The role of Vax1 in craniofacial development

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King's College London

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The Role of Vax1 During Craniofacial Development

Finn Edward Geoghegan

A Thesis submitted for the Degree of Doctor of Philosophy at the University of London

2018

Department of Craniofacial Development and Stem Cell Biology
King’s College London
Declaration

No part of the work referred to in the present thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning
Abstract
Holoprosencephaly (HPE) is a clinically heterogeneous developmental anomaly affecting the CNS and face, in which the embryonic forebrain fails to divide into distinct halves. Numerous genetic loci and environmental factors are implicated in HPE, but mutation in the sonic hedgehog (Shh) gene is an established cause in both humans and mice. Vax (ventral anterior homeobox) genes are a family of homeobox-containing genes which have been identified in mice, humans, Xenopus, chicken and zebrafish. They are transcription factors which are induced by Shh and ventralize the forebrain. In humans, it has been shown that a VAX1 mutation is associated with microphthalmia, agenesis of the corpus callosum, and orofacial clefting.

Skeletal and histological analysis of Vax1-/- mice revealed a series of craniofacial defects predominantly affecting the neurocranial and facial midlines as well as the visual and olfactory systems. These included anomalies of the ventral forebrain, a single maxillary incisor, premaxillary hypoplasia, cleft palate and the presence of an ectopic second pituitary gland. During early facial development, Vax1 was expressed in the developing ventral forebrain and facial structures in adjacent and partially overlapping areas of expression to Shh and in a broadly reciprocal manner to its' transcriptional target Pitch1. In the absence of Vax1 function, there was an overall reduction in the size of craniofacial regions, in particular of the developing forebrain. Moreover, mutant mice did have a midline cavity originating from the embryonic forebrain and extending through the nasal cavity to expand this region and prevent approximation of the palatal shelves. This was combined with a decrease in cellular proliferation in this region and reduced Shh signaling activity. This phenotypic and molecular analysis was strongly indicative of the role of Vax1 downstream of Shh during craniofacial development.

Vax1-/- mice also demonstrate lobar HPE. Shh expression was reduced in both Gas1-/- and Vax1-/- mice, both modifiers of HPE. The craniofacial phenotype of Gas1 ;Vax1 compound mutant mice displayed a more severe defect of the facial midline thus demonstrating the combinatorial role of modifying genes in the wide spectrum of phenotypic variablility in Shh mediated HPE.
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**List of Abbreviations**

III vt - third ventricle  
IV vt - fourth ventricle  
2cdp - secondary cartilage of condylar process  
AER - Apical Ectodermal Ridge  
AP - Anteroposterior  
a - atrium  
a’ - atlas  
ac - anal canal  
agp - angular process  
aip - anterior intestinal portal  
ANR - Anterior Neural Ridge  
Asn - asparagine  
Asp - aspartic acid  
ATP - Adenosine 5’-triphosphate  
at - aortic trunk  
au - autopod  
av - aortic valve  
ax - axis  
ba1 - first branchial arch  
ba2 - second branchial arch  
bc1 - first branchial cleft  
bh - body of hyoid  
bo – basioccipital  
Boc - biregional Cdo-binding protein  
β-gal - β-galactosidase  
BLAST - basic local alignment search tool  
BMP - bone morphogenetic protein  
br - bronchus
BrdU - 5-bromo-2'-deoxy-uridine
bs - basisphenoid
BSA - Bovine serum albumin
ca - capitate
cc - costal cartilage
Cdo - cell-adhesion-molecule-related/downregulated by oncogenes
cdp - condylar process
ce - centrale
cl - cervical loop
cm - cortical region of mesonephros
CNCC - cranial neural crest
cDNA - complementary DNA
cec - central canal
CNS – central nervous system
cpa - cartilage primordium of the anterior arch of C1 vertebra (atlas)
cpax - cartilage primordium of the body of C2 vertebra (axis)
cpbb - cartilage primordium of basioccipital bone (clivus)
cpc3 - cartilage primordium of the body of C3 vertebra
cpc4 - cartilage primordium of the body of C4 vertebra
crp - coronoid process
cs - coronal suture
ct - cortical region of testis
CTP - Cytidine 5'-triphosphate
CUB - Complement subcomponent C1r/C1s, EGF-related sea Urchin protein, Bone morphogenetic protein1 domain
d - diencephalon
List of Abbreviations

dd - dorsal dermomyotome
Dhh - desert Hedgehog
Dig - Digoxigenin
dl - dental lamina
Disp - Dispatched
DNA - deoxyribonucleic acid
dnt - dentary
dp - dental papilla
E - embryonic day
ECL - enhanced chemiluminescence labelling
EDTA - ethylenediaminetetraacetic acid
eee - external enamel epithelium
EGF - epidermal growth factor
EGTA - Ethylen glycol tetraacetic acid
ek - enamel knot
Eng - Engrailed
eo - exoccipital
EMT - Epithelial Mesenchymal Transformation
EMX - Empty Spiracles Homeobox 1
ept - epithalamus
ER - endoplasmic reticulum
ES - embryonic stem
ESEs - exon splicing enhancers
ESSs - exon splicing silencers
EST - expressed sequence tag
EtBr - ethidium bromide
etm - ectotympanic process
FBS - fetal bovine serum
fr - frontal
List of Abbreviations

FEZ - Frontonasal Ectodermal Zone
FNP - Frontonasal Process
fmx - frontal process of maxilla
g - genioglossus
g' - gut
Gas1 - Growth arrest-specific-1
GFP - Green Fluorescent Protein
Gli - vertebrate homologue of Drosophila cubitus interruptus gene
Glu - glutamic acid
Gln - glutamine
GnRH - Gonadotrophic releasing hormone
GTP - Guanine 5'-triphosphate
ha - hamate
hb - hyoid bone
hc - hyaloids cavity
H and E - hematoxylin and eosin
hf - hair follicle
HEK - human embryonic kidney
HERS - Hertwig's epithelial root sheath
hh - Drosophila hedgehog
Hh - Hedgehog
hin - hindbrain
Hip - Hedgehog-interacting protein-1
Hn - Hensen’s node
Hox - Homeobox
iee - internal enamel epithelium
Ihh - Indian hedgehog
IHH - Idiopathic hypogonadotropic hypogonadism
ina - incisive alveolus of dentary
inf - infundibulum
List of Abbreviations

ip - interparietal
irs - intra-retinal space
is - interventricular septum
ISEs - intron splicing enhancers
ISSs - intron splicing silencers
it - isthmus
itb - incisor tooth bud
jg - jugal
kd - kidney
kDa - kilodalton
K–S - Kolmogorov–Smirnov
LGE - Lateral Ganglionic Eminence
la - left atrium
lb - limb
lcv - left cardiac vein
lg - lung
lgb - lung bud
li - lower incisor
ll - left lip
lm - lumen of midgut
LNP - lateral nasal process
lur - lumen of urogenital sinus
lv - liver
lv' - left ventricle
ls - lamboidal suture
m - mesencephalon
m1 - first molar
m2 - second molar
m3 - third molar
MAB - 50 mM maleic acid, pH 7.5, 250 mM NaCl
List of Abbreviations

MABT - 50 mM maleic acid, pH7.5, 250 mM NaCl and 0.1% Tween 20
Mc - Meckel’s cartilage
mc - metacarpals
mcv - major caudal vein
md - mandible
med - mesonephric duct
mee - medial edge epithelial
mes - medial epithelial seam
MGE - Medial Ganglionic Eminence
microCT - micro computerised tomography
mid - midbrain
ml - mammary line
mm - medullary region of mesonephros
MNP - medial nasal process
mp - mammary placode
mRNA - Messenger ribonucleic acid
ms - metopic suture
mt - medullary region of testis
mt’ - metencephalon
mtb - molar tooth bud
mx - maxilla
mxl - maxillary incisor tooth bud
MXP - Maxillary process
MyHC - slow myosin heavy chain
n - nasopharynx
na - nasal
nc - nasal cavity
nc’ - nasal capsule
NCBI - National Center for Biotechnology Information
nce - nasal cavity epithelium
Neo - neomycin
nos - nostril
np - nasal pit
np’ - neural plate
ns - nasal septum
nt - neural tube
NTMT - 100 mM NaCl. 2 ml of 5M.
100 mM Tris (pH 9.5)
oc - oral cavity
oc’ - optic cup
oe - oral epithelium
ON - overnight
oes - oesophagus
ope - optic eminence
ORF - open reading frame
ov - otic vesicle
ot - optic vesicle
Pax - Paired-like homebox
P0 - postnatal 0
pa - pulmonary artery
pb - presphenoid bone
PBS - phosphate buffered saline
PCR - polymerase chain reaction
pe - pharyngeal endoderm
PFA - Paraformaldehyde
ph - phalanges
Phe - phenylalanine
pl - palatine
pmx - premaxilla
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<th>Abbreviation</th>
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<td>pp</td>
<td>parachordal plate</td>
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<td>pp'</td>
<td>parietal plate</td>
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<tr>
<td>ppmx</td>
<td>palatal process of maxilla</td>
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<tr>
<td>pppl</td>
<td>palatal process of palatine</td>
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<tr>
<td>pr</td>
<td>prostatic region of urethra</td>
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<td>primitive streak</td>
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<td>PTHrP</td>
<td>Parathyroid hormone-related protein</td>
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<td>pt</td>
<td>pulmonary trunk</td>
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<td>Ptch1</td>
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<td>Rathke’s pouch</td>
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<td>RPM</td>
<td>revolutions per minute</td>
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<td>rpMc</td>
<td>rostral process of Meckel’s cartilage</td>
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<td>RT-PCR</td>
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<td>rv</td>
<td>right ventricle</td>
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<td>sa</td>
<td>septal area</td>
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List of Abbreviations

sc - scapula
smg - submandibular gland
SCUBE - Signal peptide CUB EGF-like domain-containing protein
Shh - sonic hedgehog homolog
SMART - simple modular architecture research tool
Smo - Smoothened
so - somites
so' - supraoccipital
SP - signal peptide
sq - squamosal
sr - stellate reticulum
ss - sagital suture
st - seminiferous tubules
t' - telencephalon
t - tongue
tb - tympanic bulla
tg - trigeminal ganglion
tgt - tip of genital tubercle
th - thymus gland
tpa - transverse process of atlas
tr - trachea
tr' - trapezium
trr - trachea ring
Tx - Triton X
TF - tissue factor
TUNEL- Terminal transferase-mediated dUTP-biotin Nick End Labelling
TGF-β - transforming growth factor beta
tv - telencephalic vesicle
Tyr - tyrosine
tz - trapezoid
ui - upper incisor
ul - ulna
ut - urethra
UTP - Uridine 5’-triphosphate
v - ventricle
VAX - Ventral Anterior Homeobox
vc - vena cava
vd - ventral dermomyotome
vno - vomero-nasal (Jacobsen’s) organ
VEGA - The Vertebrate Genome Annotation
wb - wing bud
zps - zygomatic process of squamosal
zpmx - zygomatic process of maxilla

Genes are stated in italics and proteins in roman.

Mouse genes/ proteins are written in lower case letters (first letter upper case).

Human genes/ proteins are in upper case letters.

Mouse genes names based on the mouse genome informatics nomenclature.
1 Introduction

1.1 Ventral anterior homeobox genes.

In craniofacial development spatiotemporal domains of gene expression induce pattern formation that characterize the face. Transcription factors are decisive controlling elements of gene expression which orchestrate the translational and epigenetic regulation of craniofacial development. Morphogenesis in the developing head is dependent on major signaling pathways such as Hedgehog (Hh), Fibroblast growth factor (Fgf), Bone Morphogenic Protein (BMP) and Transforming Growth Factor (TGF-β). These pathways are responsive to extracellular and environmental factors. Signals are dispatched from cell-surface receptors to transcription factors, which in turn, recruit cofactors, activators or repressors, and chromatin-modifying enzymes, thus regulating gene expression to specify cell fate (Gou et al., 2015).

Transcription factors are proteins that bind to DNA and regulate the transcription of genes. A single transcription factor may regulate the expression of many target genes. Given the enormous complexity involved in craniofacial development, a particular gene may participate in several developmental processes (Price et al., 2012). The use of the same transcription factors for both tissue morphogenesis and axonal growth and guidance may be an example of this. The use of mouse genetics to observe the effect of a loss of function of a transcription factor is one method that can be utilised to illustrate the importance of transcription factors in eye and forebrain. It is becoming clear that many transcription factors have multiple roles in forebrain morphogenesis, axonal pathway and facial development (Pratt and Price, 2006).

Homeobox genes share a conserved sequence motif of about 180 base pairs called the homeobox, which was first found in Drosophila homeotic genes (McGinnis et al., 1984; Scott and Weiner, 1984). The homeobox encodes a homeodomain within the protein which has DNA binding activity (Müller et al., 1988). The homeobox gene family is of ancient origin with the common feature of the homeobox. Most homeobox genes encode transcription factors that act through sequence-specific DNA binding, which is mediated by the homeodomain. (Gou et al., 2015). This is a motif with a helix-loop-helix-turn-helix structure. This structure recognises specific AT-rich sequences for DNA binding. Vax (ventral anterior homeobox) genes are a family of homeobox-containing genes, which have been identified in mice, humans, Xenopus, chicken and zebrafish. These genes interact with protein cofactors to regulate expression of other
genes which effects change in cell behavior or activity. Homeobox genes are found in almost all Eukaryotes, and have diversified into 11 gene classes and over 100 gene families in animal evolution (Holland, 2013).

Vax1 was identified in a study of forebrain induction as a homeobox-containing gene with high sequence homology with members of the Emx and Not gene families,- transcription factor families that play a critical role in development of the telencephalon (Hallonet et al., 1998). The mouse and Xenopus genes share a high sequence homology and a similar pattern of expression during neurulation. The high sequence homology between Vax1, Not1 and Emx genes suggest that it could function in the specification and formation of rostral and ventral forebrain structures (Hallonet et al., 1998). Vax1 was identified in the distal region of mouse chromosome 19, in the vicinity of Emx2, in a region which shares a region of homology with human chromosome 10q25-26 (Hallonet et al., 1998; Marazita et al., 2004; Beaty et al., 2010). A second member (Vax2), was subsequently identified on chromosome 6 of the mouse in the vicinity of Emx1 in a region which shares a region of homology with human chromosome 2 (Barbieri et al., 1999).

1.1.1. Locus and molecular structure of Vax1 and Vax2
The Vax gene family is classified into Vax1 and Vax2 according to structure and expression pattern. The Vax1 and Vax2 homeodomain sequences suggest that these proteins recognize identical or at least similar target DNA sequences (Barbieri et al., 1999). Sequence similarity was also found to extend outside of the homeodomain. The amino acid sequence of the Vax2 homeodomain is identical to that of Vax1, and the corresponding homeobox nucleotide sequence has a 85% identity. An approximately 110 amino acid long stretch including the homeodomain is conserved between the two proteins but the other regions were quite divergent except for the c-terminal ends. (Barbieri et al., 1999).

The Vax1 gene spaces at least 4.3 kb whilst Vax2 is at least 26kb in length. Both genes consist of 3 exons with conserved positions of exon/intron junctions in their cDNA sequences. The intron lengths vary considerably between Vax1 and Vax2; 1.3kb and 2.0kb for introns 1 and 2 of Vax1, whilst those for Vax2 are 21kb and 4.5 kb (Ohsaki et al., 1999). The regions of Vax1/2 homology may contribute to biochemical functions (e.g. DNA binding, transcriptional activation and/or protein–protein interaction), but show no significant amino acid sequence similarity with
other known proteins. With regard to the homeodomain sequence, *Emx* is the most closely related vertebrate homeobox gene family to the *Vax* genes (The exon/intron organization of these genes is also conserved). The linkage of *Vax1-Emx2* on mouse chromosome 19 and the possible linkage of *Vax2* and *Emx1* suggest that these clusters arose from the same ancestral *Vax-Emx* cluster derived by tandem duplication of the *Vax/Emx* ancestors (Hallonet *et al.*, 1998) Although the linkage and structure of these gene clusters were maintained during evolution, *cis*-regulation of the *Vax* and *Emx* genes has evolved differently (Ohsaki *et al.*, 1999). The *Vax1* and *Vax2* proteins also show significant sequence similarities (approximately 65–70% identity) with the homeodomains of the Emx and Not families. The sequence homology is with these groups are confined to the homeodomain and does not extend outside this region, which clearly indicates that the *vax* genes represent an independent group of homeobox genes (Barbieri *et al.*, 1999).

1.1.2 *Vax1* gene expression

In the mouse, *Vax1* gene expression is first detected at embryonic day (E) 8 at the rostral level of the medial neural plate including the anterior neural ridge (ANR) and surrounding ectoderm (Hallonet *et al.*, 1998). At E9, *Vax1* is expressed in the ectoderm in a transverse band between the two olfactory placodes, medially contacting the neural tube. From E10-12, persistent expression is seen in the rostral oral ectoderm and in the rostral and medial regions of the olfactory placodes. From E9-14, expression is confined to the derivatives of the neural plate expressing *Vax1* at E8; the optic disk and stalk, later the optic nerve, the optic chiasm, the suprachiasmatic area, the hypothalamic cell cord, the whole preoptic area, the septum, the entopeduncular area and the basal ganglia. Laterally, there is confinement of expression at the lateral (LGE) and medial (MGE) ganglionic eminences (Hallonet *et al.*, 1998).

In *Xenopus*, expression of *vax1* is very similar to the mouse during early neurulation (Hallonet *et al.*, 1998). *vax1* transcription is first detected at the early gastrula stages. In the adult frog, *vax1* expression is restricted to neural derivatives and the testes. Similarly to the mouse, *vax1* expression in the anterior-most region of the open neural plate and mid-anterior and mid-lateral anterior ridge. At stage 19 (late neurula stage), *vax1* expression is present in the derivatives of these regions as predicted by fate maps (Eagleson and Harris 1990; Eagleson *et al.*, 1995). These are in the anterior and rostral ventrolateral part of the forebrain neuroepithelium, the primordium striatum, the optic stalk, the chiasmatic ridge and the anterior hypothalamus. At
stage 26 (tailbud), there is extension of expression laterally and caudally with enlargement of the forebrain vesicle. \textit{vax1} expression at stage 34 (tadpole) is specific to the optic disc, the optic stalk and the anterior hypothalamus (Wilson and Houart, 2004).

In zebrafish, \textit{vax1} and \textit{vax2} are expressed in the anterior forebrain, including the optic stalks, ventral retina and preoptic area territories that probably all originate from a similar region of the anterior medial neural plate (Varga \textit{et al.}, 1999). A low level of \textit{vax1} mRNA is first detected in the anterior neural keel at 7 somites. By 12 somites, expression is more robust in the medial forebrain and forming eyes (Take-uchi \textit{et al.}, 2003).

1.1.3 Vax2 gene expression

\textit{Vax2} is the second member identified in the Vax gene subgroup. It is strongly expressed in the developing eye of vertebrates (Barbieri \textit{et al.}, 1999). \textit{Vax1} shares some expression domains with \textit{Vax1} (such as the optic nerve). However, its area of expression is restricted to the developing eye, as determined in mouse, human, and Xenopus embryos. \textit{Vax2} is consistently expressed throughout gestation, starting at E9 in mouse in the inferior portion of the optic vesicle and later in the optic cup and neural retina; however, it is not detected in the dorsal regions of the above mentioned structures. Therefore, the expression of \textit{Vax2} seems to define a territory: the ventral part of the developing neural retina in which fundamental processes of eye morphogenesis take place; namely, the beginning of the invagination of the optic vesicle and the formation and subsequent closure of the optic fissure (Barbieri \textit{et al.}, 1999).

In the mouse, \textit{Vax2} is first expressed in the developing eye at E9 in the ventral half of the optic vesicle extending from the infero-nasal to the infero-temporal retina across the ventral furrow. This expression remains consistent during early gestation (Barbieri \textit{et al.}, 1999). At E12.5, \textit{Vax2} is also detected from the inferior neural retina along the entire optic nerve and stalk and extends in the hypothalamus from the supra-chiasmatic area to the anterior preoptic area. Medially, expression continues in the ventral telencephalon along the lamina terminalis to the septum. At E16.5, there is strong expression in the ventral neural retina but not in the ventral forebrain (Hallonet \textit{et al.}, 1998).
1.1.4 Vax gene expression in the eye

Vax1 and Vax2 demonstrate early expression in the optic vesicles, with Vax1 ultimately becoming restricted to the optic stalk and Vax2 localizing to the ventral retina (Ohsaki et al., 1999). Several homeobox genes are expressed in the eye field of the neural plate and in the optic vesicle. Expression of Vax1 and Vax2 suggests that this group of genes participates in different aspects of retina development. Vax1 and Vax2 expression in the optic vesicle would suggest a role in development of the two-layered optic cup (Ohsaki et al., 1999). This is generated by invagination of the optic vesicle, the outer layer gives rise to the retinal pigment epithelium (RPE) and the inner layer, the neuroretina. Vax1 is expressed in the optic stalk and in the outer layer of the optic cup, but Vax2 is expressed in the inner layer of the optic cup (Ohsaki et al., 1999). Vax1 and Vax2, are transiently co-expressed from E9.5 to E11.5 at the junction between the optic stalk and the presumptive neural retina and central to the axial positioning of the developing eye (Mui et al., 2005). At E13.5, both Vax1 and Vax2 genes exhibit non-overlapping patterns of expression. Vax1 is expressed in the optic stalk while Vax2 is expressed in the developing neural retina, where it exhibits both a steep high-ventral-to-low-dorsal gradient and a shallower high-nasal-to-low-temporal gradient.

Figure 1.1 Molecular cascade involved in partitioning of the visual system in the eye and optic nerve.

Vax1 and Pax2 are induced in the optic stalk by midline signals, such as Shh, which confine Pax6 and Rx expression in the optic cup (redrawn from Hallonet et al., 1998).
Together, Vax1 and Vax2 co-operatively control eye-field polarization, neuroepithelial cell proliferation, and retinal differentiation. Vax proteins cooperatively ventralize the developing eye field by acting at the midpoint of mouse embryogenesis (Mui et al., 2005). They allow for the development of the optic nerve by inhibiting the development of the retina, and they achieve this through their ability to repress the Pax6 gene and its promotion of retinal differentiation.

Figure 1.2 Genetic pathways involved in the regulation of Vax genes in patterning of the early eye primordium. Cyc/Nodal signals from axial tissues promote medial fates in the anterior neural plate (including optic stalks and preoptic area), in part through the regulation of Hh expression. Hh proteins promote both Pax2.1 and Vax gene expression. Within the forebrain, Bel and Fgf activity is required for Hh to promote Vax expression. It is also probable that Fgf signals act more widely to pattern optic stalks as well as the preoptic area. Both Pax2.1 and Vax genes are required for closure of the choroid fissure, whereas Vax genes have a more prominent role than Pax2.1 in limiting retinal differentiation to the eye cup. (redrawn from Takeuchi et al., 2003).
1.2 Vax1 knockout mice

Targeted mutation of Vax1 in mice causes a number of defects in the developing eye, including dysgenesis of the optic nerve and coloboma (a failure of the eye to close at the choroid fissure) (Hallonet et al., 1999). Mice lacking function of Vax1 demonstrate additional defects associated with the brain and craniofacial region. In particular, these mice have lobar holoprosencephaly (HPE) associated with defects in the basal telencephalon, impaired cleavage of the forebrain and craniofacial defects, including fusion of the maxillary incisors, a duplicated pituitary and cleft palate (Bharti et al., 2011; Hallonet et al., 1999; Soria et al., 2004) as well as exhibiting failure of closure of the optic disks leading to coloboma and loss of eye-nerve guidance properties of anterior midline cells in the developing forebrain and generation of GABAergic interneurons within the cortex (Taglialatela et al., 2004). These axon guidance defects do not result from Vax1 expressing midline cells but instead correspond to reduced expression of attractive guidance cues in these cells (Bertuzzi et al., 1999). Retinal axons fail to penetrate the brain in significant numbers and fail to form an optic chiasm. The axons in multiple commissural tracts of the anterior central nervous system (CNS) including the corpus callosum and the hippocampal and anterior commissures, fail to cross the midline. Subsequent analysis of these mice has shown that in the CNS Vax1 plays an important role in regulating cell lineages in the CNS and controls a number of genetic cascades involved in developing neuronal circuitry and forebrain morphology in the mouse embryo (Bertuzzi et al., 1999).

Vax1 mutant embryos display brain and craniofacial malformations from E10.5 (Hallonet et al., 1999). These embryos show variable deficient growth of structures in the medial anterior forebrain, namely the medial ganglionic eminence, preoptic area and septum. The telencephalic phenotype of Vax1 mutants varies from a total absence of growth of medioventral structures to a growth recovery of dorsolateral structures fusing medially. The medioventral defects typically include a defective cleavage of the dorsal forebrain into bilateral vesicles resulting in HPE. Reduced growth and/or absence of midline structures are also observed at the craniofacial level, such as fused or abnormal maxillary incisors (Hallonet et al., 1999).

Vax1 mutant mice have been shown to display a second, more rostrally-positioned, Rathke's pouch. The pituitary is a small-sized, major endocrine gland located at the base of the brain that is derived from an evagination of the oral ectoderm, (Rathke’s pouch), and a part of
1. Introduction

the diencephalic neuroectoderm (infundibulum). This pouch is completely separated from the first pouch and matures into an ectopic adenohypophysis containing all relevant cell lines and also exhibits posterior pituitary markers (Bharti et al., 2011).

The developmental interactions between these tissues are regulated by many factors, including transcription factors such as *Pitx1* and *Pitx2* and their target, *Lhx3*, which are specifically expressed in the oral ectoderm/Rathke’s pouch; others, such as *Six3/6*, *Sox2*, *Hesx1* and *Pax6*, which are found in both oral and neuroectoderm; and growth factors such as *Fgf8*, *Fgf10*, *Bmp2*, *Bmp4*, *Shh*, and *Notch*, which are expressed in the neural or oral ectoderm, or in both tissues (Bharti et al., 2011; Hoffmann et al., 2016).

**Figure 1.3 Absence of Vax1 induces Fgf10 expression in the rostral neuroectoderm**

A model of pituitary biogenesis in which Vax1 plays an important role as a repressor of Fgf10 expression in the neuroectoderm rostral to the infundibulum, thereby restricting its expression to the infundibulum. In the absence of Vax1, Fgf10 is expressed at the site of second pouch induction. However, there is no Fgf10 expression between the primary and second pouch (Redrawn from Bharti et al., 2011).
1.2.1 Vax genes in dorso-ventral (DV) patterning

Canonical Wnt signaling (which plays a prominent role in patterning the DV axis of the CNS) is frequently opposed by Shh. A recent study has described a mechanism for this reciprocal repression whereby Vax gene expression is activated by Shh, which in turn, activates expression of the potent Wnt signaling inhibitor, dnTcf7l so that the Vax proteins function in DV specification of the forebrain through their ability to induce antagonists of canonical Wnt signaling (Vacik et al., 2011).

