	5' UTR of <i>ALX4</i>	Has an <i>Eco</i> RI tail; pair ALX4-F3/-R3 amplifies the first exon of <i>ALX4</i>
ALX4-R3	ACCAGTTTCAAGGGATGCGGAAG	3' of exon 1 of <i>ALX4</i>	Pair ALX4-F3/-R3 amplifies the first exon of <i>ALX4</i>
ALX4-F2	CCCCCTGACATTCCTCTCTT	5' of exon 2 of <i>ALX4</i>	Pair ALX4-F2/-R2 amplifies the second exon of <i>ALX4</i>
ALX4-R2	GCTTACCAGCCTCACTCCAGGT	3' of exon 2 of <i>ALX4</i>	Pair ALX4-F2/-R2 amplifies the second exon of <i>ALX4</i>
ALX4-F4	GGGAACAGTTTGACTGCCTGAA	5' of exon 3 of <i>ALX4</i>	Pair ALX4-F4/-R4 amplifies the third exon of <i>ALX4</i>
ALX4-R4	CTCCTCCAAGGGGCTCATTCTCA	3' of exon 3 of <i>ALX4</i>	Pair ALX4-F4/-R4 amplifies the third exon of <i>ALX4</i>
ALX4-F5	GAGCCCCCTCCACACCACACT	5' of exon 4 of <i>ALX4</i>	Pair ALX4-F5/-R5 amplifies the fourth exon of <i>ALX4</i>
ALX4-R5	GGAATTCACCTGGCTTTCTCCACTGCCTGT	3' UTR of <i>ALX4</i>	Has an <i>Eco</i> RI tail; pair ALX4-F5/-R5 amplifies the fourth exon of <i>ALX4</i>
ALX4-F7	TAACAAGTTCAGCCCCAGTCGTC	Exon 1 of <i>ALX4</i> ; 5' of poly(P/Q) repeat	One mismatch compared to the final sequence
ALX4-R7	CGCTGCAAGTAAAGATGCGGTTG	Exon 1 of <i>ALX4</i> ; 3' of poly(P/Q) repeat	
ALX4-F8	GCCTCAATGGCTACGAGCTCAACG	Exon 4 of <i>ALX4</i>	
ALX4-R8	ACTGTGCTCCTTGGCCTTCATGC	Exon 4 of <i>ALX4</i>	
ALX4-F10	AGCTGGAGGAGCTGGAGAAGGTCT	Exon 2 of <i>ALX4</i>	
ALX4-R10	GTCTGTCTCATGGCCAGCTGTT	Exon 2 of <i>ALX4</i>	
ALX4 Q140X	CCTTCCTAGAGTTTG		Detects the mutation 418C→T
ALX4 R218Q	CGGAACCAGACCACC		Detects the mutation 653G→A
ALX4 Q246X	GCCAGCTATTCCTCGC		Detects the mutation 736C→T
ALX4 R35T	CCTTTTACGGCATT		Detects the variant 104G→C
ALX4 P102S	TGCGGCGACGGCTGG		Detects the variant 304C→T
ALX4 708C-A	CAGACCTACCCAGCC		Detects the variant 594C→A
ALX4 842G-A	TGTATGCACGGGAAC		Detects the variant 729G→A
ALX4 892-11G-A	CTGTTTACGCTTTG		Detects the variant 778-11G→A
ALX4 996C-T	GGGTGAGAAGGGGCA		Detects the variant 879C→T
ALX4 1188C-T	GGCCACATGACTGC		Detects the variant 1074C→T
ALX4 1578C-T	TGTGCCCAGGAGCAG		Detects the variant 1464C→T
404M15-1-F	TCAACCCCCAATCCCATCTAC	Distal end of clone RPCI-3 404m15	
404m15-1-R	CCCTGAACTCTGAGAATTGCCC	Distal end of clone RPCI-3 404m15	
404M15-3-F	GGCTGCATGTGATTGTGGATGAGG	Proximal end of clone RPCI-3 404m15	
404M15-3-R	AACAGCCGGAACGTGTAGAACCCC	Proximal end of clone RPCI-3 404m15	
102D9T7-F	GTGTACATTTATGTAACCAC	Upstream of <i>EXT2</i>	
102D9T7-R	TCATAATCACCCATAACTGG	Upstream of <i>EXT2</i>	
EXT2-EX3-F	GCGTTTATTTTCCCCTGTC	In/around exon 3 of <i>EXT2</i>	
EXT2-EX3-R	TAGCACAAGCCTGTCACAC	In/around exon 3 of <i>EXT2</i>	
EXT2-EX8-F	TCTCGCTTGCTCACTTAAAC	In/around exon 8 of <i>EXT2</i>	