Furthermore, genetic studies in mice, chicks, and zebrafish demonstrate that Vax genes are involved in the control of (DV) patterning of the ventral forebrain and its derivatives, including the corpus callosum, optic chiasm, optic nerve and retina, and that they drive the differentiation of these ventral structures by inhibiting the differentiation of dorsal structures (Vacik et al., 2011; Takeuchi et al., 2003).

1.2.2 Vax genes in ventral patterning of the developing eye

The optic vesicle- evaginated neuroepithelium, buds from the ventral diencephalon (Chow and Lang, 2001). As the eye develops, during mid-gestation, a defined dorsoventralization occurs, with the optic disk separating the ventral optic stalk from the more dorsally positioned presumptive retina. The optic stalk, which is apposed to the midline source of the ventralizing morphogen Shh, develops into the optic nerve (Martí and Bovolenta, 2002). It has been demonstrated that levels of Shh are critical to dorsoventral patterning of the eye (Chiang et al., 1996; Ohkubo et al., 2002).
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From E9.5 to E11.5 both Vax1 and Vax2 are coexpressed. Vax1 extends from the ventral optic stalk into the ventral retina, and Vax2 extends from the ventral retina into the ventral optic stalk (Ohsaki et al., 1999). At E9.5, the developing eye has progressed to the optic vesicle stage, and has already been patterned along the dorsoventral axis through the action of sonic hedgehog (Hallonet et al., 1998; Mui et al., 2002; Takeuchi et al., 2003). Invagination of the optic vesicle takes place between E9.5 and E10.5 by which time the optic cup is formed. At this stage, the diminishing expression of Vax1 mRNA in the ventral retina is also graded along the nasotemporal axis from high in the ventral nasal retina to low in the ventral temporal retina. This Vax1 gradient is therefore similar to the high-nasal-to-low-temporal gradient of Vax2 in the ventral retina, which, unlike Vax1, persists into late embryogenesis (Mui et al., 2002). By E11.5, the basic structure of the eye is apparent, with the ventral optic stalk having invaginated into the interior of the developing optic nerve and the RPE having extended ventrally around the eye.

In Vax1 knockout mice, the boundary between optic cup and optic stalk is poorly defined with regions normally occupied by optic stalk exhibiting retinal features including retinal pigment epithelium. Although RGCs form in these mutants, their axons navigate abnormally and, instead of approaching the midline to form the optic chiasm, become stalled shortly after

Figure 1.4 Genetic interaction between Vax genes during eye development
Schematic dorsal–ventral (DV) section of the neuroepithelium of the optic vesicle as it develops from the ventral diencephalon at E9.5. Shh expressed at the ventral midline, has patterned this tissue along the DV axis by this time, such that structures closest to Shh are most ventral. pvOS, pNR, pRPE, and pdOS are presumptive ventral optic stalk (red), neural retina (green), retinal pigment epithelium (yellow), and dorsal optic stalk (blue), respectively. Graded expression of the Vax1 and Vax2 genes within these tissues is displayed in decreasing gradients (B) DV section similar to that in A, at E10.5, after the optic vesicle has invaginated to form the optic cup (redrawn from Mui et al., 2005).
leaving the eye (Bertuzzi \textit{et al.}, 1999; Hallonet \textit{et al.}, 1999). This defect is most likely to reflect a requirement for \textit{Vax1} in producing the correct environment for navigating axons as \textit{Vax1} is not expressed by RGCs. Furthermore, \textit{Netrin-1}, which is normally expressed at the optic nerve head and where the optic nerve connects to the brain and is believed to guide axons along their path is missing (Pratt and Price, 2006). The importance of the overlapping expression of \textit{Vax1} and \textit{Vax2} from E9.5 to E11.5 is demonstrated by the phenotype of double-mutant embryos. In the absence of both proteins, the optic nerve is transformed in its entirety into fully differentiated retina and this transformation results from the loss of ventralizing activity in the developing eye field, and that ventralization is mediated in part through Vax repression of the \textit{Pax6} gene, a potent inducer of retinal development. (Mui \textit{et al.}, 2005).
1.2.3 *Vax genes and hedgehog signaling*

Shh is a secreted protein that plays a key role in development, mediating signaling activities of the notochord and prechordal mesoderm and floor plate (Chiang *et al.*, 1996). It exerts its effects in a concentration-dependent manner, spreading dorsally from these points and acting as a ventralizing morphogen. In the absence of Shh, signal activity is held in quiescence through the G-protein and principle Shh receptor Patched (Ptch1) repressing activity of a further transmembrane protein and Shh-agonist Smoothened (Smo). Shh binding to Ptch1 releases the inhibition of Smo, which then transduces the signal intracellularly. Downstream of Smo, a multimolecular network transduces the hedgehog signal to modify the activity of Gli protein transcription factors (glioma-associated oncogene family members; Gli1-3) (Varjosalo and Taipale, 2008).

There is evidence in the developing eye that *VaxI* lies downstream of *Shh* in the hedgehog signaling pathway (Figure 1.1, 1.2) (Hallonet *et al.*, 1999; Takeuchi *et al.*, 2003). Shh is the dominant ventralizing signal in the developing eye field as it is elsewhere in the embryonic CNS; it patterns the field through induction of the *Pax2* gene proximally and the *Pax6* gene distally (Ekker *et al.*, 1995; Macdonald *et al.*, 1995). In reciprocal gain-of-function experiments, injection of Shh into xenopus embryos expands the *vaxI* territory. In zebrafish, loss of *shh* leads to severe coloboma and the loss of *vaxI* and *vax2* expression. Inactivation of both *vax* genes results in severe coloboma supporting the model that Shh is active upstream in eye development (Take-uchi *et al.*, 2003; Wilson *et al.*, 2004). Also in zebrafish, it has been shown that Hh signals acting through Smo act downstream of the Nodal pathway to promote *vax* gene expression (Take-uchi *et al.*, 2003) in the eye. However, in the absence of both Nodal and Hh signals, *vax* genes are expressed, revealing that other signals including Fgf’s contribute to their regulation (Take-uchi *et al.*, 2003). Targeted mutation of *VaxI* in mice also causes a fully penetrant cleft of the secondary palate as well as disruptions to midline structures but the basis of these malformations is not known.

In the absence of *Shh*, *VaxI* and *Vax2* are not expressed in the eye (Wilson *et al.*, 2004). By the study of *VaxI* homozygous mutants, it has been indicated that *VaxI* and *Pax2* expression in the optic stalk requires midline signals, such as Shh (Hallonet *et al.*, 1999). Also, *Shh* overexpression leads to dorsal expansion of the *Vax2* expression domain (Sasagawa *et al.*, 2002).
Vax2 has been thought to play an important role in eye development because of both its expression patterns and functional studies carried out in frog and chicken (Take-uchi et al., 2003). In zebrafish it has been demonstrated, in the developing eye, that Hh signals acting through Smoothened act downstream of the Nodal pathway to promote vaxl gene expression. Overexpression of Hh induces vaxl and vax2 whereas expression of vaxl and vax2 is lost in the Hh pathway mutants (Take-uchi et al., 2003).

In the developing eye of Vaxl and Vax2 double mutants, the loss of ventral optic stalk is not due to the loss of the primary ventralizing morphogen; Shh levels remain high at the ventral midline in the double mutants in contrast to the situation in Foxg1 (BF1) mutants, where a portion of midline Shh expression is lost (Mui et al., 2005; Huh et al., 1999). This maintained expression of Shh is in keeping with the demonstration that the Vax genes are downstream of Shh signaling and subject to Shh induction (Take-uchi et al., 2003).

In the absence of both Nodal and Hh signals, vax genes are expressed, revealing that other signals including Fibroblast Growth Factors (Fgf) contribute to their regulation (Takeuchi et al., 2003). Targeted mutation of Vaxl in mice also causes a fully penetrant cleft of the secondary palate as well as disruptions to midline structures but the basis of these malformations is not known.
1. Introduction

In the absence of Shh, Vax1 and Vax2 are not expressed in the eye (Wilson et al., 2004). By the study of Vax1 homozygous mutants, it has been indicated that Vax1 and Pax2 expression in the optic stalk requires midline signals, such as Shh (Hallonet et al., 1999). Also, Shh overexpression leads to dorsal expansion of the Vax2 expression domain (Sasagawa et al., 2002) Vax2 has been thought to play an important role in eye development because of both its expression patterns and functional studies carried out in frog and chicken. In zebrafish, it has been demonstrated, in the developing eye, that Hh signals acting through Smoothened act downstream of the Nodal pathway to promote Vax1 gene expression pathways. Over-expression of Hh induces vax1 and vax2 whereas conversely, expression of vax1 and vax2 is lost in the Hh pathway (Takeuchi et al., 2003).
1.3 Craniofacial development

The vertebrate craniofacial complex and the skull is anatomically complex and phylogenetically diverse. The head's primary functions include roles in feeding, respiration and as supporting structures for the brain and sensory organs. It arises as a result of intricate and coordinated interactions between distinct embryonic tissues which are mediated and controlled by numerous molecular signaling cascades. The embryonic and postnatal development of this region is a dynamic and multi-phased process. Disruptions or mutations in many of these signaling cascades may result in disruptions of the normal development of the face and in congenital craniofacial malformations. These types of malformations may occur as part of a syndrome or can be isolated, individual anomalies. Craniofacial anomalies may be caused by external, environmental factors. Together, these constitute approximately 30% of all human birth anomalies and may have a significant impact on the affected individual's quality of life (Epstein et al., 2004).

Craniofacial anomalies have traditionally been corrected surgically, which can be onerous for the patient as interventions (surgical and others, such as orthodontic treatment) occur over several stages throughout childhood and may last several years in duration. This can have a psychological impact on the developing child. Surgical correction is often deferred until the majority of growth is complete. Other complications involved in this approach include a lack or deficit in the amount of the necessary tissue required for repair (alveolar and palatal bone in cleft palate repair for example). It has been stated that a ‘paradigm-shift’ in the understanding of craniofacial development over the last 4 decades has opened the possibility for cell-based tissue engineering which could be incorporated into or as a replacement for conventional treatment in the future. Thus, there is huge value in a continually improved understanding of the molecular and cellular mechanisms of normal and abnormal developmental biology of the craniofacial complex (Solomon et al., 2013).

Head structure patterning systems are controlled by multiple conserved growth and transcription factors. Amongst these are paracrine factors which we can subdivide into 4 well known major gene groups (according to their structure): Hh, Fgf, Wnt and Tgfb. These groups encode secreted signaling proteins that regulate a wide range of cellular functions and developmental processes such as cell proliferation, survival, fate determination, differentiation, polarity, adhesion, migration, morphogenesis, and patterning. Their activity is not restricted to
the craniofacial region but also to the control of a number of other early developmental processes during embryogenesis such as gastrulation, axis specification, neural tube formation and limb development. As a result of this complex and intricate interplay of roles and activities, in mice with loss-of-function mutations of these genes, a range of developmental defects and frequently early lethality often results. This can make investigation of the roles of said genes in later stages complicated (Francis-West et al., 1998; Depew et al., 2002). Alternative experimental methods, such as chimeric mutant mice and conditional knockout mutant mice as well as inhibition and over-expression culture studies have illustrated the vital importance of these genetic interactions are for normal growth and morphogenesis in the craniofacial region. Mutant mice strains with phenotypic features resembling those observed in humans represent an important tool in understanding the genetic understanding of human syndromes (Hayhurst and McConnell, 2003; Francis-West et al., 2003; Cobourne, 2004; Rice, 2005; Geng and Oliver, 2009).

1.3.1 Origin and development of the head

The embryological and postnatal development of the craniofacial region is a dynamic, multistep process. The interaction of several embryonic cell lineages; ectoderm, endoderm, mesoderm and cranial neural crest cells (CNCCs) that arise at the interface of ectoderm and neural tissues during formation of the neural tube. Each of these tissue components produces distinct derivatives. Ectoderm differentiates into the epidermis and sensory neurons, mesoderm gives rise to parts of the craniofacial bones, musculature and circulatory system. The endoderm forms the mucosa of the lining the pharynx. CNCCs are critical for normal facial development (Francis-West et al., 2003). The skeletal elements from the skull are derived from both the mesoderm and the CNCCs (Le Douarin et al., 2004). The functions of neural crest cells include coordination of the different visceral activities (chomaffin cells of adrenal medulla), externally as melanocytes and in craniofacial skeletal development (Le Douarin et al., 2004).

The neural crest cell contribution may be direct; providing cells or, indirect; providing a necessary, often inductive, environment in which other cells develop (Hall, 2008). CNCCs undergo epithelial-to-mesenchymal transition and migrate ventrolaterally to their final destinations in the neck and craniofacial regions (Trainor and Nieto, 2003). CNCCs populate the branchial arches, giving rise to maxillary and mandibular primordia as well as the hyoid and more posterior components. They also populate the frontonasal prominence and calvaria. These
give rise to cartilage, bone, connective tissue, glia and muscles (Anderson, 1997). Strong evidence exists to show that CNCCs are developmentally 'plastic', their fate not being predetermined before they reach their final destination. Progenitor cells are instructed by signals from other tissues in order to generate craniofacial skeletal elements (Cordero et al., 2011).

The pharyngeal endoderm, the branchial arch ectoderm and the isthmic organiser at the midbrain-hindbrain boundary are some of the tissues that provide instructive signals for the CNCCs (Baker et al., 2002; Couly et al., 2002; Trainor and Nieto 2003; Le Douarin et al., 2004). Histological analysis of early mouse embryos, transplantation, vital dye labelling experiments and by use of the Wnt1-Cre/R26R transgenic mouse, it has been shown that the facial skeleton and anterior cranial base are entirely of CNCC origin and that the posterior cranial base skeleton is derived from the paraxial and somitic mesoderm (Nichols, 1981; Tan and Morriss-Kay 1986; Serbedzija et al., 1992; Chai et al., 2000; Jiang et al., 2002; Pietri et al., 2003).

Around E7.5 in the mouse, there are the first clear signs of head development, where the rostral end of the neural plate begins to enlarge to form head folds. At approximately the same time, neurulation begins with the appearance of the neural plate (NP) in the midline rostral to the regressing neural streak. As the NP begins to thicken at its lateral part, it folds and separates from the surface ectoderm to form a hollow neural tube that runs parallel and dorsal to the notochord (Geng and Oliver, 2009).

The narrow caudal part of the neural tube develops into the spinal cord whereas the rostral end expands and subdivides into the three vesicles of the brain; the rhombencephalon, mesencephalon and telencephalon (Francis-West et al., 2003; Rossant and Tam 2004). Further development of the prosencephalon is instructed by an important signaling molecule centre, the prechordal plate (PrChP) (Figure 1.6). The PrChP forms a thickened layer of endodermal cells located beneath the forebrain at the rostral end of the notochord. The PrChP induces the forebrain to develop and proliferate and guides evagination of the two sets of vesicles from the dorsolateral walls of the rostral prosencephalon. The first set give rise to the optic vesicles and the second to the telencephalic vesicles. These bilateral vesicles become separated by an interhemispheric sulcus. These olfactory bulbs also develop as evaginations from the anterior aspect of the telencephalon. The removal of the PrChP results in a failure of the prosencephalon
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and eye field to subdivide and consequently leads to HPE and cyclopia. (Muenke and Beachy 2000).

The rhombencephalon is further divided into 7 rhombomeres (R1-R7) that give rise to three distinct streams of neural crest cells expressing different combinations of homeobox containing Hox- genes. The most rostral stream that produces trigeminal crest arises from caudal midbrain, R1 and R2 to populate the first branchial arch. Precursors of the second branchial arch derive from R4 and form hyoid crest. R6 and R7 subsequently form postotic crest and populate the more caudal branchial arches. R3 ad R5 are important in segregation of the different streams of CNCC as they only produce low levels of CNCCS that integrate with adjacent streams (Lumsden et al., 1991; Schilling and Kimmel 1994; Le Douarin et al., 2004). CNCCs populating the FNP migrate from more rostral parts of the midbrain and caudal forebrain (Francis-West et al., 2003).

Figure 1.6  Morphology, tissue components and regional organization of the vertebrate neural plate and neural tube

(A) the view in B presents a cross section of the prosencephalic neural plate (np) at the level indicated by the dotted line in A and illustrates the relationship of the neuroectoderm (ect) with the non-neural ectoderm head mesenchyme (mes), prechordal mesendoderm (PrChp) and forground endoderm (endo). (B) Dorsal view of the anterior neural plate showing the anterior neural ridge (anr), the neural ridge (nr), and the non-neural ectoderm. The approximate positions of the primordia of the prosencephalon (pr), mesencephalon (mes) and rhombencephalon (rh) are also demarcated. (C) Neural plate schema showing approximate axial positions of several forebrain primordial structures: the chiasmatic (cs) and optic stalks (os) are located just posterior to the telencephalic domains, followed by the hypothalamus that overlies the prechordal mesendoderm. Lateral to the chiasmatic and optic stalks is the primordia of the eyes. Loss of Shh signaling from the prechordal mesendoderm results in a lack of eye field. (D) Lateral schema of the neural tube demonstrating the topographic relationships of the structures described in (A-C). The anterior neural ridge forms the roof plate of the telencephalon (adapted from Rubenstein and Beachy, 1998).
The branchial arches (pharyngeal arches) are structures formed as bilateral swellings on both sides of the embryo. Vertebrates have five distinctive branchial arches numbered I-IV and VI (Figure 1.7). Branchial arch V regresses early in development and as a result doesn't give rise to any derivatives. The arches are separated externally by four pharyngeal grooves and internally by pharyngeal pouches. Each arch therefore forms a unit whose outer layer is covered by ectoderm and the inner, the oropharyngeal side, is lined by endoderm. Mesoderm forms and inner core of the branchial arch that becomes surrounded by CNCCs which occupy the arches. The branchial arches each develop its own subset of muscles, cartilage, ganglions and vessels. Ectodermal (epithelial) and endodermal (oropharyngeal) tissues of each arch express instructive signaling molecules which initial crosstalk with the CNCCS occupying the arch. These signaling molecules induce proliferation and patterning of the arches. (Graham et al., 2004; Graham, 2005; Chai and Maxson, 2006). Branchial arches are transient structures. Their development; segregation, identity, and polarization are dependent on signals from the endoderm and can even take place in the absence of CNCCs (Hunt and Krumlauf, 1991; Couly et al., 2002). CNCCs respond to these signals by expressing different Hox genes (Hunt and Krumlauf 1991). The mesoderm is required for maintaining the normal levels of homeobox gene expression within the branchial arches (Figure 1.7) (Gavalas et al., 2001).

Figure 1.7  Schematic drawing of the pharyngeal region at E10.0)
Left panel (lateral view), Right panel (Ventral view). The pharyngeal arches are comprised of mesoderm (green), neural crest cells (light blue arrows and light blue), endodermal pouches (red), medial pharyngeal endoderm (pink), and lateral ectoderm (darker blue). Grown zebrafish possess 6 endodermal pouches and 7 pharyngeal arches. Ventral view. ep. endodermal pouches; eye, developing eye; ov, otic vesicle; PA, pharyngeal arches; R, rhombomere (redrawn from Kopinke et al., 2006).
1.3.1.1 Development of the early forebrain

During development, the forebrain acquires precise subdivisions occur along the DV and the anteroposterior (AP) axis (Shimamura and Rubenstein 1997). Different morphogens are required for this complex organization and for cell type differentiation. As the ventral forebrain and retina develop, they are under the control of similar extracellular cues. Recent contributions of cell biochemical, animal model and genetic studies have revealed some of the signaling pathways essential for this region (Gongal et al., 2011). During early embryogenesis, the mouse blastocyst develops into a bilayered conical structure with the epiblast inside and the visceral endoderm outside (Geng and Oliver, 2009). During development, towards the end of gastrulation, the embryo contains the three primary germ layers: the ectoderm, mesoderm, and endoderm. The axial mesoderm consists of the PrCP and the notochord posteriorly. The neural plate folds upon itself to form the neural tube. The anterior end then expands and bifurcates to form the telecephalon. At the end of somatogenesis, the forebrain is comprised of the telencephalon, diencephalon and the hypothalamus. The dorsal telencephalon develops into the cerebral cortex, the ventral will develop into the basal ganglia and the olfactory bubs will form the most anterior portion of the cerebrum in mouse (Figure 1.8).

![Figure 1.8](image_url)

**Figure 1.8  Development of the mammalian forebrain**

At early somite stage (E8.5 for mouse; CS10 for human), the neural ectoderm has been specified into different regions along the anterior-posterior axis and the axial mesoderm is underlying the midline of the neural ectoderm. ANC, anterior notochord; PFB, prospective forebrain (or ANE); PH, prospective hindbrain; PM, prospective midbrain; PNC, posterior notochord; PSC, prospective spinal chord. (E) Neural tube closure occurs at around the 15-somite stage (E9.0 for mouse; CS11 for human). The forebrain gets further regionalized into telencephalon, diencephalon, and prospective hypothalamus (PH). OV, optic vesicle. (F) Approximately at E10.5 in the mouse or at CS14 in human embryos, the expanding telencephalon bifurcates dorsally to form the two hemispheres and gets patterned into dorsal telencephalon (DT) and ventral telencephalon (VT) (Geng and Oliver, 2009).
1.3.1.2 Development of the dentition

The first branchial arch, appears around E8 in the mouse and divides into two. The caudal division forms the mandible and the rostral forms the maxillary process. The mandibular processes approximate early in the midline to establish the lower jaw. Once oral-aboral and disto-proximal polarities of the mandibular are established, chemical signals (including Wnt, Fgfs, Shh, Bmps) are produced from the oral epithelium to the underlying mesenchyme to initiate tooth formation. Tooth development begins as thickenings (dental lamina) in the oral epithelium in regions of the proximo-distal axis of both jaws around E11-E11.5. These thickenings then invaginate into the under ectomesenchyme that is condensing and proliferating around the developing tooth (Francis-West et al., 2003).

The stages of tooth development are named after their resemblance to the shape of the invaginating epithelium including bud (E13-14), cap (E14-E14.5), bell (E17-18) and late bell stages (Figure 1.9). The incisors begin development before molars. Tooth morphogenesis is regulated by signals emanating from the epithelial thickenings known as enamel knots visible at the bell stage. The knots express a number of signaling molecules necessary for normal cusp formation in molars. In incisors, these knots die via physiological cell death. Under the control of distinct instructive signal, the oral epithelium differentiates into enamel producing ameloblasts, whereas the neural crest-derived mesenchyme gives rise to the dental pulp, cementum, periodontal ligaments, alveolar bone and dentine producing odontoblasts (Zhang et al., 2005).

Figure 1.9 The signals mediating the initial steps in tooth development

Molecular signaling during tooth crown development. Expression sites of transcription factors (italic) and signaling molecules (bold). Institute of Biotechnology, University of Helsinki
1.3.1.3 Development of the nasal processes

Development of the human face begins in the fourth week of embryogenesis with migrating neural crest cells that combine with the core mesoderm and the epithelial cover to establish the facial primordial. At E9.0 in mouse, the primitive mouth, or stomatodeum is bound rostrally by the developing forebrain and caudally by the swelling mandibular arches (first pharyngeal arch). By E9.5, the facial primordial consist of 5 separate prominences. At the rostral side of the stomodeum is a symmetrical, unpaired frontonasal, which is situated ventrolaterally to the forebrain and is populated my mesenchymal cells derived from the fore- and mid-brain neural crest (Hinrichsen, 1985).

From E10.0-10.5, the frontonasal prominence widens as the forebrain gives rise to the telencephalic vesicles. The surface ectoderm thickens on the ventrolateral part of the frontonasal prominence which give rise to the nasal placodes. The frontonasal process grows and bulges around the nasal placodes, resulting in the formation of nasal pits and the swelling horseshoe shaped lateral and medial nasal processes (Figure 1.10) (Sperber, 2002). Rapid growth of the mesenchyme in the maxillary processes, have pushed the nasal pits medially while the medial nasal processes have grown ventrolaterally converting the nasal pits from round depressions into dorsally pointed slits. By approximately E11.0, rapid growth of maxillary (MXP) and medial nasal processes (MNP) push the lateral nasal processes (LNP) further rostrally and bringing the distal ends of the MXP and MNP into direct contact. Fusion between the nasal processes occur initially at the posterior part of the nasal pits and proceed anteriorly (Gaare and Langman, 1977).
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Figure 1.0 Early facial development of the mouse.
Scanning electron micrographs of an early mouse embryo show development of the face from a series of rudimentary processes. In particular, the lateral part of the upper lip is formed from the maxillary processes, whilst the philtrum is derived from the paired medial nasal processes, which fuse in the midline. e, eye; fnp, frontonasal process; md, mandibular process; mx, maxillary process; lnp, lateral nasal process; mnp, medial nasal process; np, nasal placode (Cobourne & Dibiase 2015)

Morphogenesis of the face in the chick differs from mammals as the medial nasal process sometimes appears as a single entity called the frontal or frontonasal process. As the epithelial fusion between the processes (MXP, MNP, and LNP) continues towards the start of the 7th week of gestation in humans (E11.5-E12.0), the MXP, growing quickly, push the MNP and LNP mediofrontally. These morphogenetic changes gradually convert the nasal pits to nose chambers and to nasal ducts as the fusion process between the medial and lateral nasal processes complete. The choanal membranes at the dorsal ends of the capsule perforate to connect the nostrils to the posterior oral cavity. The nostrils then proceed to transform to small slits and their lower edges are remodelled by the fusion between the medial nasal and primary palate maxillary processes during the last stages of upper lip formation.