Designation	Sequence (5'→3')	Location	Comments
EXT2-EX8-R	ACCCATGCCTCCTTACTTATC	In/around exon 8 of <i>EXT2</i>	
EXT2-EX9-F	TGGGGAGAGAATGGAGATAAAC	In/around exon 9 of <i>EXT2</i>	
EXT2-EX9-R	ATGACTGAGAAAAATGGAGGC	In/around exon 9 of <i>EXT2</i>	
EXT2-EX10-F	TCCCCTGACACAGTTCTACC	In/around exon 10 of <i>EXT2</i>	
EXT2-EX10-R	TGCTCTTTTGAAATTGTCCCC	In/around exon 10 of <i>EXT2</i>	
EXT2-EX11-F	CCCAACTCAGTAAGCTATTACC	In/around exon 11 of <i>EXT2</i>	
EXT2-EX11-R	TCAGTTTGTACCTTGCC	In/around exon 11 of <i>EXT2</i>	
EXT2-3GN-F	GAGGAATGCTGATACCCCTCTG	In/around exon 14 of <i>EXT2</i>	
EXT2-3GN-R	GTGGCTAAAGCCCAAACTG	In/around exon 14 of <i>EXT2</i>	
MSX2-E1FB	CCGCAGCAAAAAAGTTTGAGT	5' UTR of <i>MSX2</i>	Pair MSX2-E1FB/-E1RB amplifies the first exon of <i>MSX2</i>
MSX2-E1RB	GTACCTCAGCCCTGGAACAC	3' of exon 1 of <i>MSX2</i>	Pair MSX2-E1FB/-E1RB amplifies the first exon of <i>MSX2</i>
MSX2-E2F	TTGCTAATCCGCTCCTCTCTCTG	5' of exon 2 of <i>MSX2</i>	Pair MSX2-E2F/-E2R amplifies the second exon of <i>MSX2</i>
MSX2-E2R	AAACAAGCATCCATCATGGAG	3' UTR of <i>MSX2</i>	Pair MSX2-E2F/-E2R amplifies the second exon of <i>MSX2</i>
MSX2-E1FC	GACAAGAAGCCGCCAAGGA	Exon 1 of <i>MSX2</i>	
MSX2-E1RC	CGCCGT GCCCGACAGCAGC	Exon 1 of <i>MSX2</i>	
MSX2-E2FB	CAGGAGGCAGAACTGGAAAAAG	Exon 2 of <i>MSX2</i>	
MSX2-E2RB	GGTTTGCAGCCATTTTCAG	Exon 2 of <i>MSX2</i>	
MSX2 417-418DELCA	GAGGAAACAAGACCAATC		Detects the mutation 417_418delCA
MSX2 505-508DUPATTG	TCCATTGATTGCAG		Detects the mutation 505_508dupATTG
#719	GCCCTGGAGTTCCTGACG		Detects the mutation 475_480delCGCAAG
MSX2-CA-F	ACGAAGCAACTTACCCATTATA		Forward primer for MSX2-CA
MSX2-CA-R	TCCCCTCTCAACTGAAAGCACTTAG		Reverse primer for MSX2-CA
mALX4 WT-F	CCTGACTTGTGGTGTCACTGC		Pair mALX4 WT-F/-R amplifies the wild-type allele of mouse <i>Alx4</i>
mALX4 WT-R	CAGCATTCTAGGTCTCCACAG		Pair mALX4 WT-F/-R amplifies the wild-type allele of mouse <i>Alx4</i>
mALX4 TD-F	TCTATAGATCTCTCGTGGGATCA		Pair mALX4 TD-F/-R amplifies the disrupted allele of mouse <i>Alx4</i>
mALX4 TD-R	CAGCATTCTAGGTCTCCACAG		Pair mALX4 TD-F/-R amplifies the disrupted allele of mouse <i>Alx4</i>
mALX4-F	CCCCCTGGAAAAGACCGATTCTGA		Pair mALX4-F/-R amplifies part of mouse <i>Alx4</i> cDNA that spans different exons in RT-PCR
mALX4-R	GGGGGATGACATCAGGAGTTGCAG		Pair mALX4-F/-R amplifies part of mouse <i>Alx4</i> cDNA that spans different exons in RT-PCR
SXG-2	TCTGGACGAAGAGCATCAGG		Pair SXG-2/-5 amplifies the disrupted allele of mouse <i>Msx2</i>
SXG-4	CCCTCTCTGCTCTAGGAC		Pair SXG-4/-5 amplifies the wild-type allele of mouse <i>Msx2</i>

Designation	Sequence (5'→3')	Location	Comments
SXG-5	GCCTGAGGGCAGCATAGGCT		Common primer for <i>Msx2</i> typing
mMSX2-F	AGACGGAGCACCGTGGATACAGGA		Pair mMSX2-F/-R amplifies part of mouse <i>Msx2</i> cDNA that spans different exons in RT-PCR
mMSX2-R	AGGGAGGGGGATGCTTTTATGCAA		Pair mMSX2-F/-R amplifies part of mouse <i>Msx2</i> cDNA that spans different exons in RT-PCR
mTWIST-F	GGCTCAGCTACGCCTTCTCCGTCT		Pair mTWIST-F/-R amplifies part of mouse <i>Twist1</i> cDNA that spans different exons in RT-PCR
mTWIST-R	TCCTGTCAGTGGCTGATTGGCAAG		Pair mTWIST-F/-R amplifies part of mouse <i>Twist1</i> cDNA that spans different exons in RT-PCR
mRUNX2-F	GACATCCCCATCCATCCACTCCAC		Pair mRUNX2-F/-R amplifies part of mouse <i>Runx2</i> cDNA that spans different exons in RT-PCR
mRUNX2-R	CCCCACCTCTTAGGGTTGCAAGA		Pair mRUNX2-F/-R amplifies part of mouse <i>Runx2</i> cDNA that spans different exons in RT-PCR
mGAPDH-F	TGAAGGTCGGTGTGAACGGATTGGC		Pair mGAPDH-F/-R amplifies part of mouse <i>Gapd</i> cDNA that spans different exons in RT-PCR
mGAPDH-R	CATGTAGGCCATGAGGTCCACCAC		Pair mGAPDH-F/-R amplifies part of mouse <i>Gapd</i> cDNA that spans different exons in RT-PCR
(CA) ₁₀	CACACACACACACACACA	N/A	Hybridisation probe for detection of alleles of short tandem repeat loci
(GATA) ₄	GATAGATAGATAGATA	N/A	Hybridisation probe for detection of alleles of short tandem repeat loci
pUC-F	GTTGTAAACGACGGCCAGT	N/A	Generic primer in plasmid polylinkers
pUC-R	CACACAGGAAACAGCTATG	N/A	Generic primer in plasmid polylinkers

Appendix D

Linkage Analysis Files

Two-point lod scores between the PFM phenotype and four short tandem repeat loci from the proximal part of chromosome 11p (*D11S1393*, *D11S903*, *D11S2095*, and *D11S554*) were computed in families 2 and 1 using MLINK — the underlying assumptions are elaborated in section 2.4. Each line in the primary pedigree files, cited below, conforms to the following convention: family name; identifier of individual (laboratory identifier if a sample was available — see appendix B; if not, fictitious, untyped samples were appended — the suffixes f, s, and c after the number denote father of, spouse of, and children of respectively); identifier of individual's father (zero, if samples from both parents were unavailable); identifier of individual's mother (zero if samples from both parents were unavailable); sex (1, male; 2, female); affection status (0, unknown; 1, unaffected; 2, affected); liability class; typing data for marker *D11S1393*; typing data for marker *D11S903*; typing data for marker *D11S2095*; and typing data for marker *D11S554*. Annotation is integrated in the corresponding parameter files, and in the accompanying reports the multiple pairwise LOD scores (1, disease locus; 2, *D11S1393*; 3, *D11S903*; 4, *D11S2095*; 5, *D11S554*) are tabulated over several values of θ . The maximum cumulative LOD score when both families are considered is 2.93 at $\theta=0.0$ with marker *D11S2095*.

FAMILY 2 PEDIGREE FILE

Family2	1595	0	0	2	2	1	1 4	1 2	1 4	1 6
Family2	1596f	0	0	1	1	1	0 0	0 0	0 0	0 0
Family2	2064	1596f	1595	2	1	2	3 4	2 2	4 4	5 6
Family2	1596	1596f	1595	2	2	1	1 3	1 2	1 4	1 5
Family2	1563f	0	0	1	1	1	0 0	0 0	0 0	0 0
Family2	1597	1596f	1595	2	1	2	1 1	1 1	1 3	1 1
Family2	2062f	0	0	1	1	1	0 0	0 0	0 0	0 0
Family2	2063	1563f	1596	2	1	2	1 3	2 4	2 4	5 5
Family2	1562	0	0	2	1	1	1 3	1 2	3 4	3 4
Family2	1563	1563f	1596	1	2	1	1 2	1 1	1 2	1 2
Family2	2062	2062f	1597	1	2	1	1 2	1 3	1 2	1 7
Family2	1598	2062f	1597	2	2	1	1 2	1 3	1 2	1 7
Family2	1564	1563	1562	2	2	1	1 1	1 1	1 3	1 3
Family2	1565	1563	1562	1	0	2	1 3	1 2	1 4	1 4
Family2	1566	1563	1562	2	2	1	1 1	1 1	1 3	1 3
Family2	2064c1	2064s	2064	2	1	3	0 0	0 0	0 0	0 0
Family2	2064c2	2064s	2064	1	1	3	0 0	0 0	0 0	0 0
Family2	2064c3	2064s	2064	1	1	3	0 0	0 0	0 0	0 0
Family2	2064s	0	0	1	1	1	0 0	0 0	0 0	0 0
Family2	2063c1	2063s	2063	1	1	3	0 0	0 0	0 0	0 0
Family2	2063c2	2063s	2063	1	1	3	0 0	0 0	0 0	0 0
Family2	2063s	0	0	1	1	1	0 0	0 0	0 0	0 0

FAMILY 1 PEDIGREE FILE

Family1	1542	0	0	2	2	1	1 3	1 2	1 1	7 8
Family1	1540	1540f	1542	1	2	1	3 4	2 3	1 3	3 8
Family1	1543	0	0	2	1	1	1 3	4 4	1 1	3 9
Family1	1541	1540	1543	1	2	1	1 3	2 4	1 1	3 8
Family1	1546	1540	1543	2	2	1	1 3	2 4	1 1	3 8
Family1	1540f	0	0	1	1	1	0 0	0 0	0 0	0 0