At approximately E12.5 formation of the upper lip is complete, the medialization of the nose chamber and the filling of the medial groove (the groove between the medial nasal processes) occurs followed by outgrowth of the intermaxillary segment into the oral cavity to form the anterior palate (Cobourne, 2004).
1.3.1.4 Development of the Vomeronasal organ (VNO)

Discovered by Rusch (1703) in humans and in other mammals by Jacobson (1811), the VNO is a paired tubular structure located along the base of the nasal septum originating from the Vomeronasal placode at the medial wall of the olfactory pit, encompassed inside a bony or cartilaginous capsule which opens into the base of the nasal passage. The VNOs are receptors units for the accessory olfactory system (AOS) – which is comprised of the VNO and vomeronasal nerves, the Grüneberg ganglion, the septal organ of Masera as well as the accessory olfactory bulb (Munger et al., 2009). It is separate from the main olfactory system (MOS) which is composed of the main olfactory epithelium (MOE) and the main olfactory bulb, which are involved in odorant detection (Eckler et al., 2011). It monitors more specific chemical signals than the MOS, in particular species-specific pheromones (Wysoki, 1979). Function may vary dependent on species, these chemical signals are related to reproductive behaviours (Winans and Powers, 1977) as well as functioning in sexual communication (Wysocki, 1979). Sexual dimorphism in the AOS in vertebrates such as salamanders (Dawley and Bass, 1988) and rats (Segovia and Guillamón, 1982) has been studied. During midgestation, the MOE and the VNO both originate from the olfactory pits. A proposed genetic mechanism has been investigated whereby both these functionally and anatomically distinct organs arise from a common olfactory pit (Eckler et al., 2011). The zinc-finger transcription factor *Fezf1* regulates the maturation and identity of MOE sensory neurons whereas *Fezf2* is responsible for the survival and proliferation of VNO progenitors.
1.3.1.4.1 Developmental stages of the VNO:
Six stages have been used to summarize the stages that characterize the growth and development of the VNO in the rat (Garrosa et al., 1998):

**Prenatally:**
1. Anlage
2. Early morphogenesis
3. Late morphogenesis

**Postnatally:**
4. Initiation of secretory activity
5. Cytoarchitectural maturity
6. Complete histogenesis

Although other mammal species follow a different timing according to its own rate of maturation. This staging-system has been used to compare the different developmental stages amongst different mammalian species. It has been acknowledged that relative discrepancies exist in the literature in the age-timing of different rodents (Garrosa et al., 1998). The primordium of the murine olfactory organ is the paired olfactory placode which can be identified in early development as a patch of thickened ectoderm on the ventrolateral aspect of the head (E9.0) which then sinks subsurface to form an olfactory pit (E10.0).

In vertebrates the primordium lies close to the forebrain prior to olfactory nerve development (Cuschieri and Bannister, 1975). Olfactory axons arise from the placodal cells and are directed towards the forebrain (His and Cajal, 1889) and neural differentiation such as β-tubulin and neural cell adhesion molecule (NCAM) expression can be seen. By E.11, the olfactory pit becomes deeper and has formed secondary recesses. The VNO is also recognizable as a thickening of the epithelium of the medial wall olfactory pit which invaginates to first form a groove. The cellular composition of these buds is varied and includes cells at different stages of maturation (Anlage stage).

As the lips fuse, the VNO has become a recognized tubular form due to its growth and closure along the base of the nasal septum (Early morphogenesis stage). Nerve fibers can be traced from the base of the vomeronasal epithelium caudally along the margin of the cartilage in
the nasal septum (Cuschieri and Bannister, 1975). From E11.0-E13.0, the VNO becomes a distinct structure at the base of the nasal septum. The two epithelia (sensory and non-sensory) differentiate and become distinguishable from one another to the point where the VNO cross section appears kidney-shaped along most of its length, showing a crescent shaped lumen, which is also laterally concave (which partially encompasses a developing blood vessel). The vomeronasal nerve appears as a distinct structure which reaches the developing olfactory bulb and basal forebrain (Daikoku et al., 1993). During the late morphogenesis stage, the VNO completes its morphogenesis in that it acquires its adult shape. However, a long period is required prior to maturation. The length increases rapidly until birth. During this stage, the nasal cavity is formed definitively and in conjunction with the elevation of the palatal shelves horizontally determines the vascular pattern of VNO. It is supplied by the middle group of radially running long septal branches of the olfactory artery, a branch of the anterior cerebral artery (Szabó and Mendoza, 1988).

The postnatal stages (4-6) describe the postnatal stages before complete histogenesis is reached. During these stages the vomeronasal glands begin to secrete (stage 4) as well as ossification of the capsule with a concurrent increase in VNO size until adulthood followed by a decrease in the number of sensory cells (Wilson and Raisman, 1980). In humans, the VNO commences development at about 37 days post ovulation as epithelial evaginations bilaterally and antero-inferiorly to the nasal septum (Bossy, 1980). During development, these fuse laterally and form tubular structures. The VNO organ appears rudimentary in new-born humans (Kölliker, 1877). Examination of the nasal septum revealed the presence of vomeronasal cavities in approximately 70% of adults (Johnson et al., 1985). In contrast to the situation in other mammals, the organ is not supported by a rigid tube of bone or cartilage. There is no erectile tissue around the cavities to draw in the stimulus. As in other mammals, the VNO system plays a role in the migration of luteinizing-hormone-releasing hormone (LHRH) neurons from the olfactory placode towards the brain during the development of the embryo (Schwanzel-Fukuda et al., 1996). Cells immunoreactive for LHRH are detected in the VNO organ at 8–12 gestational weeks and later (at 12–19 weeks) along the nerve fascicles arising from the vomeronasal organ. No LHRH-positive cells can be seen close to the vomeronasal organ in foetuses older than 19 weeks (Kjaer and Hansen, 1996).
1.4 Sonic hedgehog signaling during craniofacial development

The Hedgehog signaling pathway plays a fundamental role in coordinating normal vertebrate development (Ingham and McMahon, 2001; Briscoe and Therond, 2013) and maintaining appropriate postnatal tissue homeostasis (Petrova and Joyner, 2014). In mammals, there are three Hedgehog-family members, Shh, Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Shh has the predominant role in developmental processes, such as in patterning, morphogenesis and proliferation of different tissues (McMahon et al., 2003). Shh is also expressed in adult tissues for tissue homeostasis. Shh has therefore been associated with a range of developmental disorders as well as in oncogenesis (Villavicencio et al., 2000). In recent years, the complexity of vertebrate Shh signaling has been highlighted by the identification of multiple novel proteins that are involved in regulating both the release and reception of this protein.

1.4.1 Isolation of the hedgehog gene

*Drosophila* hh was identified in a classical screen of genetic mutations which disrupted the larval body plan of the fly. Hedgehog (*hh*) was originally identified as a segment polarity gene. Its name comes from the mutant phenotype of the larvae, where the neatly patterned rows of denticles are disrupted and instead a lawn of denticles form, resembling the spines of a hedgehog.

Following the identification of *hh* in *Drosophila*, homologs of the *hh* have been discovered in a number of other species such as the mouse (Echelard et al., 1993), chick (Riddle et al., 1993), zebrafish (Currie and Ingham, 1996) and xenopus (Ekker et al., 1995). When expression patterns are compared, it is evident that Hedgehog function is conserved throughout evolution. Drosophila has a single *hedgehog* gene, three homologs are present in mouse, with two present in the chick, namely *Shh* (Riddle et al., 1993) and *Ihh* (Vortkamp et al., 1996). *Dhh* in the mouse is the most closely related to *hh*. *Ihh* and *Shh* are products of later gene duplication (Echelard et al., 1993).

The mammalian *Hh* genes have specific patterns of expression suggesting distinct and various functions during development and in later life (Echelard et al., 1993). *Dhh* is present in Schwann cells and Sertoli cells of testis where is participates in spermatogenesis (Clark et al., 2000). *Ihh* is found in the gut and pre-hypertrophic chondroblasts (Bitgood and McMahon, 1995). Studeis of *Ihh* focus on its role in chondrogenesis and bone formation (Vortkamp et al.,
During embryogenesis, *Shh* has a very specific expression pattern (Bitgood and McMahon 1995). It is expressed in areas that are important signaling centres such as the node, notochord, prechordal plate, ventral forebrain, midbrain, branchial arches, and in the zone of polarizing activity (ZPA) in the limb (Echelard *et al.*, 1993; Chang *et al.*, 1994; Bitgood and McMahon, 1995)

### 1.4.2 Biochemistry of the Shh pathway

The hedgehog ligand is initially synthesised as a 46 kDa precursor with two distinct domains: the N-terminal domain is processed to a 19 kDa fragment (Hh-N) following proteolytic cleavage that is executed by the C-terminal domain within the endoplasmic reticulum (Lee *et al.*, 1994; Ekker *et al.*, 1995; Currie and Ingham, 1996). The C-terminus acts as a cholesterol transferase to covalently attach a cholesterol group to the carboxy end of the Hh amino terminal fragment, Hh-N (Porter *et al.*, 1996). The Hh-N molecule is further modified by the subsequent addition of a palmitoyl group at Cys-24 resulting in a hydrophobic molecule that is referred to as Hh-Np (Hh-N-processed) (Chamoun *et al.*, 2001).

The processing of Hh-N takes place in the secretory pathway and is mediated by a palmitoylacyltransferase which is coded for by the Skinny hedgehog gene (*Ski/Skn*). This gene is also known as *sightless (sit) rasp and hedgehog aceyltransferase (Hhat)*. Mice deficient in *Skn* exhibit similar defects to *Shh* mutants: they lack a differentiated floor plate and display neural tube defects. These data indicate that the palmitoyl addition is essential for SHH function. The addition of cholesterol and palmitate increases the efficacy of SHH-Np while addition of hydrophilic adducts to the N terminus reduces the activity of SHH. Lipidation of the Hh signaling protein is an essential step in producing a functional signaling molecule (Ramsbottom and Pownall, 2016).
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Figure 1.11  Hh signaling cascade

The Shh ligand is initially synthesised as a precursor with two distinct domains: the N-terminal “hedge” and C-terminal “hog” domain, which undergoes autoproteolysis to give N- and C-terminal fragments. The C-terminal fragment acts as a cholesterol transferase to attach cholesterol to the N-terminal fragment. Skinny hedgehog attaches palmitate to the N-terminus of Hh to give rise to the fully processed form. Following synthesis, Hh is delivered from the endoplasmic reticulum to the cell membrane, where it is released from the cell via a number of different mechanisms. (1) Hh binds Disp in a cholesterol-dependent manner, and through the combined action of Disp and Scube2, is released from the cell. Heparan sulfate proteoglycans (HSPGs) act as assembly points for multiple components. Hh monomers are able to form multimeric complexes aided by association with HSPGs and Scube2. Hh is released following proteolytic processing by sheddases, which remove cholesterol and palmitate. These complexes are more soluble than the monomeric form and so are able to diffuse away from the cell; (2) Unprocessed Hh may re-enter the cell in a Disp-dependent fashion (2a) and be internalised by endosomal sorting complexes required for transport (ESCRT) proteins, which sort Hh proteins into intra-luminal vesicles (2b); These vesicles subsequently fuse with the plasma membrane and are released from the cell (2c); (3) Association of Hh with HSPGs results in loading of Hh into lipoprotein particles. Glypicans (Glycosylphosphatidylinositol (GPI)-linked HSPGs) may be cleaved and released along with Hh. (from Ingham et al., 2011).
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1.4.3 Primary cilium

In recent years, the primary cilium has been identified as a key organelle involved in the essential regulation of normal Shh signaling (Figure 1.11) (Caspary et al., 2007; Huangfu and Anderson, 2005; Rohatgi et al., 2007; Singla and Reiter, 2006). Cilia are 1–3μm membrane-bound, microtubule-based structures found on many cells in the vertebrate body (Davis et al., 2011). Cilia can be categorized into two groups via their structure and function; both groups share at their base a centrosome-derived basal body that anchors the cilium within the cytoplasm. The axoneme in motile cilia is organized in a ‘9 + 2’ arrangement, where the double microtubule core in the middle is surrounded by nine microtubule doublets arranged in a circular pattern. Motility is achieved through the protein–protein interactions between the central core and the surrounding doublets (Nonaka et al., 1998). Primary cilia, also known as non-motile cilia are arranged in a ‘9 + 0’ structure lacking the central microtubule core. Even though primary cilia are considered non-motile, it has been reported that primary cilia at the embryonic node can generate a rhythmic movement, though not in the same motion of motile cilia (Nonaka et al., 1998).

Smo, Ptch1, Gli1-3 and the negative regulator Sufu have been detected at the primary cilium Ptch1 is proposed to be localized to the base of the cilium in the absence of its ligand and to inhibit signaling by preventing Smo localization to the cilium (Figure 1.11). Upon ligand binding, simultaneous removal of Ptch1 and localization of Smo to cilia occur. β-arrestins are involved in facilitating Smo interaction with the Kif3a kinesin motor protein, regulating Smo localization to primary cilia (Kovacs et al., 2008; Rohatgi et al., 2007). However, it has also been proposed that Smo may move through a lateral transport pathway from the plasma membrane to the ciliary membrane (Rohatgi et al., 2009). Furthermore, phosphorylation of Smo carboxyl terminal tail in the mouse by the serine/threonine kinases GIRK2 and CK1α was found to activate the receptor and promote its ciliary accumulation (Davis et al., 2011).
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Figure 1.12  Hh signaling pathway

In the absence of Hedgehog (HH) ligand (OFF, left panel) the Patched (Ptc1h) receptor predominates in the primary cilium and represses Smo through an uncharacterized (but indirect) mechanism that prevents Smo entry into the cilium. Gpr161 is also localized to the cilium and may contribute to activation of PKA by generating an increase in cAMP. The Gli proteins Gli2 and Gli3 are maintained in the cytosol by SuFu and PKA, CKI and GSK3β, which leads to their partial degradation through proteolytic processing into repressor forms (GliR) that suppress HH target gene transcription in the nucleus. Hhip1 (pink), a negative regulator of the pathway binds HH and is found as both membrane-associated and soluble forms. In the presence of ligand (ON, right panel) HH binding to Ptc1h takes place, which is facilitated through the formation of a Lrp2/Boc/Cdo/Gas1 multi-molecular co-receptor complex and results in the aggregation of Ptc1h away from the cilium and the relief of Smo inhibition, allowing Smo to enter the cilium in a movement mediated by oxysterols. Evcl and Evc2 placed at the cilium base bind Smo to relieve the inhibition of Gli proteins through the Kif7-mediated anterograde transport of the Gli-SuFu complex to the tip of the cilium. Gpr161 is removed and internalized from the cilium, which prevents its inhibitory activity (not shown). The production of Gli activators (GliA) derived from the full-length Gli proteins occurs, and accumulation of GliA in the nucleus enables activation of HH target genes, such as Ptc1h and Hhip1, and the inhibition of Gas1. In secreting cells, the HH ligand precursor undergoes a series of internal modifications before reaching an active, multimeric form. Hhat is necessary for the post-translational palmitoylation of HH; in the absence of Hhat, HH secretion is decreased. HH first traverses the lipid bilayer via its interaction with Disp1 and then is extracted from the membrane via interaction with Scube2. Exactly how Scube2 facilitates the subsequent movement of HH away from its source remains unclear (Xavier et al., 2016).

Activation of Smo is believed to antagonize SuFu, which in turn negatively regulates the Gli transcription factors (Figure 1.12). The kinesin Kif7, a vertebrate homolog of Drosophila Costal2 regulating the activity of Cubitus interruptus (the homolog of the Gli transcription factors) (Ayers and Théond 2010; Ingham et al., 2011), is proposed to play a major role for coordinating and maintaining the elements of the Hh pathway at the primary cilium. Kif7 is believed to act downstream of Smo and upstream of Gli2. Its activity depends on the presence of the primary cilium and it has both negative and positive roles in Shh signal transduction. In the absence of
Shh, Kif7 is localized to the cilium base where it forms a complex in particular with Gli proteins and promotes processing of Gli repressor forms. Upon ligand stimulation, Kif7 translocates to the cilium tip and would block Sufu, which results in Gli proteins accumulation and activation, Shh signal reception and transduction (Endoh-Yamagami et al., 2009; Liem et al., 2009; Ruat et al., 2012).

In a simplified model, this involves release of the Shh signal from the surface of signaling cells as a dual-lipidated protein modified by the addition of cholesterol and palmitate groups at the C and N-terminal regions, respectively. Release from the cell perimeter is achieved through the combined activity of Dispatched (Disp), a multipass sterol-sensing domain (SSD) protein and Scube2 (Signal peptide CUB EGF-like domain-containing protein), a secreted glycoprotein; both of which interact with Shh through its cholesterol moiety (Creanga et al., 2012; Tukachinsky et al., 2012). Once released, Shh can signal within tissues at both short and long-range, with transport achieved through the formation of soluble multimers, lipoproteins and exovesicles. Correct spatial gradients of Shh in relation to its production site are achieved by several complex mechanisms involving the cholesterol modification of the newly translated precursor molecule, the actions of enhancers, activators and repressors of the Shh signaling pathway (Xavier et al., 2016).

Shh proteins mediate their action via a receptor complex associating two transmembrane proteins: Ptc1, the Shh receptor, which displays a transporter-like structure, and Smo, a putative member of the G protein-coupled receptor superfamily, which transduces the Shh signal downstream of Ptc1. The repression exerted by Ptc1 on Smo is relieved when Shh binds Ptc1 and a complex signaling cascade is initiated leading to the activation of the transcription factors of the Gli family (Gli1-3) and to the transcription of target genes including Ptc1 and Gli1 themselves (Figure 1.12) (Roessler et al., 2003). Canonical Shh pathway activation leads to the inhibition of Gli processing into their transcriptional repressor forms and to the synchronous accumulation of their activator forms. Downstream intracellular signaling is subsequently coordinated directly through the cilium and regulated by variation in the balance of Gli transcription factors; these provide a readout for transcriptional activation or repression, primarily through Gli2 and Gli3, respectively (Hui and Angers, 2011). Increased Gli1 activation is a direct response to pathway activation and amplifies the Hh response. Gli2 and Gli3 function mainly as transcriptional activator and repressor, respectively, even if both can show the opposite
activity in specific contexts (Roessler et al., 2003; Riobo and Manning 2007). Amongst the direct transcriptional targets of Shh are Gli1, Ptc1 and Hedgehog-interacting protein (Hhip1), a membrane glycoprotein that can also bind and sequester Shh ligand (Chuang and McMahon, 1999). Thus, a caveat of signal activation is that Ptc1 (and Hhip1) rapidly accumulate on the cell surface; and pathway activity is therefore buffered in a ligand-dependent manner, through the relative availability of bound and unbound receptor (Chen and Struhl, 1996; Casali and Struhl, 2004).

A number of additional receptor proteins have been identified that can physically associate with Shh at the cell surface. These include the low-density lipoprotein receptor-related protein, Lrp2 (otherwise known as gp330/megalin) (Saito et al., 1994); the GPI-linked membrane glycoprotein Growth arrest-specific 1 (Gas1) (Martinelli and Fan, 2007), the negative regulator Hedgehog-interacting protein (Hip), which is found in a soluble and membrane associated form in brain regions (Coulombe et al., 2004) and the Ig/fibronectin single-pass membrane-spanning cell adhesion proteins Cdon (cell adhesion associated, oncogene regulated) and Boc (Boc cell adhesion associated, oncogene regulated) (Kang et al., 1997; 2002). The structurally related Boc and Cdo are integral membrane proteins conserved from Drosophila to rodent whereas Gas1, a glycosylphosphatidylinositol anchored plasma membrane protein, is specific to Hh signaling in vertebrates. A likely model proposes that Gas1, Cdo and Boc form a physical complex with Ptc1 and function as essential coreceptors that mediate multiple cellular responses to Hh. However, this requirement of Hh coreceptors depends on the cell type and the stage of development (Allen et al., 2007; Allen et al., 2011).

A non-canonical Shh pathway has also been described. It induces synchronous Ca^{2+} spikes and IP3 transients at the neuronal primary cilium through the activation of Smo (Belgacem and Borodinsky, 2011). There are multiple cell surface proteins that interact with Shh during normal signal transduction. In particular, these proteins are intimately involved in both the production and reception of Shh during craniofacial development (see Figure 1.12) (Xavier et al., 2016).
1.4.4 *Shh* production

*Drosophila dispatched* (*disp*), encodes a twelve-pass transmembrane protein required to transport lipid-modified Hh protein (Burke *et al*., 1999). Of the two *Disp* homologues in mouse (*Disp1* and -2), it is believed that only *Disp1* is involved in signaling (Nakano *et al*., 1989; Ma *et al*., 2002; Tian *et al*., 2005). Long-range Hedgehog activity absolutely depends on *Disp1* in vertebrates (Ma *et al*., 2002; Nakano *et al*., 2004; Tukachinsky *et al*., 2012). DISP1 interacts with human *SHH* via its cholesterol anchor, and this interaction is necessary for appropriate *SHH* secretion. However, DISP1 alone is not sufficient to release *SHH* from cells, this process also requires SCUBE2 function in order to overcome the insolubility conferred by *SHH* cholesterol modification (Tukachinsky *et al*., 2012). Truncating mutations of *DISP1* have been identified in two independent human families, where affected individuals demonstrated clinical features of microform HPE (Roessler *et al*., 2009).

The Scube gene family consists of three independent evolutionarily conserved members (Yang *et al*., 2002; Wu *et al*., 2004; Woods and Talbot, 2005; Hollway *et al*., 2006; Haworth *et al*., 2007; Xavier *et al*., 2009; Wu *et al*., 2011; Xavier *et al*., 2013). These genes encode secreted and cell surface-associated proteins that share a domain organization of at least five recognizable motifs, including multiple EGF (epidermal growth factor-like) and N-linked glycosylation sites, and a C-terminal CUB domain (Grimmond *et al*., 2000). *Scube1-3* demonstrate dynamic patterns of expression in the vertebrate embryo with transcripts predominating in the notocord, CNS and somites from the earliest stages of development (Grimmond *et al*., 2001; Hollway *et al*., 2006; Tu *et al*., 2006; Tu *et al*., 2008; Xavier *et al*., 2009; Tsai *et al*., 2009; Xavier and Cobourne, 2011).

Biochemical characterization demonstrate that Scube2 coordinate with Disp1 during Shh release from secreting cells, specifically disrupting cholesterol-mediated membrane-anchoring (Creanga *et al*., 2012; Ingham, 2012; Tukachinsky *et al*., 2012) and facilitating Shh protein removal from the plasma membrane (Ingham, 2012). A severe loss of *Shh* target gene expression is observed when all *Scube* family members are knocked down; however, defects in Shh-sensitive cell types are also evident when *scube1* or *scube3* are knocked down in combination with *scube2*. Recent findings in zebrafish indicate that *scube2* is the most important family member in modifying Hh signaling (Johnson *et al*., 2012).
1.4.5 *Shh* reception

The Smo gene encodes a seven transmembrane G protein-coupled receptor which does not bind Shh (Stone *et al*., 1996b) or participate in the binding of Shh to Ptch1 (Chen and Struhl, 1998). It appears that the principle function of Smo involves inhibition of the intra-cellular tumor-suppressor protein Sufu and the protein kinase A (PKA), casein kinase 1a (CK1a) and glycogen synthase kinase 3b (GSK3b), which all promote proteolytic processing of Gli transcription factors to their repressor forms (Figure 1.11) (Sharpe *et al*., 2015).

The mechanism by which Ptch1 regulates Smo activity in Hh signaling is unclear but obviously significant as this is a frequently deregulated interaction in two Hedgehog-driven cancers, medulloblastoma and basal cell carcinoma (BCC) (Mukhopadhyay and Rohatgi, 2014). It is believed that Ptch1 regulates Smo through the concentration or localization of a small molecule ligand (Taipale *et al*., 2002) and that enrichment of Smo at the primary cilia membrane is correlated with activation of signaling (Ocbina and Anderson, 2008; Rohatgi *et al*., 2009; Ocbina *et al*., 2011; Firestone *et al*., 2012; Barakat *et al*., 2013; Nedelcu *et al*., 2013).

The expression of Smo shows considerable overlap with Ptch1 in the craniofacial region, being adjacent to Shh-expressing cells in the developing lens, jaws, tongue, taste buds and teeth (Stone *et al*., 1996; Choi *et al*., 2014). Between E11.5-15.5 transcriptional activity is observed in the epithelium and mesenchyme of both maxillary and mandibular processes and strong expression is detected in Meckel's cartilage and the whisker follicles (Du *et al*., 2012). Smo mutants do not survive beyond E9.5, exhibiting significant ventral cyclopia and HPE, consistent with an essential role in Shh signal transduction. Targeted deletion of Smo in CNCCs has demonstrated the importance of normal Shh responsiveness in this cell population during early craniofacial development (Jeong *et al*., 2004). In particular, many CNCC-derived skeletal and non-skeletal components are absent in these mice, associated with facial truncation secondary to reduced growth and development of the pharyngeal arches. The abnormal pharyngeal arch and facial patterning is associated with combinatorial loss of Fox gene expression, suggesting that this patterning is mediated through a Shh-Fox gene network. Inactivation of Smo in the otic epithelium leads to cochlear and saccular agenesis as a consequence of impaired Shh signaling in the ventral otocyst; however, no vestibular phenotypes are observed, suggesting that Shh signaling in the dorsal otocyst is dispensable for vestibular morphogenesis (Brown and Epstein,
Early loss of *Smo* in the lens leads to a distinct microphthalmia (Choi *et al.*, 2014), a phenotype similar to mice lacking function of the Shh co-receptors *Cdo* and *Gas1* (Lee *et al.*, 2001; Zhang *et al.*, 2009).

*Ptch1* is first detected in the developing mouse embryo at E7.5 with transcripts present in the ventral neural tube and later, the somites, limb buds and developing craniofacial region (including pharyngeal arches, palate, tongue and tooth buds) (Goodrich *et al.*, 1996; Hahn *et al.*, 1996). In humans, protein-truncating *PTCH1* mutations are responsible for autosomal dominant Basal Cell Nevus Syndrome (BCNS; also known as the Nevoid Basal Cell Carcinoma Syndrome (NBCCS) or Gorlin–Goltz syndrome) (Wicking *et al.*, 1997), which is characterized primarily by multiple recurrent BCCs, recurrent odontogenic keratocysts of the jaws, palmar/plantar pits, ectopic calcification of the falx cerebri and less commonly, cleft lip/palate and tooth agenesis (Kimonis *et al.*, 1997; Kimonis *et al.*, 2004; Kimonis *et al.*, 2013).