FAMILY 2 PARAMETER FILE

```

5 0 0 5 <<5 LOCI, NO RISK CALC, NOT SEX-LINKED, MLINK
0 0.0 0.0 0 <<NO MUTATION, ZERO MUT RATE IN MALES, ZERO MUT RATE IN FEMALES, NO DISEQUILIBRIUM
1 2 3 4 5 <<ORDER OF 5 LOCI
1 2 <<DISEASE LOCUS, 2 ALLELES
0.99999 0.00001 <<FREQ OF NORMAL ALLELE, FREQ OF MUTANT ALLELE
3 <<NO OF LIABILITY CLASSES
0.0 1.0 1.0 <<PENETRANCES FOR NORMAL/NORMAL, NORMAL/MUTANT, MUTANT/MUTANT --- LIABILITY CLASS 1
0.0 0.80 1.0 <<PENETRANCES FOR NORMAL/NORMAL, NORMAL/MUTANT, MUTANT/MUTANT --- LIABILITY CLASS 2
0.0 0.50 1.0 <<PENETRANCES FOR NORMAL/NORMAL, NORMAL/MUTANT, MUTANT/MUTANT --- LIABILITY CLASS 3
3 4 <<CO-DOMINANT LOCUS, 4 ALLELES --- D11S1393
0.25 0.25 0.25 0.25 <<EQUIFREQUENT ALLELES
3 4 <<CO-DOMINANT LOCUS, 4 ALLELES --- D11S903
0.25 0.25 0.25 0.25 <<EQUIFREQUENT ALLELES
3 4 <<CO-DOMINANT LOCUS, 4 ALLELES --- D11S2095
0.25 0.25 0.25 0.25 <<EQUIFREQUENT ALLELES
3 7 <<CO-DOMINANT LOCUS, 7 ALLELES --- D11S554
0.143 0.143 0.143 0.143 0.143 0.143 0.142 <<EQUIFREQUENT ALLELES
0 0 <<NO INTERFERENCE, EQUAL MALE AND FEMALE REC FRACTIONS
0.1 <<INITIAL REC FRACTION (PARAM OVERRIDDEN IN ANALYSIS)
1 0.1 0.45 <<0.1 INCREMENT VALUE FOR REC FRACTION, END AT 0.45 (PARAMS OVERRIDDEN IN ANALYSIS)

```

FAMILY 1 PARAMETER FILE

```

5 0 0 5 <<5 LOCI, NO RISK CALC, NOT SEX-LINKED, MLINK
0 0.0 0.0 0 <<NO MUTATION, ZERO MUT RATE IN MALES, ZERO MUT RATE IN FEMALES, NO DISEQUILIBRIUM
1 2 3 4 5 <<ORDER OF 5 LOCI
1 2 <<DISEASE LOCUS, 2 ALLELES
0.99999 0.00001 <<FREQ OF NORMAL ALLELE, FREQ OF MUTANT ALLELE
2 <<NO OF LIABILITY CLASSES
0.0 1.0 1.0 <<PENETRANCES FOR NORMAL/NORMAL, NORMAL/MUTANT, MUTANT/MUTANT --- LIABILITY CLASS 1
0.0 0.99 1.0 <<PENETRANCES FOR NORMAL/NORMAL, NORMAL/MUTANT, MUTANT/MUTANT --- LIABILITY CLASS 2
3 4 <<CO-DOMINANT LOCUS, 4 ALLELES --- D11S1393
0.25 0.25 0.25 0.25 <<EQUIFREQUENT ALLELES
3 4 <<CO-DOMINANT LOCUS, 4 ALLELES --- D11S903
0.25 0.25 0.25 0.25 <<EQUIFREQUENT ALLELES
3 4 <<CO-DOMINANT LOCUS, 4 ALLELES --- D11S2095
0.25 0.25 0.25 0.25 <<EQUIFREQUENT ALLELES
3 7 <<CO-DOMINANT LOCUS, 7 ALLELES --- D11S554
0.143 0.143 0.143 0.143 0.143 0.143 0.142 <<EQUIFREQUENT ALLELES
0 0 <<NO INTERFERENCE, EQUAL MALE AND FEMALE REC FRACTIONS
0.1 <<INITIAL REC FRACTION (PARAM OVERRIDDEN IN ANALYSIS)
1 0.1 0.45 <<0.1 INCREMENT VALUE FOR REC FRACTION, END AT 0.45 (PARAMS OVERRIDDEN IN ANALYSIS)

```

FAMILY 2 REPORT**L O D T A B L E R E P O R T**

Order	0.0	0.01	0.05	0.1	0.2	0.3	0.4	

1=2	1.47	1.44	1.34	1.20	0.91	0.59	0.28	a
1=3	0.82	0.80	0.72	0.60	0.38	0.17	0.04	a
1=4	2.33	2.29	2.13	1.93	1.48	1.01	0.50	a
1=5	1.72	1.70	1.57	1.41	1.08	0.71	0.35	a

= = Test Interval

a = LOD Scores (LOG 10)

FAMILY 1 REPORT**L O D T A B L E R E P O R T**

Order	0.0	0.01	0.05	0.1	0.2	0.3	0.4	

1=2	0.60	0.59	0.56	0.51	0.41	0.29	0.16	a
1=3	0.60	0.59	0.56	0.51	0.41	0.29	0.16	a
1=4	0.60	0.59	0.56	0.51	0.41	0.29	0.16	a
1=5	0.60	0.59	0.56	0.51	0.41	0.29	0.16	a

= = Test Interval

a = LOD Scores (LOG 10)

Appendix E

Regulatory Approvals

E.1 Research in the Genetic Basis of Enlarged Parietal Foramina in Humans

Genetic research on PFM involving human subjects was approved by the Central Oxford Research Ethics Committee – permission granted to Professor A. O. M. Wilkie under project number COREC 99.059 – and a series of standard documents related to the study is appended in the following pages. For families who were recruited locally through the Oxford Craniofacial Unit informed consent was given by the individuals or their parents, as appropriate, before obtaining a blood or buccal sample. Documents one and two are specimens of the invitation letters addressed to parents of affected children attending the Unit and other family members respectively. They were accompanied by document three, a copy of the information sheet providing answers to the most likely questions. At the same time, permission in writing was sought for a clinical examination from the parents and any other approachable relatives — documents four and five respectively. Four versions of the main consent form follow, covering all scenarios: index subject (child); his/her parent; other family member; and child of the latter (documents six to nine). In the last case, a buccal sample was requested to minimise inconvenience. When patients decided to proceed, the final document (document ten) advised their GP of their participation.

Other patients and families were referred through clinical geneticists elsewhere in the UK. In these cases, the information sheet and an appropriate consent form were sent to the contact clinician while emphasising the research nature of the project.

An equivalent approach was followed for the substantial number of samples that originated from overseas — namely from Poland, Spain, The Czech Republic, Germany, The Netherlands, Brazil, and Australia. The essential literature

was forwarded to the local clinician who was responsible for its translation and/or communication and for obtaining informed consent.

Document 1: Invitation Letter (Parent of Proband)

**Oxford Craniofacial Unit
The Radcliffe Infirmary NHS Trust
Woodstock Road
Oxford OX2 6HE**

Tel: 01865-224100

Dear Parent

Genetic basis of parietal foramina COREC 99.059

One of the doctors working with me in the Oxford Craniofacial Unit, Dr Andrew Wilkie, a consultant in genetics, is conducting a study of Parietal Foramina (gaps in the skull) to try to find out the cause of the condition. He hopes to include as many people as possible and will be contacting all parents with an affected child who attend the Oxford Craniofacial Unit. As may have been explained to you, parietal foramina may be caused by an alteration in one of the many thousands of genetic instructions which we carry from the time of conception, but no one knows which instruction is altered or why. If the alteration could be identified, this might help us to understand why parietal foramina occur.

I am writing to you at Dr Wilkie's request to invite you to take part in this study, which would involve:

- 1 Permission to examine your child's notes and X rays.
- 2 A visit to an outpatient clinic appointment in Oxford (if possible, both parents and the affected child should attend). Dr Wilkie would ask about your family history and your child's problems (if any), carry out a short examination of the head, take a photograph and take a small blood sample from you and your child. (This would be used to analyse the chromosomes, make a source of living cells, and for extraction of the genetic material or DNA.) In addition, a single skull X ray would be obtained from yourself and your partner if this has not been performed previously. If it is known that other relatives on one side of the family have the same condition, Dr Wilkie would ask if you could contact them to enquire if they would like more details about the study. He would reimburse any travel costs incurred by you and your family.

There is no obligation to take part in this study and it is unlikely that this work would have any medical implications or benefit for yourself or your family. If you decide you would like to be involved, Dr Wilkie would inform you if any discoveries were made and you would be most welcome to contact him at any time to discuss his progress. However it is important to understand that it may take some years before he comes close to identifying the cause of Parietal Foramina and there may be no immediate answers. Further information on the study is provided on a supplementary sheet.

Whether or not you would like to join the study, I would be very grateful if you would sign the attached form and return it in the enclosed pre-paid envelope. Please feel free to contact Dr Wilkie if you require any further information before making a decision, he would be happy to answer any questions. He can be contacted at the Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS or on 01865 222619.

Yours sincerely

Mr S Wall MBBCh(rand) FRCS FRCPCH FCS(SA) Plast

Consultant in Plastic and Reconstructive Surgery

Document 2: Invitation Letter (Relative)

UNIVERSITY OF OXFORD

Tel. Oxford (01865) 222619

INSTITUTE OF MOLECULAR MEDICINE

Fax Oxford (01865) 222500

JOHN RADCLIFFE HOSPITAL

email awilkie@worf.molbiol.ox.ac.uk

HEADINGTON

OXFORD OX3 9DS

Dear relative

Genetic basis of parietal foramina COREC 99.059

As you are probably aware, some members of your family are affected with an disorder termed Parietal Foramina, which is associated with enlarged gaps in the skull at birth which tend to close up with age. Parietal foramina may be caused by an alteration in one of the many thousands of genetic instructions which we carry from the time of conception, but no one knows which instruction is altered or why. If the alteration could be identified, this might help us to understand why parietal foramina occur. We are conducting research in Oxford to try to identify the alteration to the genetic blueprint that causes this condition. Various family members have previously donated samples for this work, and we are inviting further family members to participate in the research. Your sample would provide further information for the study, even if you are not affected with the condition yourself.

I am writing to invite you to take part in this study, which would involve a visit to an outpatient clinic in Oxford. I would ask if anything unusual had been noted about the shape of your skull, carry out a short physical examination, take a photograph and take a small blood sample from you. In addition, a single skull X ray would be obtained from you if this is not already available. If it is known that other relatives have the same condition, I would ask if you could contact them to enquire if they would like more details about the study. I would reimburse any travel costs incurred.