Homozygous *Ptch1* mutant mice die between E9.0-10.5, with gross phenotypic changes evident at E8. The neural tube fails to close completely and there is overgrowth of the head folds, hindbrain and spinal cord. Embryonic lethality is thought to be secondary to abnormal cardiac development, which has hampered analysis of any potential craniofacial phenotype (Goodrich *et al.*, 1997). However, conditional inactivation of *Ptch1* in CNCC (*Wnt1Cre; Ptch1<sup>c/c</sup>*) has established a direct role in the pathogenesis of craniofacial anomalies. Loss of *Ptch1* function in CNCC causes mid-facial expansion and an early defect in the nasal pit, which culminates in cleft lip (Metzis *et al.*, 2013). Interestingly, the wider mid-facial morphology observed in these mutants has been explained by changes in cell packing, specifically a more loosely packed cellular network within the mesenchyme. However, cleft lip occurs through a failure of coordinated fusion between medial nasal process, lateral nasal process and maxilla. In *Wnt1Cre; Ptch1<sup>c/c</sup>* embryos, these prominences fail to meet; which is correlated to defective nasal pit invagination, with *Ptch1* required in a non-cell-autonomous manner for maintenance of cell shape in the invaginating nasal pit epithelium (Metzis *et al.*, 2013). Unfortunately, even conditional disruption in CNCC leads to lethality at E12.0; after primary, but before secondary palate development (Ferguson, 1988; Metzis *et al.*, 2013). It is likely that abnormalities of the secondary palate, such as high-arched and cleft palate would also be observed in these mice, if embryos survived until later in embryogenesis.
Ptch1 is also an important molecule involved in co-ordinating brain and facial development during embryogenesis. The capability of the C-terminal domain of Ptch1 to regulate Caspase-9 (Casp9)-associated mitochondrial cell death directly affects the dimensions of the forebrain and the nasal processes (Aoto and Trainor, 2014). Furthermore, the X-linked inhibitory apoptosis protein (XIAP), which is observed in primary cilia in a Hedgehog-dependent manner, acts as an inhibitor of Ptch1-induced cell death. Ptch1-induced apoptosis by XIAP in primary cilia protects Ptch1 from translocation into mitochondria, providing an important survival mechanism in this organelle which also help co-ordinate brain and facial development. This supports the observation that mitochondrial dysfunction may be a developmental risk factor associated in the pathogenesis of HPE (Aoto and Trainor, 2014).

N-ethyl-N-nitrosourea (ENU)-induced mutagenesis provides an unbiased forward genetic approach for identifying novel alleles important for embryogenesis. In a recent screen for recessive mouse mutations affecting craniofacial morphology, a single nucleotide change was identified at the 3'-end of *Ptch1* exon 13. These mice (*Ptch1*<sup>DL</sup> termed DL: Dogface-Like) display abnormal skull and snout morphology and craniosynostosis of the lambdoid suture. Skeletal defects related to the pathology of BCNS are also present, including defects in development of the scapula, ribcage, secondary palate, cranial base and cranial vault. The general overgrowth, rhabdomyosarcomas and medulloblastomas observed in *Ptch1* heterozygous mice (Goodrich *et al.*, 1997; Hahn *et al.*, 1998) are not seen in *Ptch1*<sup>DL</sup> mice. This mutation demonstrates a new hypomorphic allele of *Ptch1* which the potential to aid in further assessment of the role of Hedgehog signaling in multiple developmental events, particularly those regulating facial shape (Feng *et al.*, 2013). An additional ENU screen in mice has also produced a further *Ptch1* mutant allele (Sandell *et al.*, 2011; Kurosaka *et al.*, 2014). Genome sequencing revealed a T to A nucleotide change in intron 15 of *Ptch1*, which created a new splice acceptor site, resulting in a premature stop codon in exon 16 and generation of a truncated protein. Mice carrying this mutation were named *wiggable* due to the presence of excessive leaf-like laminae or folia in the brain, which resemble a wig. *Ptch1*<sup>wiggable</sup> embryos die *in utero* at around E12.0 as a result of various defects, including open neural tube and hypertelorism of the face, consistent with a gain-of-function in Hedgehog signaling (Sandell *et al.*, 2011; Kurosaka *et al.*, 2014). In an effort to rescue the phenotype observed in *Ptch1*<sup>wiggable</sup> mice, compound mutant mice with disrupted function in Hedgehog acyl transferase (Hhat) have been generated (*Hhat<sup>creface</sup>*)
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(Kurosaka et al., 2014). Hhat is responsible for modifying Hedgehog proteins through the addition of palmitic acid (Ohlig et al., 2011) and Hhatcreface mice have a phenotype consistent with a loss of Shh signaling. Interestingly, Hhatcreface; Ptc1wiggable mice present with cleft lip and fissure of the premaxillary bone at E16.5, implying that Hhat and Ptc1 play an important role in regulating Hedgehog signaling during lip development (Kurosaka et al., 2014).

In addition to induced and engineered Ptc1 mutants, spontaneous mutants have also been reported. A recessive Ptc1 mouse mutation, mesenchymal dysplasia (mes) causes excess skin, increased body weight and mild preaxial polydactyly, with these mice also having a shortened face, wide-set eyes and dome head (Makino et al., 2001). Ptc1D11 is another spontaneous mutation caused by an aberrant recombination event during production of a Ptc1 null allele. The Ptc1hD11 locus presumably results in a weak Ptc1 allele, with homozygous animals being sterile, but otherwise normal (Oro and Higgins, 2003).

In humans, PTCH1 maps to chromosome 9q22.3 and is widely assumed to be a tumor-suppressor gene (Hahn et al., 1996). Mutation analysis of PTCH1 in both familial and sporadic HPE cases has revealed four different missense mutations in five unrelated individuals. These findings demonstrate that mutations in different components of the Shh pathway that lead to a common effect on Shh signaling can each result in the same phenotype: decreased Shh activity causing HPE and increased activity causing tumors (Goodrich et al., 1999; Ming et al., 2002).

Vertebrates have an additional Patched receptor (Ptc2), which encodes a structural homologue of Ptc1 (Motoyama et al., 1998). Ptc2 mutant mice are viable, fertile and do not display obvious developmental defects, although males develop skin lesions associated with alopecia and ulceration, with progressing age (Nieuwenhuis et al., 2006). However, Ptc2 is a target of Hedgehog signaling and participates in ligand-dependent feedback inhibition, which in conjunction with Ptc1 (and Hhip1) is an important antagonist of pathway activity, in the neural tube at least (Holtz et al., 2013).

Hhip1 encodes a type I transmembrane glycoprotein involved in the extracellular regulation of Hedgehog signaling, which like Ptc1 and -2 is upregulated in response to signal (Chuang and McMahon, 1999; Chuang et al., 2003; Bosanac et al., 2009; Bishop et al., 2009). During embryogenesis Hhip1 is expressed in the mesenchyme of numerous tissues including
lung, gut, whisker, hair and rugae (Echelard et al., 1993). However, targeted disruption of HHip1 leads to lethality a few hours after birth due to respiratory failure secondary to abnormal lung formation (Chuang et al., 2003).

Gas1, Cdon and Boc are a group of Hedgehog co-receptors that retain the ability to interact directly with Shh (Lee et al., 2001a; Okada et al., 2006; Tenzen et al., 2006; Martinelli and Fan, 2007; McLellan et al., 2008) and form high-affinity individual complexes with Ptch1 on the surface of receiving cells (Bae et al., 2011; Izzi et al., 2011). Boc and Cdon are also able to bind Gas1, although these interactions are unlikely to be tripartite (Bae et al., 2011; Izzi et al., 2011); whilst, Cdon and Boc can complex with each other through their extra- and intra-cellular domains (Kang et al., 2002).

Collectively, these co-receptors have an important role during vertebrate hedgehog signaling; the binding of Shh to Ptch1 alone is insufficient for pathway activation (Izzi et al., 2011) and mice with targeted disruption of Gas1, Cdon and Boc lack all Shh transduction except for some basic rudimentary activity (Allen et al., 2011). A current view is that these individual tissue-specific receptor complexes bind Shh, which lead to de-repression of Smo and activation of the pathway. This view is consistent with previous findings of both redundancy and specific requirements for these co-receptors in different developmental roles.

The role of these receptors during early craniofacial development has been investigated extensively through the generation of single and compound mouse mutants, which demonstrate a variable and background-dependent severity of phenotype. Loss of Cdon can produce a semilobar-type HPE characterized by cebocephalic face (single nostril, ocular hypotelorism and maxillary hypoplasia) or a less severe microform HPE with philtral dysgenesis and maxillary incisor agenesis (Cole and Krauss, 2003; Zhang et al., 2006; Hong and Krauss, 2012). Loss of Gas1 results in a more consistent microform HPE associated with maxillary incisor fusion and cleft palate (Seppala et al., 2007), whilst Boc mutant mice lack HPE but do have misguided commissural axon guidance, cerebellum reduction and reduced ipsilateral retinal ganglion cells (; Izzi et al., 2011; Sanchez-Arrones et al., 2013). As might be expected, Gas1; Cdon mutants have a more severe semilobar HPE associated with a single external nares, fusion of the nasal processes and absence of maxillary and mandibular skeletal elements (Allen et al., 2007; Seppala
et al., 2014); whilst Cdon; Boc mice on a Cdon-resistant background have lobar HPE with more severe craniofacial anomalies (Zhang et al., 2011). More recently, the craniofacial region of Gas1; Boc mutants have been analysed, demonstrating an allele dosage-dependent phenotype. In particular, Gas1; Boc mice have lobar HPE associated with disruption of the corpus callosum, oro-facial clefting and maxillary incisor agenesis (Seppala et al., 2014). Significantly, loss-of-function mutations in GAS1 and CDON have also been associated with HPE in humans (Ribeiro et al., 2010; Bae et al., 2011).

Low-density lipoprotein (LDL) receptor-related proteins (LRPs) are multifunctional cell surface receptors structurally related to the LDL receptor-family. LRP2/megalin encodes an endocytic transmembrane protein found primarily on the apical surfaces of epithelia within the kidney, lung and brain (Kantarci et al., 2007). Lrp2 has been shown to play a critical role in murine forebrain development (Spoelgen et al., 2005), representing an auxiliary docking site for Shh in the ventral midline of the rostral diencephalon. It locally sequesters Shh, enabling target cell reaction to low concentrations of ligand over a critical time period during early neurulation (Christ et al., 2012). This role in relation to Shh activity in the forebrain is consistent with craniofacial abnormalities observed when Lrp2 activity is disrupted. Genetic deficiency of this multifunctional receptor in mice results in a HPE phenotype, characterized by abnormal development of the forebrain, absence of olfactory apparatus and abnormalities of facial structures that originate from forebrain-derived CNCC and contiguous mesoderm (Willnow et al., 1996). In humans, mutations in LRP2 are seen in subjects presenting with Donnai-Barrow (DBS) syndrome (Kantarci et al., 2007), which have microform HPE characterized by corpus callosum anomalies and enlarged globes, with small colobomas at the optic nerve heads (Kantarci et al., 1993; 2007; Rosenfeld et al., 2010).

Gpr161 is part of the large G protein-coupled receptor (GPCR) superfamily, involved in mediating the ability of cells to sense and respond to their environment (Matteson et al., 2008). The binding of ligands to GPCRs activates heterotrimeric G proteins at the plasma membrane where most of their effectors are intrinsically or transiently associated (Gainetdinov et al., 2004; Garcia-Regalado et al., 2008).
Gpr161 is expressed in several tissues during murine development, initially being restricted to the neural folds along the antero-posterior axis. Later, transcripts are detected in the ventricular zone of the developing CNS, forelimb, hindlimb, retina and throughout all stages of lens development (Matteson et al., 2008). Positional cloning has demonstrated that the previously characterized hypomorphic vacuolated lens (vl) mutation (Wilson and Wyatt, 1986, 1988) is caused by a deletion in Gpr161 (Matteson et al., 2008). Different neural tube phenotypes are observed in mouse embryos displaying vl mutations, with around half displaying frank lumbar-sacral spina bifida; whilst in the remainder, the neural tube closes but dorsal phenotypes are observed, including a thinning of the midline neuroepithelium and epidermis, dilation of the dorsal ventricle and ectopic neuroepithelial cells in the ventricle (Matteson et al., 2008; Wilson and Wyatt, 1986, 1988). The vl mutation leads to lethality in half of homozygous mutants; however, all surviving adult vl/vl mice display congenital cataracts and no obvious signs of spina bifida (Matteson et al., 2008).

A null mutation of Gpr161 has been shown to produce extensive craniofacial abnormalities and open forebrain/midbrain regions (Mukhopadhyay et al., 2013). In humans, whole-exome sequencing of a family with two siblings displaying pituitary stalk interruption syndrome (PSIS) revealed a unique homozygous missense mutation in GPR161. PSIS is a defect of the pituitary gland characterized by a triad of very thin/interrupted pituitary stalk, ectopic (or absent) posterior pituitary gland and hypoplasia/aplasia of the anterior pituitary. Patients with PSIS may present midline defects and various pituitary deficiencies, ranging from isolated growth hormone deficiency to combined pituitary hormone deficiency (Gutch et al., 2014; Karaca et al., 2014).

The craniofacial abnormalities observed in mice and humans upon disruption of Gpr161 function are consistent with a relationship between this protein and Shh signaling. Gpr161 modulates Shh pathway activity at the primary cilium, localizing to this cellular compartment via intraflagellar transport and acting as a negative regulator of signaling. This inhibition takes place through ‘tuning’ of protein kinase A-dependent basal repression machinery in Shh signaling, modulating cyclic adenosine monophosphate levels. Interestingly, Gpr161 is also removed from the cilium in a Shh-dependent manner (Mukhopadhyay et al., 2013; Hwang and Mukhopadhyay, 2014; Mukhopadhyay and Rohatgi, 2014).
Ellis-van Creveld (EvC) syndrome (also called chondroectodermal dysplasia) is an autosomal recessive chondrodysplasia, characterized by severe truncation of the limbs, postaxial polydactyly, natal teeth and dysplastic nails (Ellis and van Creveld, 1940). Two-thirds of patients have congenital heart malformations, mainly in the form of atrial-ventricular canal defects. Positional cloning has identified mutations in the \textit{EVC} and \textit{EVC2} genes as the cause of EvC syndrome (Galdzicka \textit{et al.}, 2002; Ruiz-Perez \textit{et al.}, 2000; Ruiz-Perez \textit{et al.}, 2003). The phenotype associated with mutations in either of these two genes is the same, indicating that \textit{EVC} and \textit{EVC2} act in a common pathway (Blair \textit{et al.}, 2011). Mutations in \textit{EVC} and \textit{EVC2} have also been linked to Weyers acrofacial dysostosis (WAD), an autosomal dominant disorder characterized by a similar phenotype to EvC, but with milder expression; including dental defects (hypodontia, irregular and small teeth, single central incisor), as well as postaxial polydactyly of the hands and feet, hypoplastic nails and a mild shortness of stature (D'Asdia \textit{et al.}, 2013; Ruiz-Perez and Goodship, 2009; Ruiz-Perez \textit{et al.}, 2000).

Studies involving targeted deletion in mice has demonstrated that the protein products of \textit{Evc} and \textit{Evc2} form a ciliary transmembrane complex (\textit{Evc-Evc2}), which is required for Hedgehog signal transduction in a tissue-specific manner (Ruiz-Perez \textit{et al.}, 2007; Blair \textit{et al.}, 2011; Caparros-Martin \textit{et al.}, 2013; 2015). \textit{Evc-Evc2} promotes Sufu/Gli3 dissociation and Gli3 recruitment to the cilium tip through direct interaction with Smo (in a distinct compartment in the primary cilium termed the \textit{EvC} zone) and modulates Hedgehog signaling (Dorn \textit{et al.}, 2012; Yang \textit{et al.}, 2012). Inactivation of \textit{Evc}, \textit{Evc2}, or both genes does not affect cilia formation, but has similar key features of the chondrodysplasia phenotypes seen in EvC patients, due to a defective response to Ihh (Ruiz-Perez \textit{et al.}, 2007; Caparros-Martin \textit{et al.}, 2013; Nakatomi \textit{et al.}, 2013).
1.4.6 Sonic hedgehog in mouse development

Shh is predominantly expressed in the epithelium of the developing craniofacial structures. Many of the structures which rely on Shh signaling during their development form through a similar reciprocal process of molecular communication and interaction between the epithelium and the underlying mesenchyme.

Shh expression is first detected in the node during the late streak stage where it is involved in left-right axis formation of the embryo. It is then exclusively expressed in the midline including the endoderm of the prechordal plate. At E8 Shh is expressed in the ventral midline of the midbrain, from where it extends rostrally into the forebrain and caudally into the hindbrain and notochord. At this stage, it has a vital role as an inductive signal for inductive Shh expression in the floor plate. At E9.5, Shh transcripts are present in the pharyngeal endoderm and ventral hindgut extending into the optic stacks and forebrain by E10 (Echelard et al., 1993).

At later stages of development of the primordial face, Shh is expressed in discrete domains of the frontonasal, maxillary and mandibular ectoderm. During tooth formation (E11.5-E12.5), Shh is expressed strongly in the dental lamina in the oral epithelium. In later tooth development, there is strong, consistent expression in the epithelial components of the tooth germ such as the enamel knot, internal enamel epithelium, stratum intermedium, stellate reticulum, ameloblasts and Hertwig's epithelial root sheath (Hardcastle et al., 1998; Dassule et al., 2000; Cobourne and Sharpe 2003).

Shh is also expressed during palatogenesis (E12.5-13.5) as 'stripes' in the oral epithelium of the vertically extending palatal shelves. In later stages expression becomes localized in the rugae (Bitgood and McMahon 1995; Economou et al., 2012). Recent studies have demonstrated evidence of an activator-inhibitor system during rugae development (Economou et al., 2012). Developing rugae, marked by stripes of Shh expression, appear at two growth zones where the space between previously laid-down rugae increases indicative of the involvement of a Turing-type reaction-diffusion mechanism in rugae development during palate formation.

Shh has diverse roles in craniofacial development which is reflected in the expression pattern in the ventral forebrain and regions of the facial ectoderm (Echelard et al., 1993; Bitgood
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and McMahon 1995). A number of studies have demonstrated this and confirmed these important Shh transcription sites as organizing centres for midline formation during development of the forebrain and face (Chiang et al., 1996; Helms et al., 1997; Marcucio et al., 2005; Helms et al., 2005; Hu and Marcucio 2009; Petryk et al., 2015) as well as in other facial structures including the teeth (Dassule et al., 2000; Cobourne and Sharpe 2003), salivary (Jaskoll et al., 2004) and pituitary glands (Treier et al., 2001).

1.4.7 Gas1

Gas1 encodes a glycosylphosphatidyl-inositol (GPI)-linked membrane glycoprotein that plays a role in cell cycle regulation, differentiation and apoptosis. The mouse Gas1 gene has been mapped to chromosome 13 where it encodes a 37 kDa single axon integral membrane protein with putative transmembrane domains at each end (Del Sal et al., 1992).

Gas1 is expressed in the craniofacial region of the mouse from E7.5 in the ANR, in later stages is expressed strongly in the mesenchyme of the first and second branchial arches as well as in the otic and optic vesicles. Expression in the otic vesicle is transient but not so in the optic vesicle where there is expression throughout development (Lee and Fan, 2001). Furthermore, the overlying ectoderm also expresses Gas1 and is later found in the RPE, the ciliary body and in the adjacent periorbital mesenchyme (Lee et al., 2001).

Gas1−/− mice demonstrate the presence of a number of features associated with microform HPE, including midfacial hypoplasia, premaxillary incisor fusion, and cleft palate, in addition to severe ear defects; each with varying levels of penetrance. However, gross integrity of the forebrain remained intact (Seppala et al., 2007). It has previously been shown that there is a positive interaction between Shh and Gas1 as demonstrated by decreased Shh signaling activity in the FNP and palatal shelves of Gas1−/− mice. Furthermore, compound Shh+/−; Gas1−/− mice have previously been generated which demonstrated an worsened midline phenotype and shown that Gas1 positively regulates Shh signaling (Seppala et al., 2007).

Gas1−/− mice mimic human microform HPE. Indeed, in humans GAS1 has been mapped to 9p21.3-q22m a region associated with congenital anomalies of the craniofacial region, including deafness and CL/P (Del Sal et al., 1994; Allen et al., 2011; Gregory et al., 2014).
1.5 Genes and molecular pathways critical during the spatiotemporal development of the forebrain, the frontonasal process (FNP) and derivatives

The complex structure of the forebrain originates in the anterior neural plate where inductive cues act on the neuroepithelium results in formation of the different regions of the brain. *Shh* which is first expressed in the axial mesendoderm (Shimamura and Rubenstein, 1997) is required for the development of the prosencephalon. *Shh* induces the expression of *Nkx 2.1* whose function is required for the formation of the ventral forebrain.

![Figure 1.13](image)

**Figure 1.13 Sequence of Shh expression in the embryonic craniofacial complex**
Schematic representations of *Shh* expression in the craniofacial complex, where *Shh* expression is denoted by red coloring, *Shh* is expressed in forebrain (fb) tissues and pharyngeal endoderm (pe). At later stages, the prosencephalon has divided into the telencephalon (tel) and the diencephalon (di); *Shh* expression is localized to the diencephalic neuroectoderm (ne). At later midgestational stages, a new domain of *Shh* is initiated in the telencephalic neuroectoderm (tel ne), which leads to a new domain of *Shh* is observed in the surface ectoderm (se), also called facial ectoderm. From this stage on, *Shh* expression remains constant in the neuroectoderm and facial ectoderm (mb, midbrain). Red, *Shh* expression; purple, surface ectoderm; light blue, neuroectoderm; brown, mesoderm. Adapted from Cordero et al., 2005.

The anterior ventral forebrain (including the septum, preoptic area, optic chiasm and ganglionic eminences) arises from a rostral and medial region of the neural plate in contact with or expressing inducing signals such as Fgf8, Shh, and BMPs. (Shimamura and Rubenstein 1997). These structures are sensitive to *Shh* expression as demonstrated by malformations in the basal forebrain in *Shh*/*+* mice. Mutations in human (Belloni, 1995; Roessler and Muenke 1998) and mouse (Chiang et al., 1996) leads to abnormalities in neural plate patterning and result in HPE and cyclopia (Cohen and Sulik, 1992; Barr and Cohen, 1999;). Later in facial development, *Shh* is expressed in the ectoderm of the FNP and in the MXP (Hu and Helms, 1999). *Fgf8*, which is expressed in the ANR (Crossley and Martin, 1995) is able to induce *BF1* (Shimamura and Rubenstein, 1997), which is required for development of the telencephalon and for expression of *Dlx2*, a telencephalic marker.
The timing of Shh signal inhibition can differentially affect the morphogenesis of the brain and face and is a crucial factor in the morphogenesis of the brain and face (Varjosalo and Taipale 2008). Studies indicate that the later the inhibition during forebrain and facial morphogenesis, the less severe the craniofacial phenotype. As the head develops and the molecular scaffolding for these structures form, the resultant phenotype is less obvious (Cordero et al., 2004; Marcucio et al., 2005; Marcucio et al., 2015).

In a series of elegant studies looking at the role of Shh at various developmental stages utilising teratogens that inhibit Shh such as cyclopamine and ethanol, Shh signaling was disrupted at different development stages in the case of cyclopamine (Taipale et al., 2002) and in a subsequent study involving different levels of signal disruption in differing mouse backgrounds utilising ethanol (Marcucio et al., 2005; Hong and Krauss 2012; Kietzman et al., 2014). From these experiments, it was possible to identify several of the developmental processes that rely on Shh activity at various developmental stages.

Although different animal models have been used in investigations of Shh signaling in craniofacial development, the chick provides a particularly useful study model to investigate the roles of local Shh signaling centers during vertebrate development due to the ability to manipulate in ovo. A number of informative studies involving transplantation, overexpression and inhibition studies techniques have demonstrated temporally distinct roles of Shh in the developing face (Helms et al., 1997; Ahlgren and Bronner-Fraser 1999; Ahlgren et al., 2002; Cordero et al., 2004; Marcucio et al., 2005). If the teratogen cyclopamine is delivered early in chick development during neurulation or after induction of Shh expression in the prosencephalon, both the prosencephalon and eye field fail to divide causing cyclopia and facial anomalies that represent manifestations of the underlying brain anomaly. If a teratogen exposure occurs at a later stage- after the initial regionalization of the forebrain with the resultant formation of Shh expression centres in the telencephalon, both eyes form. However other features such as severe hypotelorism, midface hypoplasia and a wide clefting of the palate result (Marcucio et al., 2005; Cordero et al., 2004).

The growing CNS has more than just a physical effect on facial development; the developmental processes that control the formation of the CNS and facial prominences are highly integrated; so that changes in one process often have downstream effects on the other.
Studies in the chick have revealed a signaling relay system that operates in the brain to control Shh expression in the telencephalon and adjacent surface cephalic ectoderm (Marcucio et al., 2011). It has been demonstrated that inhibiting Shh signaling in the developing CNS and thus, forebrain prevents the initiation of Shh expression in the basal telencephalon, which subsequently inhibits the subsequent induction of Shh expression in the Frontonasal Ectodermal Zone (FEZ). The FEZ is a signaling center that regulates facial development - essentially a region identified a molecular boundary in the FNP ectoderm, defined by the juxtaposed domains of Fgf8 and Shh, which presaged the initial site of frontonasal process outgrowth (Marcucio et al., 2005).

Furthermore, Shh signaling initiation in the CNS, which has been shown to lead to ventralization of the forebrain, results in expansion of Shh expression in the basal telencephalon, and alterations in the pattern of Shh expression in the FEZ (Helms et al., 1997). In both these studies, significant variation in facial morphology resulted. It was also demonstrated that by varying the level of Shh signaling in the brain, phenotypic variation is produced that is continuous with normal variation (Marcucio et al., 2011). Thereby, inhibition of Shh in varying increments in the developing CNS produces phenotypes that are consistent with the range of phenotypes that appear in patients with loss-of-function mutations in Shh, as well as activation of Shh in varying increments in the developing CNS produces phenotypes that are consistent with the range of phenotypes that appear in patients with gain-of-function mutations in the Shh pathway.

In fundamental terms of facial development, all of these phenotypes are associated with changes in the level and pattern of expression of Shh in the FEZ. Shh signaling in the CNS directly affects facial development by controlling the induction and organization of the FEZ, (Hu et al., 2003). These experiments confirm a non-linear relationship between SHH signaling and phenotype which suggest a reason behind the extreme variability in phenotype that is observed among patients with mutations that cause HPE Therefore, the changes in the facial phenotype of patients with milder forms of HPE (lobar or MIH) may be a direct result of the signaling interactions among the brain, surface ectoderm, and adjacent neural crest cells. The spatial organization of the gene expression patterns in the FEZ is highly associated with facial shape and changes to the shape of the expression domains may directly result in the facial phenotypes similar to those in HPE. An example of this pattern is seen where reduced Shh signaling in the
CNS may lead to midfacial hypoplasia and clefting as a result of alterations to the initial growth patterns of the facial primordia prior to fusion of the primary palatal tissue. The surface ectoderm in the midline of the upper maxillary and nasal processes express a series of signaling molecules including: Shh, Fgf8, Wnt9b, Bmp2, Bmp4, and Bmp7 (Ashique et al., 2002; Abzhanov et al., 2004; Foppiano et al., 2007; Geetha-Loganathan et al., 2009). Shh is expressed in the ectoderm that will form the roof of the mouth and forms a boundary with cells expressing Fgf8 and Wnt9b in more dorsal ectoderm (Hu and Marcucio, 2009) This ectoderm has the ability to promote growth and patterning of the upper jaw in part by regulating expression of Bmp2, Bmp4, and Bmp7 in the adjacent mesenchyme that then controls growth of the upper jaw. This may serve to demonstrate the signaling anomalies that occur in the CNS may have a direct impact on the developing face through the regulation of the signaling interactions that occur among these adjacent tissues during early development. There are a number of other, major signaling pathways are involved in the molecular interactions between the brain and the face (Marcucio et al., 2005; Young et al., 2014; A Petryk et al., 2015). These interactions begin during the earliest stages of development and continue throughout the patterned growth of the facial primordia, which lead to development of the face and jaws.