There is no obligation to take part in this study and it is unlikely that this work would have any medical implications or benefit for yourself or your family. If you decide you would like to be involved, I would inform you if any discoveries were made and you would be most welcome to contact me at any time to discuss progress. However it is important to understand that it may take some years before we come close to identifying the cause of parietal foramina and there may be no immediate answers. Further information on the study is provided on a supplementary sheet.

Whether or not you would like to join the study, I would be very grateful if you could sign the attached form and return it in the enclosed pre-paid envelope. Please feel free to contact me if you require any further information before making a decision, I would be happy to answer any questions.

Yours sincerely,

Dr. Andrew Wilkie DM FRCP

Wellcome Trust Senior Clinical Fellow
Honorary Consultant In Clinical Genetics

Document 3: Information Sheet

UNIVERSITY OF OXFORD

Dr Andrew Wilkie
 Tel. Oxford (01865) 222619
 Fax Oxford (01865) 222500
 Email: awilkie@worf.molbiol.ox.ac.uk



INSTITUTE OF MOLECULAR MEDICINE
 JOHN RADCLIFFE HOSPITAL
 HEADINGTON
 OXFORD OX3 9DS

Information Sheet – Parietal Foramina

What are Parietal Foramina?

We are conducting research in Oxford to try to find the altered gene for parietal foramina. This is a rare condition that causes affected people to have unusually large gaps in the skull at birth. These generally close up during childhood and rarely cause any problems. Other parts of the body are usually not affected.

What causes Parietal Foramina?

We know that parietal foramina are often caused by an altered gene. We know this because of the way in which the children of affected individuals may themselves be affected. On average about half the children of affected individuals will inherit the condition and the other half will not.

How are genes passed on to our children?

Genes are passed on to our children on chromosomes. Chromosomes contain thousands of genes and we each have a total of 23 pairs of chromosomes. We would like to find out which chromosome the altered gene(s) for parietal foramina is on. Children who are affected by parietal foramina have the chromosome with the altered gene while the unaffected children have the other member of the chromosome pair that does not have the altered gene.

What new information do we need from this research?

We are very interested to see the way in which these chromosomes are inherited. This means that the chromosomes of both affected and unaffected members of a family are useful because it helps us to build up a pattern of the way in which genes, and the chromosomes that they are on, are inherited. In this way we can narrow down the part of the chromosome that carries the altered gene, and eventually identify the gene itself.

What is the purpose of the research?

The purpose of our research is to try and understand how genes instruct the body to make a normal skull at an early stage during the baby's life inside the womb.

What would I have to do if my child or I were to participate in this study?

The accompanying letter invites you/your child to take part in this study. This would involve a visit to the outpatient clinic in Oxford, a skull X-ray (unless this has been performed previously) and either a blood test (adults and first affected child in the family) or a sample brushed from the cheek (all other children). The blood and cheek cells contain the genetic material that we can use in the study. We can store this material in a freezer for long periods and set up a cell line that provides a permanent source of living cells. The research would not involve any treatment of any kind.

Are there likely to be any medical benefits for me, or my family?

It is unlikely that this work would have any medical implications or benefit for yourself and your family. However, you are welcome to discuss any questions you may have about parietal foramina and to find out about the progress of the study, at any stage.

Would the samples collected be used for any other purpose?

No, the samples would only be used to identify the parietal foramina gene. All results would be maintained confidentially.

What if I want to leave the study?

If you decide to participate in the study you would of course also be welcome to leave the study at any time and this would make no difference to your normal medical care.

Who do I contact if I want further information?

You are welcome to contact Dr Andrew Wilkie at any stage. Contact details are provided at the top of the sheet.

Document 4: Permission for Clinical Appointment (Parent of Proband)

UNIVERSITY OF OXFORD

Tel. Oxford (01865) 222619

INSTITUTE OF MOLECULAR MEDICINE

Fax Oxford (01865) 222500

JOHN RADCLIFFE HOSPITAL

email awilkie@worf.molbiol.ox.ac.uk

HEADINGTON

OXFORD OX3 9DS

Genetic basis of parietal foramina COREC 99.059

Parent's Name.....

Address.....

Date of birth.....

Have you read the Invitation Letter/Information sheet? Yes*/No*

Would you like to ask questions and discuss this study further? Yes*/No*

I would*/ would not* like to visit an outpatient clinic in Oxford with my family to discuss participation in this study, which would involve providing a blood sample and having a skull X-ray (if not already available). I understand that my family is free to leave the study at any time, without having to give a reason for leaving and without this affecting our medical care.

Signed: Date:

Name (in block letters):.....

Are all the above details correct? (if not, please amend)

If you are participating it would be helpful if you could provide a telephone number.....

Any comments.....

*please delete as appropriate

Document 5: Permission for Clinical Appointment (Relative)

UNIVERSITY OF OXFORD

Tel. Oxford (01865) 222619

INSTITUTE OF MOLECULAR MEDICINE

Fax Oxford (01865) 222500

JOHN RADCLIFFE HOSPITAL

email awilkie@worf.molbiol.ox.ac.uk

HEADINGTON

OXFORD OX3 9DS

Genetic basis of parietal foramina COREC 99.059

Patient's Name.....

Address.....

Date of birth.....

Have you read the Invitation Letter/Information sheet? Yes*/No*

Would you like to ask questions and discuss this study further? Yes*/No*

I would*/ would not* be willing to visit an outpatient clinic in Oxford to discuss participation in this study, which would involve providing a blood sample and having a skull X-ray (if not already available). I understand that I am free to leave the study at any time, without having to give a reason for leaving and without this affecting my medical care.