Other signalling pathways include Wnt signaling, which is involved in several developmental processes from cell proliferation to cell fate determination and differentiation, to cell survival (Cadigan and Nusse 1997). In craniofacial development, Wnt signaling is linked to the generation and migration of neural crest cells. Many Wnt ligands and receptors continue to be expressed in the craniofacial complex well after the neural crest cells have completed their migration. In particular, various canonical Wnt ligand genes are expressed in the facial prominences. Canonical Wnt signaling reporter transgenes such as TopGAL, BATgal, and Axom2-lacZ are highly expressed in the prominences and their derivative (Brugmann et al., 2006). Wnt9b⁻/⁻ mice show significantly retarded outgrowth of the nasal and maxillary processes due to reduced proliferation of mesenchymal cells. These cellular defects in these mice are caused by reduced FGF family gene expression and signaling activity resulting from compromised canonical WMT/β-catenin signaling (Jin et al., 2012) Similarly, using transgenic Wnt reporter embryos, a highly conserved pattern of Wnt responsiveness in the developing mouse face that later corresponded to derivatives of the frontonasal and maxillary prominences was discovered (Brugmann et al., 2007). Further exploration of the consequences of disrupting
Wnt signaling, using mice carrying compound null mutations in the nuclear mediators *Lef1* and *Tcf4* exhibited radically altered facial features resulting in a hyperteloric appearance and a foreshortened midface (Kurosaka *et al.*, 2014).

Fgfs and their receptors make up a complex family of signaling molecules that play an important role in embryogenesis. In particular, *Fgf8* is expressed broadly in the frontonasal and mandibular epithelia before outgrowth of the nasal processes. Nasal explant cultures show that it can substitute for the facial ectoderm to stimulate mesenchymal proliferation and maintain mesenchymal gene expression (Firmberg and Neubüser, 2002). It has been suggested that *Fgf8* is crucial for development of the olfactory epithelium, nasal cavity and VNO and that it also defines an anterior morphogenetic centre required for the proper morphogenesis of the entire nasal cavity (Kawauchi *et al.*, 2005). It has been demonstrated in the chick (Hu and Helms, 1999) that a molecular boundary (FEZ) exists in the frontonasal process ectoderm defined by *Fgf8* and *Shh* which predate the initial site of frontonasal process outgrowth.

BMPs are a group of secreted signaling molecules of the transforming growth factor beta (Tgfß) superfamily (Wozny *et al.*, 1988). It has been shown that *Bmp2* and *Bmp4* mRNAs were expressed in dynamic spatiotemporally regulated patterns in the developing chick facial primordial, with *Bmp4* having highly restricted expression in the distal epithelia of the medial nasal, lateral nasal, maxillary and mandibular primordial (Francis-West *et al.*, 1994).
1.6 Perturbations in SHH signaling result in human developmental conditions

Genetic disruptions in *SHH* and other components of its signaling pathway result in a range of congenital anomalies and pathological conditions in humans. In addition to HPE (SHH) these include Smith-Lemli-Opitz syndrome (OMIM#270400), (7-Dehydrocholesterol reductase (DHCR7)), Basal cell nevus syndrome (OMIM#109400) (*PTCH1*), Basal Cell Carcinoma (OMIM#155255) (*PTCH1H2*), Glioblastoma (OMIM#605462) (*PTCH1, PTCH1H2*), and medulloblastoma (OMIM#155255) (*PTCH1H2*).

1.7 Holoprosencephaly

HPE (OMIM#236100) is the most common forebrain birth defect in humans and encompasses a wide range of craniofacial and neural defects. It is commonly defined as the failure of the forebrain to divide into two distinct cerebral hemispheres during development. It is rare in live births (1/16,000) however it is believed that up to 1/250 conceptuses are affected (Geng and Oliver 2009). In humans, advancements in X-ray computerized tomography and in magnetic resonance imaging have increased knowledge of HPE. These analyses define two major classes of HPE that encompass four types. (Fernandes and Hébert, 2008) The first is ‘Classic’ HPE where the most severely affected area is the basal/ventral forebrain. The second is midline interhemispheric HPE (MIH HPE), also known as syntenencephaly, where the cortical/dorsal part of the hemispheres does not separate and the basal forebrain can be normal (Aguilella *et al.*, 2003).

Classic HPE is grouped into three forms; alobar, semilobar and lobar. (Cohen *et al.*, 2006). The former is the most severe, characterised by the presence of a small single cerebral ventricle that lacks interhemispheric division, corpus callosum and olfactory bulbs. In semilobar HPE; the frontoparietal lobes fail to separate but the interhemispheric fissure is present posteriorly and the corpus callousum and olfactory bulbs are either absent or underdeveloped. In the mildest form, lobar HPE, a distinct hemispheric fissure is present but with some midline continuity of the cingulated gyrus. Eighty percent of cases of HPE are associated with facial abnormalities. The range of presentation can vary from cyclopia and complete absence of lobar division to more subtle defects such as median cleft lip, absent olfactory bulb, agenesis of the...
corpus callosum and a single central maxillary incisor. Milder craniofacial features that occur in the absence of forebrain defects are called microform.

The second class of HPE (MIH HPE) is rarer and milder that classic HPE. The defects in separation occur only at the posterior frontal and parietal regions, the anterior and occipital lobes separate normally (Lewis et al., 2002). It has been suggested that genetic pathways important for development of the ventral forebrain are defective in classic HPE whereas in MIH HPE pathways important for the development of the dorsal forebrain are defective (Geng et al., 2009).

1.7.1 Genetic Heterogeneity of HPE
The genetics of HPE is complex and only fourteen mutated genes that underlie familial cases of HPE have been identified so far. HPE can be caused by both genetic aberrancy and by teratogens. Approximately 25% of cases are due to detectable genetic anomalies (Muenke and Cohen 2000). Karyotype analyses has identified at least 12 different genomic regions spread over 11 chromosomes (loci HPE1 to HPE12) which have been described as containing HPE candidate gene (Roessler, 1996; Dubourg et al., 2007;). Pedigree studies support autosomal dominance, recessive and X-linked inheritance. HPE can also be associated with congenital syndromes such as Smith-Lemli-Opitz and Pallister-Hall syndrome (Hall et al., 1980; Kelley et al., 1996;). Heterogeneity in familial HPE in individuals carrying the same mutation may be due to environmental or teratogenic factors such as alcohol, cholesterol, retinoic acid, diabetes, and modifier genes (Muenke and Cohen 2000). Recent work has revealed insights into the normal function of HPE genes; \textit{SHH}, \textit{PTCH1}, \textit{GLI2}, \textit{TDGF1} (or \textit{CRIPTO}), \textit{TGIF}, \textit{FGF8}, \textit{FOXH1}, \textit{ZIC2}, \textit{SIX3}, \textit{CDON}, \textit{DLL1}, \textit{GAS1}, \textit{DISP1} and \textit{NODAL}; (Hayhurst and McConnell, 2003; Krauss, 2007) which have shown important roles in three major developmental signaling pathways (nodal, sonic hedgehog and retinoic acid). The wide phenotypic variability of human HPE is seen in both sporadic cases and in pedigrees. In pedigrees, greater than 33% of mutation carriers are without clinical expression (Cohen, 1989; Ming et al., 2002). These observations may suggest that heterozygous mutation of HPE genes are insufficient to produce severe anomalies and furthermore that HPE arises from a complex of developmental, genetic and environmental factors (Ming et al., 2002; Krauss, 2007). Some possible explanations have been discussed including: (1) multiple genetic-hit models such as heterozygous mutations of two HPE genes or an interaction between a mutation in an HPE gene and an otherwise silent modifier gene, (2)
environmental insults during different stages in fetal development and: (3) stochastic events within a halpoinsufficient HPE gene background; and (4) gene-environment interactions (Krauss 2007; Zhang et al., 2011).

Mouse model studies are key to characterization of HPE. In contrast to humans, heterozygous mutations in single genes associated with Shh signaling is essential for normal development of the early forebrain (Echelard et al., 1993) and is required for formation ventral midline structures (Seppala et al., 2007). Shh mutant mice have otocephaly, with only a single forebrain vesicle and cyclopia (Chiang et al., 1996). Among familial forms of HPE, phenotypes also vary greatly. In consanguineous obligate mutation carriers, approximately 37% have HPE, 27% microforms, and 36% have no clinical manifestations (Krauss, 2007). HPE diagnosis prenatally is mainly based on ultrasound and MRI—which has decreased sensitivity to milder forms. Genetic diagnosis is not practical at present because of the aetiologic heterogeneity and pathological variability of the disorder (Bendavid et al., 2006). The causes of HPE are not completely understood but both genetic and environmental factors are believed to play an important role.

1.7.2 Mouse models of HPE

A number of mutant mouse models have become available over the last decade that exhibit holoprosencephalic phenotypes and these are now beginning to provide considerable insight into the genetic basis underlying these conditions (Hayhurst and McConnell, 2003). In particular, they are helping to identify essential components of molecular signaling pathways that are required during the complex process of craniofacial development. Disruption associated with any of these closely co-coordinated embryonic events can lead to HPE and CP phenotypes in mice and given the striking similarities between early murine and human craniofacial development, the mouse provides an excellent model to investigate the developmental basis of this condition. Most of these mouse models have been generated by genetic alterations, though some were obtained through the use of chemicals (Kolf-Clauw et al., 1996; Gofflot et al., 1999; Nagase et al., 2005; Aoto et al., 2008).

1.7.2.1 Mouse models exhibiting an alobar HPE-like phenotype

Mouse genetic models have confirmed the results from ablation and transplantation experiments in several species and show that signaling from the PrCP is vital in order for separation of the
single eye field and the patterning and morphogenesis of the forebrain. One of the signals from the PrCP is Shh which induces Nkx2.1 expression and represses Pax6 expression (Shimamura and Rubenstein 1997). In the Shh-null mouse forebrain, the most ventral structures are absent, the telencephalon fails to bifurcate, and the eyefield does not separate The secretion and long range activity requires dispatched (Disp). Mutations in SHH, GLI2, DISP1, and PTCH1 have been identified in HPE patients (Cohen, 2006; Krauss, 2007). Mutations in SHH alone account for 12.7% of HPE cases (Dubourg et al., 2007).

Mutations in genes encoding components of the nodal signaling pathway have been identified in patients with HPE (Hayhurst and McConnell, 2003; Krauss, 2007). In zebrafish, a connection has been shown between cyclopia and Nodal signaling (Sampath et al., 1998; Rebagliati et al., 1998; Gritsman et al., 1999). In mice, inactivation of the pathway causes early embryonic lethality due to defects in mesendoderm specification and gastrulation (Zhou et al., 1993; Nomura & Li 1998; Ding et al., 1998). Nodal insufficient embryos show that even though gastrulation occurs, mesodermal cells fail to migrate anteriorly and form the PrCP. In amphibians and in chicken embryos, physical ablation of the PrCP leads to cyclopia (Pera and Kessel 1997; Li et al., 1997). Transplantation of a PrCP laterally to the PrCP activates expression of the ventral midline gene Nkx2.1 and it also represses expression of Pax6- which is a marker for the dorsal telencephalon and eye field in the overlying neuroectoderm (Shimamura and Rubenstein, 1997). This would confirm the requirement of PrCP is required for eye field separation and forebrain patterning.

ZIC2 mutations account for 9% of all HPE cases (Dubourg et al., 2007). The zinc finger protein Zic2 physically interacts with Gli2 (Nagai et al., 2000). Mouse embryos homozygous for the allele exhibit alobar HPE-like phenotypes (Brown et al., 2002; Elms et al., 2003; Warr et al., 2008). Bmps belong to another branch of the TGF-β superfamily. They bind through type 1 and type 2 bmp receptors and signal through Smad complexes. Chordin (Chd) and noggin (Nog) act as secreted antagonists of BMP. Twisted gastrulation (Tsg) is a secreted protein that directly interacts with BMP and Chd.. There have been no identified components of the BMP pathway in patients with HPE, inactivation of Chd and Nog or Tsg causes PrCp defects and alobar-HPE like phenotype in mice (Table 1.1) (Dubourg et al., 2007).
1. Introduction

1.7.2.2 Mouse models exhibiting a semilobar HPE-like phenotype

In patients with HPE, 46 mutations in SIX3 have been identified- accounting for 4% of HPE cases. (Dubourg et al., 2007; Domené et al., 2008). Six3 mutant mice lack the telencephalon and anterior diencephalon (Lavado et al., 2008) It has been demonstrated that there is cooperation between Six3 and Shh in the pathogenesis of HPE (Chiang et al., 1996; Jeong et al., 2008). The HPE mutant embryos show a lack of the nasal septum, hypoplasia of the olfactory bulbs, and the presence of two posteriorly separated cerebral hemispheres (Geng et al., 2008). Several ventral telencephalon defects were shown including; the presence of a single ganglionic eminence with molecular features of the LGE, and the presence of posterior dorsal telencephalic midline structures such as hippocampus, cortical hem and choroid plexus.

Unlike the abnormal Shh expression observed in embryos showing alobar HPE-like phenotype, Shh expression in the PrCP was unaffected in the embryos with semilobar HPE-like phenotype. Shh expression was, however, missing in the midline of the ventral forebrain, in the region of the prospective hypothalamus. Nkx2.1, which is a marker for the prospective hypothalamus and is a downstream target of Shh signaling from the PrCP, was still present in these mutants. This suggests that the absence of Shh expression in the ventral forebrain is not caused by a defect in the prospective forebrain (ANE). Further studies have shown that Six3 is a direct activator of Shh expression in the ventral forebrain and that Shh maintains Six3 expression in this region (Geng et al., 2008; Jeong et al., 2008).

Cdo is a member of the Ig superfamily and it is a positive regulator of Shh. Cdo mutants have shown a semilobar HPE-like phenotype. As in Six3 mutants, Shh expression in the PrCP and Nkx2.1 expression in the prospective hypothalamus was unaffected but Shh expression was lost in the prospective hypothalamus (Zhang et al., 2006). Loss of Shh expression in the ventral forebrain is probably insufficient to cause HPE- as evidence in Nkx2.1 mutant embryos show that with normal Shh expression in the PrCP but absent Shh expression in the ventral forebrain, only ventral patterning defects occur.

As the telencephalon develops, several Fgf genes are expressed in the rostral midline of the telencephalon (commissural plate) and 3 Fgf receptor (Fgfr) genes are expressed are expressed in neural progenitor cells (Gutin et al., 2006). Reduced Fgf signaling in mouse embryos stops the anterior cerebral hemispheres from separating (Gutin et al., 2006; Storm et al.,
Ventral telencephalon defects are similar to those seen in Six3 promoted HPE (Spoelgen et al., 2005)(see above). In Shh mutant embryos, Fgf expression is downregulated in the commissural plate of Shh mutant embryos (Ohkubo et al., 2002), supporting the proposition that Fgf8 activity lies downstream of Shh during telecephalonic morphogenesis. It has also been suggested that Fgf signaling functions downstream of Shh signaling activity in the regulation of ventral telencephalic patterning.(Geng et al., 2008; Zhang et al., 2006).

Megalin (also known as lrp2) is a member of the LDL receptor related protein family. Functional inactivation of megalin causes semilobar HPE-like phenotypes (Willnow et al., 1996; Spoelgen et al., 2005). It is an endocytic receptor that binds to the amino-terminal of Shh and internalizes it. It may, therefore, be involved in regulation of the Shh pathway (McCarthy et al., 2002) Megalin is also an endocytic receptor and negative regulator for BMP4. Loss of Megalin leads to increase in Bmp4 expression and signaling in the dorsal telencephalon of mutant embryos and the loss of Shh expression in the ventral telencephalon leading to HPE (Spoelgen et al., 2005).

1.7.2.3 Mouse models exhibiting a lobar HPE-like phenotype
Very few mouse models of lobar HPE have been identified. This may be due to the relatively mild forebrain defects which have been overlooked. (Geng and Oliver 2009). However the Vax1 mouse exhibits a lobar HPE phenotype (Hallonet et al., 1999).

1.7.2.4 Mouse models exhibiting a microform HPE-like phenotype
Cdo mutant embryos have been shown to exhibit semilobar HPE (in the C57BL/6 background only ) (Zhang et al., 2006). However, in a mixed background (129/Sv/C57BL/6), Cdo mutants show only mild facial defects; including craniofacial defects, dysgenesis of the philtrum of the lip, hypoplasia of the cartilage of the nasal septum as well as a missing primary palate (Helms et al., 2005).

Shh is expressed in the facial ectoderm of the FNP and is an important signaling pathway involved in development of the facial midline.(Hu et al., 2003; Jeong et al., 2004). Cdo is also highly expressed in the FNP and positively regulates Shh activity, suggesting that Cdo may regulate facial midline development by modulating Shh signaling. (Zhang et al., 2006; Morales et al., 2006).
Gas1 encodes a membrane glycoprotein and, similarly to Cdo, behaves as an agonist of Shh signaling during facial midline development. These mice (in certain genetic backgrounds) display midface hypoplasia, solitary central maxillary incisors, and cleft palate. Additionally, both Gas1 and Cdo cooperate in promoting Shh signaling during neural tube patterning and in craniofacial development. (Seppala et al., 2007; Allen et al., 2007).

Gli2 is an downstream component of the Shh pathway and mutations in GLI2 have been demonstrated in HPE patients (Cohen, 2006; Krauss, 2007). Functional inactivation in mice causes mild defects such as the variable loss of pituitary, and lack or partial fusion of the maxillary central incisors (Hardcastle et al.; 1998 Park et al., 2000).
1.7.2.5 Telencephalon development in normal and HPE conditions in the mouse

Using data generated from analysis of animal models of HPE we can more clearly interpret the pathogenesis and wide spectrum of phenotype of HPE.

Figure 1.14 Mechanistic model of telencephalon development in normal and HPE conditions.

On the left side, the PrCP is represented by a white rectangle. The blue square around it highlights those steps known to be critical in the pathogenesis of alobar HPE. Toward the right side of the diagram, genes known to be important during subsequent steps of forebrain development are indicated. The orange rectangle highlights steps and genes important for semilobar HPE, the green rectangle highlights those important for MIH, and the gray rectangle highlights those important for microforms of HPE. Solid lines represent those processes that have been demonstrated and dashed lines represent those processes that have not yet been directly proved. To better understand the regional relationships between some of those critical genes, their normal expression patterns in the telencephalon at E9.0 and E10.5 are illustrated in B and C, respectively (Monuki, 2007). Hnf3b, hepatocyte nuclear factor 3β; Otx2, orthodenticle homolog 2; Pax6, paired box gene 6; Wnt8b, wingless-related MMTV integration site 8b (adapted and redrawn from Geng and Oliver, 2009).

At pre-gastrula stages, Nodal activity regulates formation of the primitive streak. As gastrulation initiates, the PrCP and anterior notochord are developed and maintained by the activity of Bmp antagonists such as Nog, Chd, and Tsg (Figure 1.14) (Geng and Oliver, 2009). As gastrulation
ces, the Shh-expressing axial mesoderm is formed along the embryo midline. Shh signaling derived from the PrCP acts on the overlying ANE to separate the single eye field, inducing optic stalk specification, and activating downstream genes such as *Nkx2.1* and *Six3* in the midline of the ventral forebrain (Chiang *et al.*, 1996; Chow and Lang, 2001).

Following this, the developing forebrain becomes patterned along the ventrodorsal and anteroposterior axes. On the ventral, *Six3* and *Nkx2.1* are required to activate Shh expression in the ventral forebrain (Shimamura and Rubenstein, 1997; Sussel *et al.*, 1999). In the dorsal telencephalon, *Fgf8* activity restricts *Wnt8b* to the dorsal midline. *Fgf8* also regulates *Bmp4* expression, which in turn is necessary to restrict *Fgf8* and *Shh* expression (Ohkubo *et al.*, 2002; Zakin and De Robertis, 2004). By maintaining *Fgf8* expression in the commissural plate, *Shh* regulates development of dorsal midline structures. Major signaling pathways (Shh, Fgf, Bmp and Wnt) regulate each other and work in collaboration to regulate the morphogenesis and in the specification of the telencephalon (Crossley *et al.*, 2001; Sur and Rubenstein 2005; Monuki 2007). It has been suggested that Shh signaling from the ventral telencephalon activates *Shh* expression in the facial ectoderm. This Shh activity is important for facial midline development (Jeong *et al.*, 2004; Marcucio *et al.*, 2005; Seppala *et al.*, 2007).
1.7.3 *Pathogenesis of HPE*

The aetiology of HPE (OMIM 236100) includes genetic, metabolic and environmental factors. To date, mutations in fourteen different genes have been recognised to cause HPE in humans. Approximately 18%-25% of individuals with HPE have a pathogenic variant in a single gene causing syndromic HPE. At least 25 different conditions in which HPE is an occasional finding have been described; the majority of these disorders are rare (Solomon *et al.*, 2013).

Some of the more common include the following, categorized by mode of inheritance (Dubourg *et al.*, 2007); Pallister-Hall syndrome, Rubinstein-Taybi syndrome, Kallmann syndrome (Isolated GnRH Deficiency), Martin syndrome (with clubfoot, spinal anomalies), Steinfeld syndrome (with congenital heart disease, absent gallbladder, renal dysplasia, radial defects), and Hartsfield syndrome (with ectrodactyly) are autosomal dominant inherited syndromes with features of HPE.

Pseudotrisomy 13 syndrome, (in which affected individuals have a normal karyotype and polydactyly), Smith-Lemli-Opitz syndrome, Meckel syndrome and Genoa syndrome (with craniosynostosis), Lambotte syndrome (with microcephaly, prenatal growth retardation, hypertelorism), Hydrolethalus syndrome (with hydrocephalus, polydactyly, and other anomalies) and facial clefts and brachial amelia are autosomal recessive associated inherited syndromes with features of HPE (Croen *et al.*, 1996; Olsen *et al.*, 1997).
Approximately 25%-50% of individuals with HPE have a chromosome abnormality. Chromosomal abnormalities are nonspecific and either numeric or structural. Those with HPE and a normal karyotype cannot be distinguished from those with an abnormal karyotype on the basis of craniofacial abnormality or subtype of HPE; however, individuals with HPE as a result of a cytogenetic abnormality are more likely to have other organ system involvement (Olsen et al., 1997). Mutations or aberrations in any of the HPE genes may result in a wide presentation of craniofacial phenotype, but some also show characteristics of anomalies reported in association with a specific gene mutation. For example, patients with reported ZIC2 mutations have dramatic forebrain defects with mild facial malformations (Brown et al., 2005), whereas SIX3 mutations in patients exhibit severe HPE in both CNS and face (Kurosaka et al., 2014).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome locus</th>
<th>% of individuals w/ HPE &amp; pathogenic variants in this gene</th>
<th>OMIM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Postive family History</td>
<td>Simplex cases</td>
</tr>
<tr>
<td>SHH</td>
<td>7q36</td>
<td>30%-40%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZIC2</td>
<td>13q32</td>
<td>5%</td>
<td>2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLY3</td>
<td>2p21</td>
<td>1.3%</td>
<td>Rare</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGIF1</td>
<td>18p11.3</td>
<td>1.3%</td>
<td>Rare</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLI2</td>
<td>2q14</td>
<td>Unknown</td>
<td>Unknown</td>
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<td></td>
</tr>
<tr>
<td>PTCH1</td>
<td>9q22.3</td>
<td>Unknown</td>
<td>Unknown</td>
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<tr>
<td>DISP1</td>
<td>1q42</td>
<td>Rare</td>
<td>Rare</td>
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<td>PGRS</td>
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<td>Rare</td>
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<tr>
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<td>8q24.3</td>
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<td>Rare</td>
</tr>
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<td>(CRIPTO) 3p23-p21</td>
<td>Rare</td>
<td>Rare</td>
</tr>
<tr>
<td>GAS1</td>
<td>3p23</td>
<td>Rare</td>
<td>Rare</td>
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<tr>
<td>DLL1</td>
<td>6q27</td>
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<td>Rare</td>
</tr>
<tr>
<td>CDON</td>
<td>11q24.2</td>
<td>Rare</td>
<td>Rare</td>
</tr>
</tbody>
</table>

Table 1.1 Genes associated with Autosomal Dominant Nonsyndromic HPE (Solomon et al., 2013).
Chromosomal microarray (CMA) has identified copy number variants (CNVs) in 10% to 20% of all individuals with HPE. These CNVs can include loci already known to be associated with HPE, as well other loci whose relationship to HPE is less well understood. CMA testing has replaced karyotyping as one of the first-line tests in many situations (Bendavid et al., 2009; Bendavid et al., 2010). However, karyotyping is useful and is an efficient and economical way to evaluate for many chromosome disorders that cause HPE (Solomon et al., 2013).

Cytogenetically normal HPE can arise as a result of exposure to environmental and teratogenic agents during early pregnancy. The most common teratogen in humans known to cause HPE is maternal diabetes mellitus. Infants of diabetic mothers have a 1% risk for HPE (Barr et al., 1983). Other teratogens, including alcohol and retinoic acid, have been associated with HPE in animal models, although their significance in humans is not established (Edison and Muenke, 2003). Cholesterol-lowering agents (i.e., statins) have been associated with HPE, although a causal relationship between prenatal statin use and HPE in the infant has not yet been proven (Edison and Muenke 2003; 2004a; 2004b) An animal model of maternal hypocholesterolemia has been shown to cause HPE. Preliminary studies in humans show that maternal hypocholesterolemia can be associated with HPE (Roux et al., 2000).

The pathogenesis of HPE is believed to involve multiple interacting genetic and environmental factors- however recent evidence points to the role of a single major mutation modified by variants of individually smaller effect (Ming and Muenke 2002; Kurosaka et al., 2014). Genetic modifiers and digenic mutations have also been implicated in the search for an adequate explanation for the lack of genotype-phenotype correlation. An understanding of these factors in HPE has been contributed to by a number of mouse mutant lines. In human, some isolated vases have already been identified (Solomon et al., 2013). In summary, the HPE phenotype can be influenced by several key factors namely, genetic, environmental and the timing of the exposure to the teratogen.
1.8 Aim of the thesis

The aim of this thesis was to investigate the role of Vax1 during craniofacial development. Previous studies of Vax1 expression and the Vax1 mutant mouse have demonstrated the presence of Vax1 transcripts in the craniofacial region and a significant effect of loss-of-function on the developing CNS and eye. This investigation commenced by establishing in detail the expression pattern of Vax1 in specific tissue components and organs of the craniofacial region, focusing on the facial region. In order to investigate the functional role of Vax1 in these regions, the craniofacial phenotype of Vax1/− mice was investigated using skeletal and histological analysis during embryonic development. These analyses were followed up by investigating the molecular mechanisms underlying the craniofacial anomalies identified using gene expression profiles of component members of relevant signaling pathways. Given the known associations between Vax1 and Shh signaling in the developing eye, we further investigated potential interactions between these signaling cascades during craniofacial development and the aetiology of HPE by generating compound mutant mice lacking function of Vax1 in a Gas1 (a Shh mediated HPE modifier) mutant background.
2 Materials and Methods

In this chapter the materials and methods used are described. For each experiment described the minimum of three wild type and three Vax1 homozygous mutant were used. If necessary, details of methods have also been described in the relevant results chapters.

2.1 Mouse embryos

2.1.1 Embryo production

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) and subject to the approved protocols at King's College London. This study conforms with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Wild type (outbreed wild type strain CD1) mice were used at times of different embryonic stages. Mice were mated overnight (ON) and the embryonic age (E) was estimated as 0.5 upon observing plug. The age was calculated more accurately by the counting of somite numbers (Kaufman, 1994). Four mice carrying a Vax1\textsuperscript{tm1Pgr/hm1Pgr} allele in a mixed C57BL/6 genetic background were provided by EMMA (European Mutant Mouse Archive). The colony was expanded by mating Vax1 heterozygous females and males. Vax1 homozygous embryos were obtained from mating Vax1 heterozygous mice. The Gas1 mutant mice used in this study has been well-characterized previously ((Lee et al., 2001; Martinelli and Fan, 2007; Seppala et al., 2007). Gas1 mice maintained in a mixed C57BL/6 background were mated with Vax1 mice. The genotype of individual embryos derived from Gas1\textsuperscript{+/-};Vax1\textsuperscript{+/-} double heterozygous crosses was determined by PCR as described in section 1.6.1 and in Chapter 7.

2.1.2 Embryo collection and dissection of adult hearts

Pregnant females were killed by cervical dislocation. The uterus of the mother was carefully removed and embryos were dissected out by the use of sterile forceps and tweezers and placed immediately in ice cold phosphate buffered saline (1X PBS, GibcoBRL). Embryos collected were washed in tissue culture grade 1X PBS at least two times at room temperature (RT). Embryonic tissue was immediately obtained from tails for genomic DNA (gDNA) extraction. Extracted gDNA was stored at 4°C until the performance of PCR for genotyping. DNA was finally stored at -20°C for future use if necessary. For the purpose of the heart analysis executed
in this study, after the selected mouse was killed by cervical dislocation the animal was weighed (Mettler Toledo PL1200) and had its heart surgically removed. Hearts were placed in ice cold 1X PBS and weighed (Mettler Toledo PB153-S).

2.1.2.1 Embryo storage

Heads required for in situ hybridization were fixed in 4% paraformaldehyde (PFA, Sigma) in PBS (NaCl, 120mmol/l; KCL 2.7mmol/l; phosphate buffer 10mmol/l pH 7.4, Sigma) for 24 hours at 4°C. Heads older than E17.5 were placed in fresh EDTA-based decalcification solution [2.7ml 0.5M EDTA, 4.8ml 1X PBS (made up from RNase free water) 2.5ml 4% PFA for 10ml of total volume] for 2-4 weeks depending on sample size. Fresh decalcification solution was changed once a week. Fixed heads were then washed with 1X PBS (GibcoBRL) and dehydrated through graded alcohols in RNase free H₂O. Time of ethanol washes depended upon gestation stage of the samples. Embryos were stored at −20°C in 100% ethanol until further use.

<table>
<thead>
<tr>
<th>Sample Embryonic Stage (E)</th>
<th>Washing time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E11.5</td>
<td>30</td>
</tr>
<tr>
<td>E12.5</td>
<td>50</td>
</tr>
<tr>
<td>E13.5</td>
<td>60</td>
</tr>
<tr>
<td>E14.5</td>
<td>100</td>
</tr>
<tr>
<td>E15.5</td>
<td>140</td>
</tr>
<tr>
<td>E16.5</td>
<td>180</td>
</tr>
</tbody>
</table>

Table 2.1 Head dehydration

2.2 Histology

2.2.1 Tissue processing

Embryonic tissue to be embedded was fixed in 4% (w/v) PFA in PBS (GibcoBRL) ON at 4°C. Next day the tissue was washed in PBS and dehydrated through a graded series of ethanol (washes depended upon the age of the sample). Ethanol was replaced by histoclear (BDH) and two washes were performed at RT (Table 2.1). Then the sample was placed in a 1:1 mixture of histoclear: paraffin wax at 60°C. Finally the samples were washed at least four times in paraffin wax at 60°C and embedded. Timing for the washes depended upon the age and size of the samples.
material (Table 2-2). Paraffin wax embedded samples were sectioned at 7\( \mu \)m thickness (Zeiss). Sections for \textit{in situ} hybridization experiments were mounted onto slides using RNase free H\(_2\)O, dried and stored in air-tight boxes at 4\(^\circ\)C until further use.

<table>
<thead>
<tr>
<th>Sample Embryonic Stage (E)</th>
<th>Incubation time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E11.5</td>
<td>30</td>
</tr>
<tr>
<td>E12.5</td>
<td>50</td>
</tr>
<tr>
<td>E13.5</td>
<td>60</td>
</tr>
<tr>
<td>E14.5</td>
<td>100</td>
</tr>
<tr>
<td>E15.5</td>
<td>140</td>
</tr>
<tr>
<td>E16.5</td>
<td>180</td>
</tr>
</tbody>
</table>

Table 2.2 Wax processing of samples

2.2.2 Sectioning

Paraffin wax embedded samples were sectioned at 7\( \mu \)m thickness using a microtome (Leica RM2295) with disposable microtome blades (Leica). Serial sections were mounted onto slides (superfrost plus, VWR) using RNase free H\(_2\)O (for \textit{in situ} hybridization experiments) or dH\(_2\)O for H&E staining. Slides were placed onto hot block to be dried ON at 37\(^\circ\)C and stored in air-tight boxes at 4\(^\circ\)C until further use.

2.2.3 Hematoxylin and eosin staining

Sections were dewaxed in histoclear for 10 minutes and passed through 100\%, 90\%, 70\% and 50\% of IMS for 2 minutes prior to a final rinse in dH\(_2\)O for another 2 minutes. The samples were then placed in hematoxylin (Sigma) for 1 minute, washed for 30 seconds in dH\(_2\)O (4 times) and placed in eosin (Sigma) for 40 seconds. After eosin, the slides were immersed for 2 minutes in 90\% IMS and 2 minutes in 100\% IMS twice. Finally, slides were left at RT for 4 hours to air dry and coversliped (VWR) using DePex (BDH) in a fume hood.
2. Materials and Methods

2.2.4 Photography of histological samples

Sections were viewed in light-field using a Zeiss microscope (Axioskope 2 plus) and photographed with an AxioCam HRC (Zeiss). Images were captured using Axiovision software, converted and stored as Adobe Photoshop (CS5) files.

2.3 Skeletal preparations

2.3.1 Bone and cartilage staining

Embryos were collected for skeletal analysis at E17.5. Skin and internal viscera of the body were removed and bodies were fixed in 95% ethanol for at least two weeks. Samples were stained for cartilage with 200μg/ml alcian blue 8 GX (Sigma) in 80% ethanol and 20% glacial acetic acid (BDH) for 3 days at RT. Then, samples were differentiated in 95% ethanol for one week (3 changes) and cleared in 1% KOH until bones were visible. Tissues were then stained in 0.1% aqueous alizarin 15 red-s solution (Sigma) including 10 drops of 1% KOH and incubated for 4 days at RT, washed for 30 minutes in running water and decolorised in 20% glycerol (BDH) in 1% KOH. After staining samples were passed through a graded series of 70% ethanol: glycerol: water (1:2:7, 2:2:6, 3:3:4, 4:4:2, 5:5:0, 4:6:0, 2:8:0, 100% glycerol) and stored at RT.

2.3.2 Storage and photography of skeletal preparation

Samples were stored in 100% glycerol at RT. Skeletal preparations were photographed using a Leica MZFLIII microscope with a Leica DFC300FX camera. The images were captured in Axiovision software and converted into Adobe Photoshop CS5.

2.4 Molecular biology techniques

2.4.1 Bacterial transformation

One hundred ng of plasmid DNA were added to 200μl of DH5α competent cells (Invitrogen) and left on ice for 30 minutes. The cells were heat-shocked at 42°C for exactly 90 seconds and placed on ice for 1 to 2 minutes. 800μl of LB (L-broth, 10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl, 5g yeast extract in 900ml H2O pH 7.5) were added and the culture was placed in a shaker incubator at 37°C for approximately 45 minutes. To maximize the efficiency of transformation, the cells were gently agitated (approximately 225 cycles/ minute) during the recovery period.
After incubation period 50μl, 100μl and 150μl of the transformed competent cells were spread onto LB agar plates (Oxoid) containing 100μg/μl ampicillin (Sigma). The plated were allowed to dry for few minutes at RT, inverted and incubated 37°C for approximately 12-16 hours.

2.4.2 Plasmid amplification

A single colony was picked from the agar plate and placed into a starter culture of 2-5ml LB supplemented with the appropriate antibiotic. The medium quantity used for miniprep was 5 ml and for maxiprep 200ml. Plasmid amplification was performed using a Qiagen plasmid midi/maxi plasmid kit and according to the manufacturers instructions as described in 2.6.3 and 2.6.4.

2.4.3 Miniprepation of plasmid DNA

Plasmid DNA miniprepation was performed using a Qiagen Qiaprep Spin Miniprep kit (Qiagen). A single colony was picked (to ensure genetic homogeneity) from the agar plate and placed into a starter culture of 5ml LB supplemented with the appropriate antibiotic. The culture was incubated at 37°C ON with vigorous shaking (225 cycles/ minute). The next day 1 ml aliquots were transferred into eppendorf tubes for storage at 4°C until further use. Bacterial culture was centrifuged at 2800g for 10 minutes (Sorval GSA) and pellet was resuspended in 250μl of cold buffer P1. After resuspension, 250μl of buffer P2 were added and tube was inverted several times until solution had cleared. 350μl of cold buffer P3 were added and mixture was gently mixed. The suspension was then centrifuged at 7800g for 10 minutes. The resulting supernatant was carefully collected and pipetted into Qiaprep spin columns placed in 2ml collection tubes. Tubes were then spun for 1 minute and the flow- through was discarded. The column was washed with 0.75ml of buffer PE and spun for another minute. The flow-through was discarded and columns spun for another minute in order to remove any residual wash buffer. Columns were then placed into clean eppendorf tubes and 50μl 10mM Tris-HCL pH 8.5 were passed through (approximately in a minute). Finally, column was centrifuged for 1 minute at 7800g. The collected pDNA was stored at -20°C until further use. Note that the contents of buffers P1, P2, N3 and PE were not specified in the kit manual.
2.4.4 *Maxipreparation of plasmid DNA*

Plasmid DNA maxipreparation was performed using a Qiagen Qiaprep Spin Maxiprep kit (Qiagen). A 100µl aliquote of cells from minipreparation were added to 250ml of LB medium supplemented with the appropriate antibiotic. The culture was incubated at 37°C ON with vigorous shaking (225 cycles/minute). Culture was transferred to 250ml centrifuged bottles and centrifuged at 5500g for 10 minutes at 4°C (Beckman JA-10 rotor). Cell pellet was resuspended in 10ml of buffer P1 (50mM Tris-HCl pH 8, 10mM EDTA, 100µg/ml RNase A). Cell lysis took place by adding 10ml buffer P2 (200mM NaOH, 1% sodium dodecyl sulfate (SDS), gently inverting the tube and incubating the mixture for 10 minutes at RT. When solution appeared clear neutralisation step took place by adding 10ml cold buffer P3 (3M NaOAc pH 5.5). Then the tube was gently inverted and mixture was incubated on ice for 20 minutes. The solution was then centrifuged (Beckman JA-17 rotor) at 30000g for 30 minutes at 4°C and the supernatant was filtered by passing into the tube through 2 layers of sterile gauze. A Qiagen-tip 500 was equilibrated by applying 10ml buffer QBT (750mM NaCl, 50mM MOPS pH 7, 15% isopropanol, 0.15% Triton X-100). The filtered supernatant was loaded into the equilibrated column and allowed to pass through. Column was washed 2 times with 30ml applications of buffer QC (1.25M NaCl, 50mM Tris-HCl pH 8.5, 15% isopropanol). The plasmid DNA was precipitated by adding 0.7 volumes of isopropanol (BDH) following centrifugation at 2500g in a Beckman S4180 rotor for 30 minutes at 4°C. The collected pellet was washed with 5ml of 70% ethanol and centrifuged for another 15 minutes. Supernatant was carefully decanted and pellet left to air dry on ice. The plasmid DNA was redissolved in 300µl 10mM Tris-HCl pH 8.5 and stored at -20°C until further use.

2.4.5 *Bacterial cell storage and recovery*

Bacteria were stored at -80°C in a 2:1 ratio with glycerol mixture (65% glycerol, 0.1M MgSO4 (BDH) and 25mM Tris-HCl pH 8). Bacterial recovery took place by scraping a few cells from the frozen vial using a sterile loop. The sample was then plated onto LB agar plates (5g tryptone, 2.5g yeast extract, 5g NaCl, 7.5g agar all supplied by Oxoid) supplemented with the appropriate antibiotic and incubated ON in 37°C incubator.
2. Materials and Methods

2.4.6 Restriction digestion of plasmid DNA

Plasmid DNA was digested in 15-20µg aliquots using restriction enzymes and buffers from Promega and Roche Diagnostics. Digests were incubated at 37ºC for approximately 2 hours and restriction enzymes comprised up to 12% of the total reaction volume. The unit concentrations were used according to the manufacturer instructions and buffer volume was 10% of the total reaction.

2.4.7 Agarose gel electrophoresis and visualisation

In order to confirm linearization of the plasmid a 1% agarose gel was prepared by adding the appropriate amount of agarose (Sigma) into 1X TAE (40mM Tris-HCl, 1mM EDTA pH 8) and boiling for 2 minutes. After cooling, 0.2mg/µl of ethidium bromide (EtBr, Sigma) were added. As EtBr is carcinogenic the necessary health and safety precautions were taken. Molten agarose was poured onto the appropriate gel tray and wells of varying sizes were formed in the gel due to the presence of Teflon combs. When the gel was set it was transferred into the electrophoresis tank loaded with 1X TAE. In order to identify the size of product on the gel a 1Kb DNA ladder (Fermentas) was loaded alongside the DNA sample. Products were loaded next to the ladder into the wells with 6X agarose loading dye stock solution: 0.25% bromophenol blue (sigma); 0.5 xylene cyanol FF (BDH); 15% ficoll (sigma); in H₂O and samples run for approximately 1 hour at 60 Volts. Product visualization was carried out under a UV transillumination (UVP).

2.4.8 Plasmid DNA purification by gel extraction

Plasmid purification was performed using Quiagen QIAquickgel extraction kit. 1% agarose gel was placed under the UV transillumination and the required DNA fragment was excised with a sterile sharp blade (Swann-Morton). Fragment of interest was placed into a clean 1.5 ml eppendorf tube of known weight. Gel weight was calculated and 3 volumes of volatilisation buffer with pH indicator buffer QC was added into the tube (e.g. 100mg=100µl). The tube was then incubated at 50ºC for 10 minutes allowing gel to dissolve in the buffer. The correct colour of the solution indicated that the pH remained at approximately 7.5. Then, a single gel volume of isopropanol (BDH) was added to the sample and mixed. The mixture was transferred to a spin column and centrifuged at 14000g for 1 minute. The flow-through was discarded and the column was washed with 0.5 ml buffer QG. A further wash with 0.75ml of buffer PE was carried out and
the column was spun 2 times for 1 minute each at 14000g in order to remove any residual ethanol. DNA elution was performed by adding 50µl buffer EB (10mM Tris-HCl pH8.5) into the centre of the column membrane and spinning at 14000g for 1 minute. The eluted DNA was collected in a clean tube and stored at -20°C until further use. Note that the manufacturer did not specify the contents of buffers QG and PE.

### 2.4.9 Genomic DNA isolation

Isolation of high molecular weight DNA from mouse embryonic tissue and mouse adult tails was performed using the GenElute Mammalian Genomic DNA Purification Kit (Sigma) according to the manufacturers’ instructions. Two small pieces (0.5-0.6cm) were cut from embryonic tails or adult ears and transferred to a microcentrifuge tube with 180µl lysis solution and 20µl proteinase K (10mg/ml). Samples were then incubated at 55°C ON in a rocking oven. After full digestion 200µl lysis solution and 200µl ethanol (100%) were added into the mixture and transferred to binding column. Mixture was centrifuged at 6500g for 1 minute. In order to remove any contaminants, column was transferred to a new collection tube, 500µl wash solution were added to column and centrifuged at 6500g for 1 minute. Another 500µl wash solution was added to column and centrifuged at 12000g for 3 minutes allowing column to dry. Then, column was transferred in a new collection tube, 200µl elution solution were added and centrifuged at 6500g for 1 minute. Genomic DNA was finally stored at -20°C for further use.

### 2.4.10 Polymerase chain reaction

2.4.10.1 Vax1 mice

Selective amplification of DNA fragments was performed by polymerase chain reaction (PCR). PCR reaction was performed in the PTC-200 Peltier Thermal Cycle (MJ Research).

Three primers (Sigma) were used:

- Vax1 wild type: 5’CGTAATCAATTGCAACAGCGAGG
- Vax1 targeted: 5’ACCACAGATGAAACGCCGAG
- Vax1 wild type/targeted: 5’AGAAGGAGGGTGGGAAAAGAAG
A multiplex reaction was performed which was designed to detect both wild type and targeted alleles (this reaction involves all three primers). In order to ensure the genotyping results, in addition to the multiplex PCR reaction, a second reaction is performed that only detects the wild type allele (this reaction involves primer \textit{Vax1} wild type and \textit{Vax1} wild type/targeted). For this reaction the amount of primer \textit{Vax1} targeted that is used for the multiplex reaction is replaced by RNase free H$_2$O. The reaction mix and conditions are shown in Table 2.3 and Table 2.4 PCR cycling conditions, respectively. Figure 2.1 Schematic of genotyping procedures illustrates the position of the primers in relation to the cassette and shows the expected gel pattern for both PCR reactions. All solutions were from Qiagen, unless otherwise stated. Two bands were generated after PCR reaction a 244 base pairs wild type and a 488 base pairs mutant.

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>1µg</td>
</tr>
<tr>
<td>\textit{Vax1} wild type (Sigma)</td>
<td>20pmol</td>
</tr>
<tr>
<td>\textit{Vax1} targeted (Sigma)</td>
<td>20pmol</td>
</tr>
<tr>
<td>\textit{Vax1} wild type/targeted (Sigma)</td>
<td>20pmol</td>
</tr>
<tr>
<td>10X Taq buffer</td>
<td>1X</td>
</tr>
<tr>
<td>dNTPs</td>
<td>25mM</td>
</tr>
<tr>
<td>RNase free H$_2$O</td>
<td>Up to 25µl total volume</td>
</tr>
<tr>
<td>Hot-start Taq DNA polymerase</td>
<td>5U/µl</td>
</tr>
<tr>
<td>Final Volume</td>
<td>25µl</td>
</tr>
</tbody>
</table>

Table 2.3 PCR mixture for 25µl of reaction

<table>
<thead>
<tr>
<th>PCR Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
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<td>7 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
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<td>10 seconds</td>
<td>35</td>
</tr>
<tr>
<td>Anealling</td>
<td>60 °C</td>
<td>30 seconds</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>68 °C</td>
<td>90 seconds</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>68 °C</td>
<td>1 minute</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>Forever</td>
<td>forever</td>
</tr>
</tbody>
</table>

Table 2.4 PCR cycling conditions

After PCR, aliquots of the mixture were loaded onto a 1,4% agarose gel and electrophoresed (180 Volts for 45 minutes) to detect amplified product. In order to identify the size of product on the gel a 100 base pairs DNA ladder (Fermentas) was loaded alongside the DNA sample. Visualization took place under UV light.
2. Materials and Methods

Figure 2.1  Schematic of genotyping procedures
(A) Position of primers in relation to the cassette (represented by an inverted triangle). (B) Expected gel pattern for all three genotypes with a multiplex or endogenous PCR. The targeted band has 488 base pairs and the wild type band 244 base pairs. WT, wild type; t, targeted; WT, T, wild type/targeted. The diagram is not drawn to scale.

2.5  Radioactive in situ hybridisation

2.5.1 Preparation of 35S UTP-labeled riboprobes

Synthesis of riboprobes labelled with α[^35]S] UTP (ICN) took place by mixing the reagents described in Table 2.5 and 3µg linearized DNA (prepared as in section 2.4.6) in a sterile screw-cap eppendorf tube and incubating the reaction at 37°C for 40 minutes. All reagents used for probe preparation were provided by Promega unless otherwise stated.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X transcription reaction buffer</td>
<td>5</td>
</tr>
<tr>
<td>1M DTT (BDH)</td>
<td>0.5</td>
</tr>
<tr>
<td>10mM GTP</td>
<td>1.2</td>
</tr>
<tr>
<td>10mM ATP</td>
<td>1.2</td>
</tr>
<tr>
<td>10mM CTP</td>
<td>1.2</td>
</tr>
<tr>
<td>10mM UTP</td>
<td>1</td>
</tr>
<tr>
<td>3µg linearized DNA</td>
<td>6.4 (maximum)</td>
</tr>
<tr>
<td>RNasin</td>
<td>0.5</td>
</tr>
<tr>
<td>α[^35]S UTP</td>
<td>7</td>
</tr>
<tr>
<td>RNA polymerase enzyme (10U/µl) T3, T7 or SP6</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.5 Transcription reaction for riboprobe synthesis
After the 40 minutes of incubation another 1µl of the appropriate RNA polymerase enzyme was added in the tube and reaction continued for 1 hour at 37°C. The DNA template was then degraded by adding 0.5µl RNasin, 1µl RNase free tRNA (10mg/ml), 0.5µl 1M DTT, 0.5µl RNase-free DNase I. The reaction was incubated for 10 minutes at 37°C. RNA precipitation was achieved by adding 160µl RNase free H2O, 4µl 1M DTT, 4µl 5M NaCl, 20µl 3M NaOAc (pH 5.2), 400µl 100% ethanol and incubation at -20°C ON.

2.5.2 Hydrolysis of radioactive riboprobes

Riboprobes longer than 300 base pairs were hydrolyzed to reduce length and therefore improve tissue penetration. The following calculation was performed to determine the length of hydrolysis time:

\[
X = \frac{(L_o - L_f)}{K L_o L_f}
\]

Where
- \(X\) = hydrolysis time in minutes
- \(L_o\) = original transcript length in Kb
- \(L_f\) = desired transcript length (300 base pairs)
- \(K\) = 0.11 (Kb min\(^{-1}\))

In order to hydrolyze the riboprobe, 50µl of hydrolysis buffer (Table 2.6) were added to the re-suspended probe and incubated for the appropriate amount of time (X minutes) at 60°C. Then, the reaction was stopped when 50 µl of neutralizing buffer (Table 2.7) were added into the tube and mixed.

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO(_3) (BDH)</td>
<td>80mM</td>
</tr>
<tr>
<td>Na(_2)CO(_3) (BDH)</td>
<td>120mM</td>
</tr>
<tr>
<td>DTT</td>
<td>10mM</td>
</tr>
</tbody>
</table>

Table 2.6 Components of hydrolysis buffer

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOAc</td>
<td>0.2M</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>1%</td>
</tr>
<tr>
<td>DTT</td>
<td>10Mm</td>
</tr>
</tbody>
</table>

Table 2.7 Components of neutralizing buffer
2. Materials and Methods

The RNA was re-precipitated by adding 15µl 3M NaOAc pH 5.2, 4µl 1M DTT, 485µl 100% ethanol and placing the RNA solution at -70°C for 1 hour. After incubation, the solution was centrifuged for 10 minutes at 78,000g and washed with 10mM DTT in 100% ethanol. The RNA tube was placed on ice for a few minutes in order for the pellet to dry and re-suspended in 50mM DTT in RNase free H₂O. Riboprobe activity was calculated by adding 1µl of probe and 2ml of Ultima Gold (Pakared) scintillation fluid in a scintillation vial, vortexing for 1 minute and placing in a scintillation counter (Beckman LS60001C). Radioactive probe activity was automatically recorded in counts per minute. Finally, the remainder of the riboprobe was stored at -80°C until further use within one month.

2.5.3 Pre-treatment of tissue sections

Slides were placed in a pre-baked slide rack (200°C oven ON) and tissue sections were dewaxed with 2 successive immersions in histoclear for 15 minutes each. The sections were then passed through a series of ethanol changes [100% ethanol, 95%, 90%, 80%, 70%, 60%, 50% and 30% (made up using RNase free water)] each for 2 minutes and then washing in RNase free water for 2 minutes. Slide treatment with 2% concentrated HCl (BDH) for 20 minutes and 2X SSC pH 4.5 for 5 minutes then took place. Tissues were permeabilised with treatment in 5µg/ml proteinase k (diluted in RNase free water, Sigma) in 100mM Tris-HCl pH 7.5, 50mM EDTA for 10 minutes at 37°C and rinsed for 2 minutes in 2mg/ml glycerol (Sigma) in RNase free 1X PBS (GibcoBRL). Following this, slides were rinsed 2 times in 1X PBS (made up in RNase free water) for 1 minute each and re-fixed for 20 minutes in fresh 4% PFA made up with RNase free water. Then, the slides were washed in RNase free 1X PBS for 2 minutes and incubated for 10 minutes in 0.1M TEA, 1/400 acetic anhydride with constant stirring at RT. Following this, slides were washed in RNase free PBS for 5 minutes and RNase free water for a further 2 minutes. The sections were finally dehydrated through a graded series of ethanol made up with RNase free H₂O (30%, 50%, 70%, 80%, 95% and 100% ethanol) and left to air dry at RT.