Signed:..... Date:.....

Name (in block letters):.....

Are all the above details correct? (if not, please amend)

If you are participating it would be helpful if you could provide a telephone number.....

Any comments.....

*please delete as appropriate

Document 6: Consent Form (Proband)

UNIVERSITY OF OXFORD

Tel. Oxford (01865) 222619

Fax Oxford (01865) 222500

email awilkie@worf.molbiol.ox.ac.uk

INSTITUTE OF MOLECULAR MEDICINE

JOHN RADCLIFFE HOSPITAL

HEADINGTON

OXFORD OX3 9DS

Genetic basis of parietal foramina COREC 99.059

Patient's Name.....

Address.....

Date of birth.....

Have you read the Invitation Letter/Information sheet? Yes*/No*

Would you like to ask questions and discuss this study further? Yes/No

If yes, have you received satisfactory answers to your questions? Yes/No

Have you received enough information about the study? Yes/No

Who, if anyone, have you spoken to? Dr/Mr/Ms.....

Do you understand that you are free to leave the study:

- at any time,
- without having to give a reason for leaving,
- and without affecting your medical care? Yes/No

I would*/ would not* like..... to participate in this study by providing a blood sample of not more than 20 ml.

Signed: Date:

Name (in block letters):.....

Relationship to child.....

Are all the above details correct? (if not, please amend)

If you are participating it would be helpful if you could provide a telephone number.....

Any comments.....

*please delete as appropriate

Document 7: Consent Form (Parent of Proband)

UNIVERSITY OF OXFORD

Tel. Oxford (01865) 222619

INSTITUTE OF MOLECULAR MEDICINE

Fax: Oxford (01865) 222500

JOHN RADCLIFFE HOSPITAL

email awilkie@worf.molbiol.ox.ac.uk

HEADINGTON

OXFORD OX3 9DS

Genetic basis of parietal foramina COREC 99.059

Parent's Name.....

Address.....

Date of birth.....

Have you read the Invitation Letter/Information sheet? Yes*/No*

Would you like to ask questions and discuss this study further? Yes/No

If yes, have you received satisfactory answers to your questions? Yes/No

Have you received enough information about the study? Yes/No

Who, if anyone, have you spoken to? Dr/Mr/Ms.....

Do you understand that you are free to leave the study:

- at any time,
- without having to give a reason for leaving,
- and without affecting your medical care? Yes/No

I would*/ would not* like to participate in this study by providing a blood sample and having a skull X-ray (if not already available).

Signed: Date:

Name (in block letters):.....

Are all the above details correct? (if not, please amend)

If you are participating it would be helpful if you could provide a telephone number:.....

Any comments:.....

*please delete as appropriate

Document 8: Consent Form (Relative)

UNIVERSITY OF OXFORD

Tel. Oxford (01865) 222619

INSTITUTE OF MOLECULAR MEDICINE

Fax: Oxford (01865) 222500

JOHN RADCLIFFE HOSPITAL

email awilkie@worf.molbiol.ox.ac.uk

HEADINGTON

OXFORD OX3 9DS

Genetic basis of parietal foramina COREC 99.059

Patient's Name.....

Address.....

Date of birth.....

Have you read the Invitation Letter/Information sheet? Yes*/No*

Would you like to ask questions and discuss this study further? Yes/No

If yes, have you received satisfactory answers to your questions? Yes/No

Have you received enough information about the study? Yes/No

Who, if anyone, have you spoken to? Dr/Mr/Ms.....

Do you understand that you are free to leave the study:

- at any time,
- without having to give a reason for leaving,
- and without affecting your medical care? Yes/No

I would*/ would not* like to participate in this study by providing a blood sample and having a skull X-ray (if not already available).

Signed: Date:

Name (in block letters):.....

Are all the above details correct? (if not, please amend)

If you are participating it would be helpful if you could provide a telephone number:.....

Any comments:.....

*please delete as appropriate

Document 9: Consent Form (Child of Relative)

UNIVERSITY OF OXFORD

Tel. Oxford (01865) 222619

INSTITUTE OF MOLECULAR MEDICINE

Fax: Oxford (01865) 222500

JOHN RADCLIFFE HOSPITAL

email awilkie@worf.molbiol.ox.ac.uk

HEADINGTON

OXFORD OX3 9DS

Genetic basis of parietal foramina COREC 99.059

Child's name.....

Address.....

Date of birth.....

Have you read the Invitation Letter/Information sheet? Yes*/No*

Would you like to ask questions and discuss this study further? Yes/No

If yes, have you received satisfactory answers to your questions? Yes/No

Have you received enough information about the study? Yes/No

Who, if anyone, have you spoken to? Dr/Mr/Ms.....

Do you understand that you are free to leave the study:

- at any time,
- without having to give a reason for leaving,
- and without affecting your medical care? Yes/No

I would*/ would not* like..... to participate in this study by providing a mouthbrushing and having a skull X ray (if not already available).

Signed: Date:

Name (in block letters):.....

Relationship to child.....

Are all the above details correct? (if not, please amend)

If you are participating it would be helpful if you could provide a telephone number.....

Any comments.....