2.5.4 Hybridization and washes of tissue sections

The slides were placed in a hybridization chamber (supported on glass rods) that contained tissue paper soaked in 100ml 2X SSC pH 4.5 and 50% formamide (BDH). Hybridization mixture comprised of 50% molecular biology grade formamide, 10% dextran sulfate (Sigma), 50mM
2. Materials and Methods

DTT, 0.5mg/ml tRNA, 0.3M NaCl, 0.02M Tris-HCl pH 8.0, 5mM EDTA, 1X Denhardt’s, α[^35]S UTP- labelled riboprobe (at a final concentration of 1x10⁴ counts min⁻¹) and RNase free H₂O was prepared the same day. Mixture was then vortexed, spun in a centrifuge for a few seconds, heated to 80°C for 2 minutes and cooled on ice prior to use. Then, 100μl of hybridization mixture were added onto each slide and a glass coverslip placed on top, to prevent samples from drying in the hybridization oven. In addition, care was taken to ensure homogenous spread of the mixture over the tissue sections. The hybridization chamber was closed and carefully sealed with Sara wrap and placed in the hybridization oven with a stable temperature of 55°C ON. The following day, coverslips were removed with care and the slides were immediately transferred to a slide rack and washed for 15 minutes at 55°C in a freshly made wash solution. This wash was repeated twice for 20 minutes each time at 55°C and 65°C. Then, the slides were removed from wash buffer and transferred to fresh RNase buffer (500mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA) twice for 15 minutes in a 37°C water bath.

Following this, the rack was transferred in 40μg/ml RNase A (Sigma) in RNase buffer for 30 minutes at 37°C and then washed in RNase buffer for 15 minutes again at 37°C. The slides were washed twice in wash solution for 20 minutes each time at 65°C and transferred into 0.1X SSC pH 4.5, 10mM DTT for another 20 minutes at the same temperature. A further wash of 0.1X SSC pH 4.5, 10mM DTT was carried out for 5 minutes at RT. Finally, the slides were dehydrated though a graded series of ethanol made up with RNase free H₂O (30%, 50%, 70%, 80%, 95% and 100% ethanol) each for 2 minutes at RT. The slides were then covered in aluminium foil and left to air dry for at least 3 hours.

2.5.5 Autoradiography

Once slides dried they were placed in a film cassette and exposed to a Kodak BioMax MR film ON at RT. Next day the film was developed and the density of the silver grain precipitation inspected. Then the slides were dipped in ilford K5 emulsion (Ilford scientific product) dissolved in 2% glycerol at 42°C under safe light and placed in a dark cassette for at least 2 hours to dry. Following this, the slides were transferred to a light proof container and left in a fridge at 4°C for an average time of 2 weeks.
2.5.6 Developing

Prior to developing the slides were placed at RT for 1 hour. In the dark room and under a safe light the slides were developed by immersion in Kodak D19 developer for 5 minutes, fixed in Kodak Unifix for 5 minutes and rinsed in tap water for 20 minutes. Following this, slides were washed 3 times in dH$_2$O, counterstained with 1% hematoxylin (Sigma), rinsed with dH$_2$O and allowed to air dry. Once dried, slides were coverslipped using depex mountant.

2.5.7 Viewing and photography

Sections were examined under an Olympus BH-2 microscope. Light- and dark-field images of sections were photographed using a Zeiss Axioscop microscope (Germany) and merged in Adobe Photoshop CS3. In Photoshop images were initially converted into RGB color mode under the image menu and the image contrast was adjusted using levels. The white pixels in the dark field image (representative of the gene expression) were selected using the magic wand tool. The selection was filled with a red color and dragged into the light field image.

2.6 Digoxigenin labeled in situ hybridization on paraffin sections.

In situ hybridization with digoxigenin labeled probes was used to visualize gene expression. Paraffin was removed from slides by washing three times in xylene for 10 mins each. Slides were then re-hydrated through a series of ethanol concentrations, twice for 2mins each (100%, 95%, 70%) and then washed in RNAse free water twice and post-fixed in 4% PFA for 10mins. PFA was removed by washing twice in PBS for 5mins each and then samples were incubated with 4μg/ml proteinase K for 8mins. After a 5 minute PBS wash, slides were fixed again in 4% PFA followed by another PBS wash. Next, acetylation was carried to remove electric charge on the slides in order to reduce background staining. This was done using 50ml H$_2$O, with 625μl triethanolamine (TEA), 130μl of 37% hydrochloric acid (HCL) and 125μl acetic anhydride. Slides were incubated for 10mins and then washed 3 times in PBS for 5mins each. Slides were again dehydrated through 70% and 95% ethanol and air-dried. During these dehydration steps 1μg of probe was added to 1ml of hybridization solution.
2. Materials and Methods

100ml hybridization solution consists of the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>50ml</td>
</tr>
<tr>
<td>50% Dextran sulphate</td>
<td>20ml</td>
</tr>
<tr>
<td>50x Denhardt’s solution</td>
<td>2ml</td>
</tr>
<tr>
<td>10mg/ml Yeast tRNA</td>
<td>2.5ml</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>6ml</td>
</tr>
<tr>
<td>1M Tris-HCL pH8</td>
<td>2ml</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>1ml</td>
</tr>
<tr>
<td>1M Sodium phosphate</td>
<td>1ml</td>
</tr>
<tr>
<td>20% Sarcosyl</td>
<td>5ml</td>
</tr>
<tr>
<td>Water</td>
<td>10.5ml</td>
</tr>
</tbody>
</table>

300μl of probe/hybridization solution was added to each slide and slides were subsequently covered with RNase free coverslips and incubated at 65°C overnight in a humid chamber. Finally, 20X saline sodium citrate (SSC), pH7 in 1litre of water was prepared ready for use the next day. 20X SSC consists of 175.3g sodium chloride and 88.2g sodium acetate dissolved in water. The next day, coverslips were removed in 5X SSC and washed in a high stringency wash, consisting of 50% formamide and 2X SSC, twice for 30mins at 65°C. Slides were then washed 3 times for 15mins each in RNase buffer. This consists of 0.5M NaCl, 10mM Tris-HCL and 5mM EDTA pH8 made up in water. After the third wash, slides were incubated in RNase buffer treated with 20mg/ml RNase A (Invitrogen) for 30mins at 37°C. After this, again samples were washed in fresh RNase buffer without RNase A. High stringency washes are then repeated twice followed by washes in 2X SSC and then 0.1X SSC at 37°C for 15mins each. Then specimens were washed in PBS Tween (PBTw), consisting of PBS and 0.1% Tween-20 for 15mins at room temperature. Finally slides were blocked for 1 hour in PBTw with 10% goat serum (GS) before incubating with alkaline phosphatase-coupled anti digoxigenin antibody (Boerhringer Mannheim), diluted at 1:5000 in PBTw with 1% GS, overnight at 4°C.

In order to visualize RNA expression, on the next day, slides are washed 4 times for 15mins in PBTw at room temperature. Then wash twice for 10mins each in NTMT buffer.

100ml NTMT buffer consists of the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5M NaCl</td>
<td>2ml</td>
</tr>
<tr>
<td>1ml Tris-HCL pH9.5</td>
<td>10ml</td>
</tr>
<tr>
<td>1M MgCl₂</td>
<td>5ml</td>
</tr>
<tr>
<td>Tween-20</td>
<td>0.1ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>82.9ml</td>
</tr>
</tbody>
</table>
Prior to addition of NTMT buffer add 0.5mg/ml levamisole in order to reduce background staining. After NTMT washes incubate slides in BM-purple AP substrate (Roche) with 0.5mg/ml levamisole and incubate in the dark at room temperature. Check slides every hour to ensure that over-staining does not occur. Once staining has finished, was slides in PBS twice for 5mins each and then counterstain in 0.005% fast red (Polyscientific) for 5mins. Finally dehydrate slides through 70%, 95% and 100% ethanol sequentially, incubate in xylene three times for 3mins each and then coverslip with DPX (Solmedia).

2.7 Whole-mount digoxigenin in situ hybridization

2.7.1 Preparation of digoxigenin-labelled riboprobes

Digoxigenin labelled riboprobes were synthesized by antisense transcription of the linearized plasmid of interest and incorporation of dig-labelled nucleotides. This was achieved by setting up the reaction mixture (outlined in Table 2.8) in a sterile screw-cap eppendorf tube and incubating at 37°C for 2 hours. An extra 1ul of the appropriate RNA polymerase was then added to the mix and returned to 37°C for a further 2 hours.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X transcription reaction buffer</td>
<td>2µl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2µl</td>
</tr>
<tr>
<td>Nucleotide mixture (GTP, CTP, ATP, Digoxigenin-UTP)</td>
<td>2µl</td>
</tr>
<tr>
<td>Linearized template 1µg</td>
<td>X</td>
</tr>
<tr>
<td>RNAsin (100U/µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>RNA polymerase enzyme (10U/µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>Nuclease free H₂O</td>
<td>For a total volume of 20µl</td>
</tr>
</tbody>
</table>

Table 2.8 Preparation of Digoxigenin-Labelled Riboprobes
2. Materials and Methods

The transcription mixture was then treated with 2µl of DNase I (Promega) for 30 minutes at 37°C. RNA was precipitated by adding 10µl 4M LiCl (BDH), 100µl RNase free H$_2$O, 300µl 100% ethanol and storing ON at -20°C. Alternatively precipitation may be achieved by storing in this solution at -80°C for 2 hours. The reaction mixture was then centrifuged for 30 minutes at 13000g. The resulting RNA pellet was washed in 70% ethanol (made up with RNase free H$_2$O) and centrifuged again for 10 minutes before being air dried for approximately 10 minutes on ice. The pellet was re-suspended in 20µl RNase free H$_2$O and probe was finally stored at -20°C until further use.

2.7.1.1 Gel electrophoresis and visualization of Dig-labelled probe

In order to ascertain if probe synthesis was successful a 1% agarose gel was prepared as outlined in section 2.4.7. 2µl of RNA product was mixed with 1ul of loading dye and 3µl of dH$_2$O and loaded into the gel. An appropriate ladder was also loaded in order to identify the size of product. Samples were run for approximately 45 minutes at 120 Volts. Product visualization was carried out under a UVP and the size of the synthesized Dig-labelled probe compared with the known size of the desired sequence.

2.7.2 Pre-hybridization treatments

Embryos were removed from –20°C and washed for 10 minutes in fresh 100% methanol at RT prior to bleaching in 5:1 MeOH/30%H$_2$O$_2$ for 5-6 hours at RT. Samples were then given 2 x 10 minutes washes in 100% ethanol before being rehydrated through a graded series of methanol/PBT washes (100%, 70%, 50%, 30% and PBT) for 10 minutes each wash. E10.5 and older embryos were then treated with 10 µg/mL proteinase K in PBT for 10-15 minutes and subsequently gently rinsed in PBT before post-fixation for 20 minutes in 4% PFA.

2.7.3 Hybridization

Samples were then rinsed once and washed for 5 minutes in PBT, 1:1 PBT Hybridization mix and finally hybridization mix alone (ensuring the embryos settled during each wash). They were then incubated horizontally in fresh hybridization mix at 65°C for 1 hour. During this time 1µg/mL DIG-labeled RNA-probe was added to hybridization mix and allowed to warm to 65°C. Finally the hybridization mix and Dig labelled RNA probe were added to the samples and incubated at 65°C ON with gentle rocking.
2.7.4 Post-hybridization washes and antibody application

Next day the probe-containing hybridization mix was removed, labelled and stored at -80°C for future use. Embryos were rinsed 3 times and washed 3 times (30 minutes each) at 65°C in prewarmed hybridization mix. This was followed by a wash in prewarmed 1:1 hybridization mix:Rnase buffer at 65°C. Samples were then rinsed twice in prewarmed Rnase buffer (37°C) and incubated for 1 hour at 37°C in Rnase buffer containing 100µg/mL Rnase. In order to inactivate endogenous alkaline-phosphotase activity samples were then placed at 70°C for 20 minutes before being rinsed twice more in Rnase buffer. The embryos were then washed in 1:1 Rnase buffer: MABT, rinsed twice in MABT and washed for 15 minutes in MABT. Embryos were then incubated for 1 hour in MABT + 2% BBR + 0.5mg/mL levamisole and placed in blocking solution (MABT + 2% BBR + 20% heat-treated serum + 0.5mg/mL levamisole) for a further hour at RT on a rocker before being placed in blocking solution + anti-dig secondary antibody (1:2000, dilution) and incubated ON at 4°C on a rocker.

2.7.5 Post-antibody washes

The next day samples were rinsed 3 times and then washed (7 x 1 hour) in MABT + 0.5mg/mL levamisole at RT and washed in the same solution at 4°C.

2.7.6 Detection of digoxigenin riboprobes

Next morning the samples were washed once in MABT, twice for 10 minutes each wash in NTMT pH9.5 (100mM NaCl, 100mM Tris-HCl pH 9.5, 50mM MgCl₂, 1% tween 20, 2mM levamisole). Samples were incubated in NTMT + 0.5mg/ml levamisole for 10 minutes before incubating in NTMT + 4.5µl/mL NBT + 3.5µl/mL BCIP + 0.5mg/ml levamisole, protected from light on a shaker until the appropriate amount of colour was achieved. When colour reaction took place, tissues were washed in NTMT pH9.5 for 10 minutes, in PBT for another 10 minutes, photographed and stored in 4% PFA at 4°C.

2.7.7 Photography of digoxigenin labelled whole-mount in situ

Samples were photographed submerged in 4% PFA in a petri dish containing a thin layer of 2% agarose gel set in the base. Photographs were taken using a Leica stereomicroscope.
2. Materials and Methods

2.7.8 Vibratome sectioning

Fixed samples were washed briefly in 1X PBS (GibcoBRL) and then embedded in desired plane in prewarmed 20% gelatin by using plastic disposable moulds. The gelatin was then allowed to solidify on ice and gelatin blocks were fixed in 4% PFA for a week at 4 ºC. For sectioning, gelatin blocks containing the sample were glued onto a metal block holder with super glue, allowed to dry, and then sectioned with a vibratome (Leica VT 1000S) while submerged in a 1X PBS bath. Individual sections (50 μm thickness) were collected with a fine brush and transferred to slides. The slides were mounted with coverslips using an aqueous mounting reagent. The mounted slides were cleaned to remove excess mounting agent before taking photographs using a Zeiss Axioscop microscope (Germany).

2.8 lacZ staining

The well characterized bacterial *Eschericha coli* lacZ gene, which encodes β-galactosidase (β-gal), is commonly used as a reporter of gene expression in transgenic organisms (Mahony *et al.*, 2002). Standard histochemical detection of β-gal involves incubation of tissue samples in the presence of a suitable chromogenic substrate. *Vax1* mutant mice were originally generated by introducing a targeted mutation of *Vax1* using homologous recombination in embryonic stem (ES) cells (Hallonet *et al.*, 1999). The mutation replaces the amino terminus of the encoded Vax1 protein (including the exon coding for the two first helices and part of the third helix of the Vax1 homeobox) with a β-galactosidase reporter (Le Mouellic *et al.*, 1990; Hallonet *et al.*, 1999). When lacZ is placed in frame with the endogenous gene, transcriptional fusion causes the expression pattern to closely resemble that of the target gene. Therefore, the spatial and temporal expression pattern of the lacZ allows the normal expression pattern of the targeted gene to be followed. (Takahashi *et al.*, 2000)

Pregnant females were killed by cervical dislocation. The uterus of the mother was carefully removed and embryos were dissected out by the use of sterile forceps and tweezers and placed immediately in ice cold 1X PBS, (GibcoBRL). Embryos were fixed in a solution made of 1% PFA and 0.2% glutaraldehyde dissolved in 1x PBS. Embryos were fixed in this solution according to its stage (Table 2.9).
2. Materials and Methods

<table>
<thead>
<tr>
<th>Sample Embryonic Stage (E)</th>
<th>Incubation time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5</td>
<td>10</td>
</tr>
<tr>
<td>E11.5</td>
<td>12</td>
</tr>
<tr>
<td>E12.5</td>
<td>15</td>
</tr>
<tr>
<td>E13.5</td>
<td>20</td>
</tr>
<tr>
<td>E14.5</td>
<td>25</td>
</tr>
<tr>
<td>E15.5</td>
<td>30</td>
</tr>
<tr>
<td>E16.5</td>
<td>35</td>
</tr>
<tr>
<td>E17.5</td>
<td>40</td>
</tr>
</tbody>
</table>

| Table 2.9 Sample fixation time |

After the specified time in fixative (Table 2.9) the tissue was washed in 1x PBS three times for five minutes. The samples were then stained overnight at 37°C, protected from light. The components of the staining solution are described in the table below:

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH 7.3 (BDH)</td>
<td>10mM</td>
</tr>
<tr>
<td>Na-dexoxycholate (Sigma)</td>
<td>0.005%</td>
</tr>
<tr>
<td>IGEPAL (Sigma)</td>
<td>0.01%</td>
</tr>
<tr>
<td>K4 (BDH)</td>
<td>5mM</td>
</tr>
<tr>
<td>K3 (BDH)</td>
<td>5mM</td>
</tr>
<tr>
<td>MgCl₂ (BDH)</td>
<td>2mM</td>
</tr>
<tr>
<td>X-Gal (Fermentas)</td>
<td>0.8 mg/ml</td>
</tr>
<tr>
<td>1x PBS (GibcoBRL)</td>
<td>for final volume</td>
</tr>
</tbody>
</table>

| Table 2.10 Components of lacZ staining solution |

The reaction was interrupted by removing the staining solution and rinsing the samples in 1X PBS (GibcoBRL). Next step involves 3 washes, of five minutes each, with 1X PBS. Embryos were then post-fixed in 4% PFA for one hour at RT and stored in 1% PFA in 4°C.

2.8.1 Viewing and photography

Whole-mount Embryos were examined and photographed on an agarose background suspended in 1X PBS, using a Leica stereomicroscope.
2.8.2 Processing of whole-mount lacZ stained tissues for section

Samples that were subsequently sectioned were washed in 1x PBS three times for 10 minutes. Following this step tissue was dehydrated through a graded series of methanol (washes depended upon the age of the sample (Table 2.9). Methanol was replaced by Isopropanol (2-Propanol - BDH) and two washes of 15 minutes were performed at RT. Then the samples were placed in tetrahydronaphthalene at RT until they sunk and moved to 60°C for 15 minutes in a fresh tetrahydronaphthalene volume. Afterwards samples were placed in a 1:1 mixture of tetrahydronaphthalene : paraffin wax at 60°C for 15 minutes. Finally the samples were washed at least four times in paraffin wax at 60°C and embedded. Paraffin wax embedded samples were sectioned at 7μm thickness (Zeiss).

2.8.3 Eosin counterstaining of lacZ stained sections

Sections were dewaxed in histoclear for 10 minutes and passed through 100%, 90%, 70% and 50% of IMS for 2 minutes prior to a final rinse in dH₂O for another 2 minutes. The samples were then placed in eosin (Sigma) for 30 seconds and washed for 30 seconds in dH₂O (4 times). Thereafter slides were immersed for 2 minutes in 90% IMS and 2 minutes in 100% IMS twice. Finally, slides were left at RT for 4 hours to air dry and coverslipped (VWR) using depex (BDH) in a fume hood.

2.8.4 Photography of lacZ stained sections

Sections were viewed in light-field using a Zeiss microscope (Axioskope 2 plus) and photographed with an AxioCam HRC (Zeiss). Images were captured using Axiovision software, converted and stored as Adobe Photoshop (CS3) files.

2.9 RT-qPCR

In order to determine the relative levels of gene expression of Shh signaling pathway genes in the Vax1+/− mice at midgestation, tissue from three Vax1 mutant and wildtype developing forebrains and facial area were carefully dissected from E10.5 samples. Immediately, RNA was isolated using the Trizol protocol and quantified using a nanodrop 2000. The RNA was then DNase treated and cDNA was synthesized as per protocol (Quarto et al. 2005). Reactions were
performed on a Rotorgene Q 2-series for 95°C for 10min then cycling at 95° - 15s, 58°C – 30s and 72° - 30s.

2.9.1 Relative quantification of gene expression

Purified and quantified RNA were treated with DNase I (Ambion, Austin, TX) to clear genomic DNA. Three micrograms of total RNA from each clone was reverse transcribed to cDNA using random primer hexamers (Invitrogen, Carlsbad, CA). The reverse transcription proceeded for 1 h at 42 °C, followed by 5 min incubation at 95 °C to inactivate the reverse transcriptase. For quantitative real-time PCR, primers were designed with Primer Express™ software (Applied Biosystems). Each primer was subjected to PCR to ensure single primary amplicon as evidenced by 2% agarose gel electrophoresis to be < 200 bp for maximum efficiency. SYBR® Green PCR Master Mix (Applied Biosystems) was used for fluorescence. Samples along with primers and Syber Green Master Mix (Applied Biosystems, Foster City, CA) were loaded in 384 well sealed plates and the reaction was run in an ABI Prism 7900 HT (Applied Biosystems) as follows: samples were cycled 45 times from 95 °C for 15 s (denaturation) to 61 °C for 1 min (annealing, extension). In addition to experimental samples, a set of negative (minus cDNA) controls were run for each primer and probe combination on each 384 well plate. B-actin (Applied Biosystems) was used for internal control. Standard curve method of quantitation was used to calculate the expression of target genes relative to the house keeping gene B-actin. Four serial dilutions of cDNA (1 : 4) were made for the calibration curve and trend lines were drawn using Ct values versus log of dilutions for each target gene and B-actin run in triplicate with correlation coefficient ($R^2 > 0.99$). Relative expressions were calculated using line equations derived from calibration curves and obtaining ratios of target gene to B-actin. For each gene, experiments were run at least three times using cDNAs obtained from three independent RNA purifications. Similar results were obtained from each experiment.(Longaker et al., 2009 Quarto, et al., 2005) Data was analyzed using the method by Livak and Schmittgen 2001.
2. Materials and Methods

2.10 Cell proliferation and death analysis

2.10.1 Incorporation of BrdU
To determine cell proliferation in palatal tissue of Vax1 heterozygote and mutant mice, a BrdU assay system was used. Pregnant heterozygote Vax1 mice were injected intraperitoneally with BrdU (Sigma, 5mg/100g body weight) and sacrificed two hours after injection. BrdU labelled embryos were isolate and fixed as described previously in this chapter.

2.10.2 Detection of BrdU
The BrdU assay was carried out by analyzing a total of three homozygous and three wildtype Vax1 mice. BrdU is a synthetic nucleoside that is an analogue of thymidine and can then replace thymidine during DNA replication. The Zymed BrdU labeling and detection kit (Invitrogen) was used to identify newly replicated cells that had incorporated BrdU in their DNA. Sections were deparaffinized in two five minute washes in reducing concentrations of ethanol (2x100%, 2x95%, 90%, 80%, 50%, 30%,dH2O) and submerged for ten minutes in quenching solution (10ml 30% H2O2 (BDH), 90ml absolute methanol (Fisher Scientific)). Following incubation, slides were rinsed three times for two minutes in 1XPBS and laid down horizontally in a hybridization chamber. 100μl of denaturing solution (provided by the kit) was spread on slides, incubated for 30 minutes at room temperature and rinsed again three times for two minutes in 1XPBS. Excess PBS was drained off and 100μl of blocking solution (provided by the kit) was applied onto the slides and left incubate for 30 minutes. Following the incubation with the blocking solution, the slides were drained and the solution was replaced by 100μl of biotinylated mouse anti-BrdU (provided by the kit) and left to incorporate for ten minutes. Anti-BrdU was then removed by washing three times for two minutes in 1XPBS and the samples were reacted with 200μl of freshly prepared color-substrate solution, diaminobenzine (DAB), to achieve a blue-black color in the cells that have incorporated BrdU. Throughout the reaction, the slides were protected from light and kept under a fume hood. The progress of the colour reaction was monitored regularly and allowed to develop from five to twenty minutes until specific staining was observed under the microscope. The reaction was stopped by rinsing the samples thoroughly in distilled water. Two drips of haematoxylin was used for counterstaining the samples that was allowed to stain for ten seconds and then rapidly washed off under running tap water. The samples were then
places into 1XPBS and rinsed again in distilled water. Slides were then dehydrated in a graded series of alcohol and left to air dry. The slides were mounted with DePex (BDH) and coverslipped (VWR). All steps were carried out in room temperature.

2.10.3 BrdU analysis

The percentage of BrdU-positive cells was calculated after counting by 1 researcher blinded to experimental group on 2 separate occasions 1 week apart, and the mean value was calculated. A total of 3 WT and 3 Vax1–/– mice were analyzed, which included a total of 50 WT and 45 Vax1–/– sections at E13.5. BrdU-positive cells were counted in the epithelium and mesenchyme of the anterior and posterior palate using an ocular scale grid orientated at the apex of the palatal projection and in the bend region of the palatal shelf (Kim et al., 1998). Specifically, this covered an area of mesenchyme that was 0.03 mm² and 0.015 mm² and bounded by 263-µm and 165-µm lengths of epithelium at the palatal apex and bend, respectively. A Student's t-test was used to analyze the significance of the difference in cell proliferation between the control and mutant mice, assuming a two tailed distribution and unequal variance in these two populations. A probability of \( p=0.05 \) means that there is a 95% chance that the difference between the two mean values is significant. Therefore, a \( P \) value less than 0.05 was considered statistically significant in this study. BrdU-positive cells were also counted in the neuroepithelium of the developing ventral forebrain using an ocular scale grid which covered an area of 0.012 mm² at five anatomically distinct sites, namely: 1, Septal area; 2, Medial ganglionic area; 3, Median eminence; 4, Hypothalamic area and 5, facial mesenchyme.