*please delete as appropriate

Document 10: GP Notification Letter**UNIVERSITY OF OXFORD**

Tel. Oxford (01865) 222619

Fax Oxford (01865) 222500

email awilkie@worf.molbiol.ox.ac.uk

INSTITUTE OF MOLECULAR MEDICINE

JOHN RADCLIFFE HOSPITAL

HEADINGTON

OXFORD OX3 9DS

Dear Doctor

Genetic basis of parietal foramina COREC 99.059

Patient's name/date of birth/address

This patient has recently attended the Oxford Craniofacial Unit as part of the above study. The aim of this study is to identify the gene alteration(s) that cause parietal foramina.

A blood sample was obtained for chromosome analysis, DNA extraction and production of a cell line. Further work will be undertaken on your patient, and other members of their family, in an attempt to identify a genetic basis for the parietal foramina. I enclose a copy of the information sheet that has been supplied to the family as part of the study.

These investigations will not affect your patient's medical care in any way. However, if you have any questions concerning the study, please feel free to contact me at any time should you require further information.

Yours sincerely,

Dr. Andrew Wilkie DM FRCP

Wellcome Trust Senior Clinical Fellow
Honorary Consultant In Clinical Genetics

E.2 Research in the Genetic Basis of Craniosynostosis in Humans

Sample collection from patients with craniosynostosis and their relatives was also conducted under an authorised project of Professor A. O. M. Wilkie, approved by the Central Oxford Research Ethics Committee — project number COREC 2742.

E.3 Animal Licences

All animal work was performed in the officially designated premises of the Department of Human Anatomy and Genetics. The sole regulated procedure carried out on mice was tail clipping for genotyping purposes, covered by the project licence PPL 30/1887 to Professor G. M. Morriss-Kay and the personal licences of L. A. Mavrogiannis (PIL 30/6041) and I. Antonopoulou (PIL 70/14687) — all issued by the Home Office.

Appendix F

Electronic Databases and Resources

Ensembl Human Genome Browser, UK (Hubbard et al. 2002)
<http://www.ensembl.org>

European Bioinformatics Institute (EBI), UK (Mulder et al. 2003)
<http://www.ebi.ac.uk>

Human Gene Mutation Database, UK (Stenson et al. 2003)
<http://www.hgmd.org>

Human Genome Browser at University of California at Santa Cruz (UCSC), USA (Kent et al. 2002)
<http://genome.ucsc.edu>

Human Genome Mapping Project Resource Centre (HGMP-RC), UK
<http://www.hgmp.mrc.ac.uk>

Human Genome Organisation (HUGO) Gene Nomenclature Committee, UK (Wain et al. 2002)
<http://www.gene.ucl.ac.uk/nomenclature>

Jackson Laboratory, USA (Blake et al. 2003)
<http://www.informatics.jax.org>

National Center for Biotechnology Information (NCBI), USA (Altschul et al. 1990; Wheeler et al. 2003)
<http://www.ncbi.nlm.nih.gov>

Oxford University Bioinformatics Centre (OUBC)
<http://www.molbiol.ox.ac.uk>

The Genome Database, USA (Fasman et al. 1997)
<http://www.gdb.org>

Appendix G

Publications Arisen from This Work

Mavrogiannis LA, Antonopoulou I, Baxová A, Kutilek S, Kim CA, Sugayama SM, Salamanca A, Wall SA, Morriss-Kay GM, Wilkie AOM (2001). Haploinsufficiency of the human homeobox gene *ALX4* causes skull ossification defects. *Nature Genet* 27:17–18¹.

Garcia-Miñaur S, **Mavrogiannis LA**², Rannan-Eliya SV, Hendry MA, Liston WA, Porteous MEM, Wilkie AOM (2003). Parietal foramina with cleidocranial dysplasia is caused by mutation in *MSX2*. *Eur J Hum Genet* 11:892–895.

Wilkie AOM, **Mavrogiannis LA** (2004). Enlarged Parietal Foramina/Cranium Bifidum. In: GeneReviews at GeneTests: Medical Genetics Information Resource [database online]. Seattle: University of Washington³.

Antonopoulou I, **Mavrogiannis LA**⁴, Wilkie AOM, Morriss-Kay GM (2004). *Alx4* and *Msx2* play phenotypically similar and additive roles in skull vault differentiation. *J Anat* 204:487–499.

¹Supplementary information available at http://genetics.nature.com/supplementary_info

²Co-first author

³Available at <http://www.genetests.org>

⁴Co-first author

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The motto comes from the first and second stanzas of the poem 'Through the mirror' in *Maria Nephele*^a. The opening image is reproduced from the article of Goldsmith in 1922^b. The author is credited with probably the first solid description of familial occurrence of enlarged parietal foramina, recorded in a large American kindred. However, the autosomal dominant mode of inheritance with incomplete penetrance passed unnoticed and the embryological background was hardly discussed. The trait was perceived as an odd yet rather benign anatomical stigma and labelled with the rather unfortunate eponymous designation 'Catlin mark'.

^aElytis O (1997). *Maria Nephele*. In: Carson J, Sarris N (Trans.), *The collected poems of Odysseus Elytis*, pp. 289–368. Baltimore: The Johns Hopkins University Press.

^bGoldsmith WM (1922). 'The Catlin mark'. *J Hered* 13:69–71.