2.11 Cell death analysis

2.11.1 Apoptosis assay.

The levels of cell apoptosis in similar sections as described in the above section and in chapter 5 were determined using In Situ Death Detection Kit, AP (Roche Diagnostics) that is based on the Terminal transerase0mediated dUTP-biotin Nick End Labeling (TUNEL) method. As cells undergoing apoptosis breakdown their DNA, the terminal transferase attaches nucleotides (sUTP-biotin) to the 3’(-OH) ends of nicked DNA. Apoptosis staining can then be revealed with Converter-AP and detected with NBT/BCIP to convert the signal to visible light. Tissue sections were de-waxed by two fifteen minute immersions in xylene, rehydrated with five minute washes
in a series of ethanol dilutions and with a final wash in 1XPBS for five minutes. Tissue sections were then permeabilised with 10g/ml proteinase K (Sigma) treatment for ten minutes at 37°C. This was followed by two washes in PBS for two minutes each, a five minute wash in 2% H₂O₂ in PBS and a further two five minute washes in PBS. At this stage, the positive control was treated for ten minutes with DNaseI (Promega) to induce DNA strand breaks. The peripheral margins of the samples were then dried and regions around the samples marked with a wax pen. 50µl of TUNEL reaction mixture (50µl enzyme solution and 450µl label solution provided by the kit) was then pipetted on all slides except for the negative control slide, but instead 50µl of label solution was added. Parafilm was used to prevent excessive evaporation of the solution and samples were placed in a humidified (PBS) chamber for sixty minutes in the dark at 37°C. After sixty minutes, the incubation solution was removed by washing the slides three times for two minutes in PBS. Areas around the sample were dried and 50µl of Converter-AP were homogeneously spread on the samples and incubated for thirty minutes in a humidified chamber at 37°C. Slides were then rinsed three times for two minutes in PBS. The final solution of 50-100µl of Substrate solution (Nitro blue tetrazolium chloride (NBT)/5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in 0.1M Tris-HCl, pH9.5, 0.1M NaCl, 0.05M MgCl₂) was pipetted onto the slides and incubated in the dark for ten minutes at room temperature. Finally, the slides were rinsed three times in PBS for two minutes, mounted immediately with AquaMount (VWR) and analyzed under the light microscope.
3 Normal expression of *Vax1* during early craniofacial development

3.1 Introduction

Previous studies of *Vax1* in the mouse have demonstrated widespread expression throughout early and later embryogenesis (Hallonet *et al.*, 1998, 1999). Furthermore, they have also indicated that *Vax1*, unlike *Vax2*, is not restricted to the developing optic system and that it is more likely to have a wider role in embryonic development (Hallonet *et al.*, 1998, 1999; Bharti *et al.*, 2011; Hoffmann *et al.*, 2016). *Vax1* transcripts have been shown to extend to the developing craniofacial structures, including the forebrain and sensory organs (Hallonet *et al.*, 1998, 1999). The composition of *Vax1* and *Vax2* homeodomain sequences suggests that these proteins recognize identical or at least similar target DNA sequences. Sequence similarity also extends outside of the homeodomain. These regions of homology may contribute to biochemical functions (such as DNA binding, transcriptional activation and/or protein–protein interactions) (Ohsaki *et al.*, 1999). We have therefore investigated the normal temporo-spatial expression domains of both *Vax1* and *Vax2* in the developing craniofacial region in detail.

3.2 Materials and methods

The early expression pattern of *Vax1* and *Vax2* was examined from E9-11.5 in wild type mouse embryos using whole-mount digoxigenin-labelled and radioactive section 35S UTP-labeled *in situ* hybridization.
3. Normal expression of Vax1 during early craniofacial development

3.3 Results

3.3.1 Normal expression of Vax1 at E9-9.5

Vax1 expression is first detected at E8.5 (8-12 somites) in the rostral and medial neural plate and anterior neural ridge. At this stage of development, CNCCs have begun to migrate with concomitant enlargement of the anterior neural folds. At E9.5 (21-25 somites) primordial structures such as the prosencephalon, the FNP and mandibular processes as well as the optic, optic and olfactory placodes have begun to develop. At this stage, Vax1 was expressed in the ectoderm in a transverse band between the two olfactory placodes, medially contacting the neural tube (Figure 3.1A-C). Vax1 was also expressed strongly in the ventral optic stalk and retina of the developing eye, with transcripts also seen in the ventral forebrain and in a medially-restricted transverse band of ectoderm situated adjacent to the forebrain (Hallonet et al., 1999). Vax1 transcripts were also detected bilaterally as localized regions of intense expression within the MNP of the early face (Figure 3.1A-C).

Figure 3.1 Expression of Vax1 in the early mouse embryo (E9.5 whole mount digoxigenin in situ hybridization)
Whole-mount digoxigenin-labelled antisense in situ hybridization of Vax1 expression in the early mouse embryo. Lateral (Panels A, B) and frontal (Panel C) views are shown. Frontal view shows dissected head only. The black arrows indicate relevant anatomical structures. ba2, second branchial arch; lb, limb bud; md, mandible; mnp, medial nasal process; op, olfactory placode; tv, telencephalic vesicle; vos, ventral optic stalk. Scale bar 250μm.
3. Normal expression of Vax1 during early craniofacial development

3.3.2 Normal expression of Vax1 at E10.5

At E10.5 (29-35 somites) expression of Vax1 continued in ventral regions of the eye and in the developing forebrain, within the ventral midline and extending laterally. At this stage of development, the FNP has subdivided into MNP and LNP and Vax1 was expressed within the FNP of the early face, supplemented by continued expression bilaterally within the MNPs (Figure 3.2A-C).

![Figure 3.2](image)

**Figure 3.2** Expression of Vax1 in the early mouse embryo (E10.5 wholemount digoxigenin *in situ* hybridization)

Whole-mount digoxigenin-labelled antisense *in situ* hybridization of Vax1 expression in the early mouse embryo. Lateral (Panel D), frontal (Panel E) and fronto-ventral (Panel F) views are shown. The black arrows indicate relevant anatomical structures. ba2, second branchial arch; lb, limb bud; md, mandible; mnp, medial nasal process; op, olfactory placode; tv, telencephalic vesicle; vos, ventral optic stalk. Scale bar 250μm.

β-galactosidase staining in Vax1 heterozygous embryos at similar stages demonstrated comparable temporal areas of Vax1 expression (Figure 3.3A, B).

![Figure 3.3](image)

**Figure 3.3** Expression of Vax1 in the early mouse embryo (E10.5 β-galactosidase staining)

β-galactosidase staining detecting normal expression of Vax1 expression in the early mouse embryo. Lateral (Panel A) and frontal (Panel B) views are shown. The black arrows indicate relevant anatomical structures, mnp, medial nasal process; tv, telencephalic vesicle; vos, ventral optic stalk. Scale bar 250μm.
To further define these expression domains, radioactive *in situ* hybridization was carried out on frontal sections of the developing face and CNS at E10.0 and E10.5, respectively (Figure 3.4-3.6). This revealed clear expression of *Vax1* within the midline ectoderm of the frontonasal region, whilst expression in the MNP was restricted to ectoderm in a region inferior to the base of the nasal pit. There was also strong expression in the developing optic stalk, the outer layer of the optic cup and in the diencephalon.

![Expression of Vax1 in the early mouse embryo (E10.0 section radioactive in situ hybridization)](image)

Figure 3.4  Expression of Vax1 in the early mouse embryo (E10.0 section radioactive in situ hybridization)

Frontal sections E10.5. Panels A, B show strong Vax1 expression in the ectoderm of the medial nasal process below the nasal pit. Panels C, D demonstrate continued expression in the facial midline and optic stalks. di, diencephalon; lnp, lateral nasal process; md, mandibular process; mnp, medial nasal process; oe, oral epithelium; opc, optic cup; os, optic stalk. te, telencephalon Scale bar 250μm.

The expression of *Vax1* remained strong in the developing central nervous system at E10.5, specifically in ventral regions of the developing forebrain. Investigation into the domains of *Vax1* expression in the developing eye, early forebrain and face at E10.5 using sagittal sections through these regions demonstrated a distinct area of *Vax1* expression in the ventral midline, specifically in the septal area (Figure 3.5A-D).
3. Normal expression of Vax1 during early craniofacial development

Figure 3.5  Expression of Vax1 in the early mouse embryo (E10.5 section radioactive in situ hybridization)
Frontal sections E10.5. Panels A,B show strong Vax1 expression in the ectoderm of the medial nasal process below the nasal pit. Panels C,D demonstrate continued expression in the facial midline, optic stalks, and in septal areas. di, diencephalon; ln, lateral frontonasal process; mnp, medial nasal process; oe, oral epithelium; opc, optic cup; os, optic stalk; sa, septal area; te, telencephalon. Scale bar 250 μm.

Figure 3.6  Expression of Vax1 in the early mouse embryo sagittally (E10.5 sectional radioactive in situ hybridization)
Sagittal sections E10.5. Panel A, show strong Vax1 expression in the neuroectoderm of ventral telencephalon and in the optic vesicle. Panels CB and C are continued expression in the facial midline. Scale bar 250μm

3.3.3 Normal expression of Vax1 at E11.5
At E11.5, wholemount in situ hybridization demonstrated some reduction and further ventralization of Vax1 expression in the developing eye, but continued expression in the facial region and central nervous system. In the MNP strong expression continued in a band of ectoderm positioned below the nasal pit, whilst weaker expression remained in the facial midline.
3. Normal expression of Vax1 during early craniofacial development

Figure 3.7  Expression of Vax1 in the early mouse embryo (11.5 wholemount digoxigenin *in situ* hybridization)
Whole-mount digoxigenin-labelled antisense *in situ* hybridization of Vax1 expression in the early mouse embryo. Frontal views (Panel F) show dissected heads only. The black arrows indicate relevant anatomical structures. lb, limb bud; lnp, lateral nasal process; md, mandible; mnp, medial nasal process; mx, maxilla; olp, olfactory placode; ov, otic vesicle; tv, telencephalic vesicle

Figure 3.8  Expression of Vax1 in the early mouse embryo (11.5 β-galactosidase staining)
β-galactosidase staining detecting normal expression of Vax1 expression in the early mouse embryo. Panel A and B show wild type lateral views. Panels C (lateral) and D (frontolateral) demonstrate Vax1 expression in the ventral forebrain. Panel E is a dorsal view demonstrating continued expression in the ventral optic stalk. (Scale bar 250μm. All photos same scale)

Expression continued at this stage in the diencephalon and in the developing vomeronasal organs (VNO) (Figure 3.8).

The domains of Vax1 expression were investigated in the midline of the early forebrain and face at E11.5 using sagittal sections through these regions (Figure 3.9A-E). This demonstrated expression of Vax1 in ventral regions of the telencephalon and diencephalon of the
forebrain (Figure 3.90F-J). *Vax1* was expressed in the median eminence and in the diencephalon adjacent to the adenohypophysis of the pituitary. It was also seen rostrally in the ventricular zone of the medial ganglionic eminence and septal area. *Vax1* was expressed within the early nasal cavity but was restricted to the floor of the medial nasal process, proximal to the region of fusion between medial and lateral nasal processes (Figure 3.90F-J).

![Figure 3.9](image)

**Figure 3.9** Expression of *Vax1* in the early mouse embryo (E11.5 sectional radioactive in situ hybridization)

Frontal sections E10.5. Panels A, B show strong *Vax1* expression in the developing VNO. Panels C, D demonstrate continued expression in the telencephalon. Scale bar 250μm.
Figure 3.10  Expression of Vax1 in the early mouse embryo (E11.5 sectional Dig insitu hybridization)
Sagittal sections E11.5 wild-type Vax1 (Panels F-J) expression in ventral region of the forebrain and nasal cavity. cp, cerebral peduncle; hn, hypothalamic neuroepithelium; if, interpeduncular fossa; ir, infundibular recess; dn, dorsomedial nucleus; me, median eminence; mge, medial ganglionic eminence; ventricular zone; or, optic recess; pit, pituitary (anterior lobe); rp, Rathke's pouch; rn, reticulotegmental nucleus; Sep, septal area. Scale bar 250μm.
3.3.4 Normal expression of Vax1 at E12.5

By 12.5, within the central nervous system Vax1 transcripts were present in the septum, MGE, LGE, and in the preoptic area. In the developing face, Vax1 was also upregulated in the developing VNO situated within the nasal cavity, with expression continuing in the midline ectoderm of the early face at E12.5. There was no evidence of any similar expression in the mandible (Figure 3.11A-D).

The expression of Vax1 was also surveyed at later stages of development between E13.5-14.5 in the developing oral cavity. No evidence of expression was seen in this region, either in the shelves of the secondary palate or the developing teeth (Figure 3.11C, D).

![Image](image_url)

**Figure 3.11** *Expression of Vax1 in the early mouse embryo (E12.5-14.5 sectional Radioactive in situ hybridization)*

Panels A,B: Frontal sections E12.5 wild-type Vax1 expression in the MGE and LGE as well as septal area of the forebrain (Sep). In the nasal cavity, Vax1 is expressed in the VNO and in the oral epithelium (oe). Panels C, E13.5; D, E14.5 Vax1 expression in the optic chiasm (oc), optic stalk (os), and preoptic area (po). No evidence of expression in palatal shelves (ps) or in the dentition. Scale bar 250μm.
3. Normal expression of Vax1 during early craniofacial development

3.3.5 Normal expression of Vax2

Vax2 expression were detected in embryos from E9.5 by whole-mount in situ hybridization. Expression was restricted to the entire inferior neural retina, starting from E9.5 (Figure 3.12A, B), when it is expressed in the ventral half of the optic cup. By E11.5, expression was strong in the ventral half of the optic vesicle, extending from the infero-nasal to the infero-temporal retina, across the ventral furrow (Figure 3.12C-F).

At E10.5, Vax2 expression continued in the optic cup and optic stalk. By E11.5, expression was strong in the ventral half of the optic vesicle, extending from the infero-nasal to the infero-temporal retina, across the ventral furrow (Figure 3.12C-F).

![Figure 3.12](image_url) Expression of Vax2 in the early mouse embryo at E9-5 to E11.5 (Whole-mount Dig in situ hybridization) Whole-mount digoxigenin-labelled antisense in situ hybridization of Vax1 expression in the early mouse embryo. (Panels A-B) E9.5; (Panels C-D) E10.5; (Panels E-F) E11.5. For each stage a sagittal (A, C and E) and a frontal (B, D and F) are shown. M, mesencephalon; nr, neural retina; Oc, optic cup; Rh, rhombencephalon; Tv, telencephalon. Scale bar 250μm.
The domains of Vax2 expression in the developing eye were investigated. Expression of Vax2 was restricted to the eye primordium, the optic vesicles and optic stalks at E9.5 and neural retinae at E10.5 (Figure 3.13A,B). Vax2 was expressed solely in the ventral halves of the optic stalks. Vax2 expression in the optic stalk diminished after E11.5 and became restricted to the inner layer of optic cup in its ventral half (Figure 3.13B,C). As the retina developed, Vax2 expression persisted in the ventral halves of all neural retina at E14.5 (Figure 3.13C).
3. Normal expression of Vax1 during early craniofacial development

3.1 Discussion

*Vax1* has a dynamic expression pattern during the early stages of craniofacial development. Indeed, the expression of *Vax1* extends to the majority of the craniofacial structures including the brain, the sensory organs and the facial processes. As development proceeds, *Vax1* expression is localized in specific areas such as the ventral forebrain as well as the optic and olfactory systems.

*Vax1* is expressed in the neural plate and in the anterior neural ridge during early development and is later confined to the ventral forebrain. *Vax1* expression is first detected at E8.5 in the rostral and medial neural plate and anterior neural ridge. Later, at mid-gestation, the expression remains confined to the ventral forebrain. High sequence homology between *Vax1*, *Not1*, and *Emx* genes suggest that it could function in the specification and formation of forebrain structures (Hallonet *et al.*, 1998). In this study, *Vax1* expression was mapped in detail during murine craniofacial development. Our data demonstrates that *Vax1* is expressed in various craniofacial tissues in the mouse including the developing forebrain, CNS, optic and olfactory systems as well as in the rostral oral epithelium.

An emphasis of this thesis is to investigate the relationship between *Vax1* and *Shh* during embryonic development. The pattern of *Vax1* expression in relation to *Shh* and *Ptch1* will be illustrated in chapter five where it is seen how *Vax1* expression partially overlaps with *Shh* as well as skirt *Ptch1* in the developing forebrain and face. *Shh* is consistently expressed in the ventral midline of the CNS, including the prosencephalon and prechordal plate (Echelard *et al.*, 1993). Significantly, *Vax1* is expressed in the midline at these stages.

Furthermore, it will be shown that *Shh* and *Vax1* are both expressed in the oral ectoderm (albeit, *Vax1* is expressed in the rostral ectoderm only) (Echelard *et al.*, 1993; Hallonet *et al.*, 1998; Rice, 2005). *Shh* is expressed in the epithelial component of the tooth germ, and plays a role in initiating, and morphogenesis of the tooth germ (as reviewed by Cobourne, 2006). During this period Rathke’s pouch invaginates from the oral ectoderm, *Shh* and Vax1 localize to the surrounding oral ectoderm but are absent from the pouch itself (Treier *et al.*, 2001; Bharti *et al.*, 2011). In the developing palate at E12.5-E13.5, *Shh* is strongly expressed in the oral ectoderm. *Vax1* is not expressed in these regions at these stages. *Vax1*−/− mice demonstrate a cleft secondary palate. This suggests an indirect role for *Vax1* in palatogenesis. In the absence of *Shh*, *Vax1* and *Vax2* are not expressed in the eye (Wilson *et al.*, 2004). Analysis of *Vax1* homozygous mutants,
3. Normal expression of Vax1 during early craniofacial development

demonstrated that Vax1 and Pax2 expression in the optic stalk requires Shh (Hallonet et al., 1999). Also, in Xenopus, Shh overexpression leads to dorsal expansion of the Vax2 expression domain (Sasagawa et al., 2002). Vax2 has been thought to play an important role in eye development as indicated in functional studies carried out in frog and chicken (Mui, et al., 2002; Mui et al., 2005).

Craniofacial morphogenesis is driven by the spatio-temporal co-ordination of gene expression, which gives rise to normal pattern formation (Gou et al., 2015). Transcription factors are important components in the regulation of gene expression via the coordination of extracellular and environmental factors and through direct epigenetic modifications (Gou et al., 2015). Vax1 is a homeobox-containing transcription factor, which plays a poorly-understood role in ventralizing the murine forebrain (Hallonet et al., 1998, 1999).

The expression of Vax1 allows speculation for roles in patterning, organ morphogenesis, and tissue differentiation (Hallonet et al., 1999; Taglialatela et al., 2004; Hoffmann et al., 2016). The role of Vax genes in Shh signaling has been previously demonstrated in the eye (Mui et al., 2005), but not been explicitly investigated in the developing forebrain and midface or, indeed as a mouse model of Shh-mediated HPE. Shh produced by the forebrain subsequently induces expression of Shh in the ectoderm of the FNP and MNP of the developing face. Shh produced by these structures is integral to patterning of the neural crest derived facial primordia which lead to specific cartilaginous structures and bones of the craniofacial midline (Schneider et al., 2001, Marcucio et al., 2003; Helms et al., 2005). Vax1 is expressed in similar areas of the forebrain and the midface at these stages. This information combined with previous animal studies would suggest a role of Vax1 as a modifier of HPE within the context of the Shh signaling pathway.

The expression patterns of Shh and Vax1 are intriguing and provide an indication for further studies of roles for Vax1 in craniofacial development and encourage the further investigation of its relationship with Shh. In chapter five, we will further analyze the pattern of Vax1 expression in relation to Shh and Ptch1 as together with other mid-gestational forebrain markers, such as Nkx2.1 and Fgf8 this will aid the understanding of the role of Vax1 during the development of the ventral forebrain, eye and nasal processes.
4. The craniofacial phenotype of Vax1−/− mice

4.1 Introduction

The expression analysis of Vax1 demonstrated the presence of transcripts in primordial structures of the head, including the facial processes, primary sensory placodes (optic and olfactory) and the brain, which was suggestive of multiple roles for Vax1 during craniofacial development. Given the prominent role of Shh signaling in morphogenesis of the head and facial patterning combined with the results of previous animal studies implicating Vax1 as a downstream effector in Shh signaling in the developing forebrain, this prompted an examination of the role of Vax1 in craniofacial development (Hallonet et al., 1999; Takeuchi et al., 2003). This was carried out by analyzing the consequences of a loss of function of this gene using mice with a targeted deletion of Vax1. A skeletal and histological investigation of Vax1−/− embryos from a range of stages was carried out.

4.2 Materials and methods

Vax1 mutant mice were originally generated by introducing a targeted mutation of Vax1 using homologous recombination in embryonic stem (ES) cells (Hallonet et al., 1999). The mutation replaces the amino terminus of the encoded Vax1 protein (including the exon coding for the two first helices and part of the third helix of the Vax1 homeobox) with a β-galactosidase reporter (Le Mouellic et al., 1990; Hallonet et al., 1999).

Vax1+/− mice were generated and maintained in a 129sv/C57BL6 mixed background and genotyped as previously described (Hallonet et al., 1999). Heterozygous mice were viable although hypofertile (Hoffmann et al., 2014) and when mated together produced litters containing the expected genotypes at Mendelian ratios. The Vax1 null allele (Vax1−/−) is therefore not lethal during embryogenesis (Hallonet et al., 1999); however, the majority of Vax1−/− mice on this background die at birth (Hallonet et al., 1999). Homozygous mutants were generated at expected Mendelian ratios at a variety of embryonic stages. All homozygous mutants exhibited craniofacial malformations, including cleft palate, coloboma, which is a failure of the eye to close at the choroid fissure, as well as growth defects in the developing ventral forebrain, areas where Vax1 is normally expressed (Hallonet et al., 1998). A detailed analysis of the craniofacial phenotype was subsequently carried out using histology and skeletal preparation techniques as
4. The craniofacial phenotype of Vax1−/− mice

described elsewhere. Phenotypic comparison of Vax1−/− mice in this study was carried out by using wild type controls.

Figure 4.1  Targeted deletion of murine Vax1

Relative positions of the exons coding for the two first helices (5′HB) and last helix (3′HB) of the homeobox are indicated by vertical bars. The map of the wild-type locus shows the deleted region in red, consisting of the start codon together with the exon coding for the first two helixes and amino-terminal part of the third helix of the homeobox and flanking intronic sequences. The map of the targeting vector shows the replacement of the deleted region by the β-galactosidase–neomycin cassette pGNA (green) (Le Mouellic et al., 1990). (Redrawn from Hallonet et al. 1999).
4.3 Results

Analysis of the craniofacial phenotype was carried out by investigating the gross anatomy at E17.5 using skeletal preparations. Comparison of the external phenotype revealed the facial appearance to be grossly normal with the presence of two external nares (Figure 4.2 A, B). However, the midface appeared truncated with a grossly more triangular appearance and cleft palate was observable through the oral cavity. From the earliest stages of development, the eyes of Vax1 mutants display coloboma, (Figure 4.2 C) (Hallonet et al., 1999).

![Figure 4.2](image)

**Figure 4.2** Gross morphology of Vax1+/+ and Vax1−/− mice at E17.5
A. Wild type, E17.5. B. Vax1 mutant, E17.5. C Vax1 mutant, E12.5; Ocular coloboma (arrow) Scale bar 500μm..

4.3.1 Skeletal analysis of the Vax1−/− craniofacial region

The craniofacial phenotype of Vax1 mutant mice was analyzed using skeletal preparation at E17.5 (n=4) (Figure 4.3, Figure 4.4, Figure 4.5). In general, the mutant skull was slightly smaller than the wild type, with some mid-facial retrusion and medio-lateral widening of the maxillary region, consistent with the gross facial appearance. Whilst the dermatocranium was normal, detailed analysis identified a number of defects associated with the mutant chondrocranium, affecting both neurocranial and splanchnocranial elements. These defects predominated along the ventral midline and varied in severity between mutants. Within the nasal cavity of the mutant, the nasal septum was either hypoplastic or absent, with accompanying hypoplasia of the paraseptal cartilages and vomer. There was a continuous, bilateral cleft of both the primary and secondary palates, seen with complete penetrance and associated with individual defects in the paired maxillary and palatine bones. Within the region of the sphenoooccipital synchondrosis there were also fenestrations associated with the basisphenoid and basisciptal bones (Figure 4.3A-C).
4. The craniofacial phenotype of Vax1<sup>−/−</sup> mice

Figure 4.3  Craniofacial analysis of wild type, heterozygous and Vax1 mutant skulls at E17.5.

Skeletal preparations of the skull differentially stained for bone (alizarin red) and cartilage (alcian blue). (A-C) Lateral (upper panel) and ventral (lower panel) views of wild type (A), heterozygous (B) and Vax1 mutant (C) skulls demonstrate a smaller size and mid-facial retrognathia associated with the mutant. In addition, the presence of only a single maxillary central incisor (red arrow), absence of the nasal septum (green arrow), cleft palate (red *) and fenestrations in the region of the sphenoid-occipital synchondrosis (yellow arrow) were also identifiable in the mutant when compared to heterozygous and wild type mice (note that the squamosal and frontal bones have been partially removed in the mutant (see Figure 4.4 for abbreviations).
Analysis of the middle ear skeletal elements showed them to be normal in the mutant, along with the *pars canalicularis* and *pars cochlearis* (Figure 4.4C, G). Gross morphology of the mutant mandible was also normal, although it was a little smaller than in the wild type (Figure 4.4D, H).

There was a range in the severity of the midline anomalies seen in association with the mutant (Figure 4.4). Specifically, there was a variable circular midline defect affecting the body of the pre-maxilla and associated with a related hypoplasia of the palatal shelves. In some cases, there was the presence of only a single midline maxillary incisor crown within the pre-maxilla. The palatal processes of the maxilla were also hypoplastic and the palatine bone severely disrupted, lacking palatal processes in many cases and exposing the underlying presphenoid.
4. The craniofacial phenotype of Vax1<sup>−/−</sup> mice

which itself also demonstrated a variable level of hypoplasia within the body and lacked medial processes, with the entire bone being essentially absent in the most severe cases.

![Figure 4.5](image)

**Figure 4.5** Spectrum of severity of HPE phenotype in Vax1 mutant.

(A, B) Highlight of the ventral maxillary region (calvarium removed) in the wild type (A) and mutant skulls (B) demonstrating a spectrum of increasing severity associated with the midline anomalies seen in the mutant (indicated by the elongated black arrow). In particular the size of the pre-maxillary midline fenestration (red arrows) and hypoplasia of the palatal processes (violet arrows), revealing a severe disruption and increasing hypoplasia of the palatal processes of the pre-maxilla (orange) arrows and maxilla (pale blue arrows). In addition the body of the palatine bone was also hypoplastic and lacked palatal processes, revealing a severe disruption and increasing hypoplasia associated with the presphenoid (yellow arrows).

In addition, the basisphenoid was wider than in the wild type and fenestrated posteriorly (yellow arrow) (Figure 4.5A, B). Within the cranial base, the basioccipital demonstrated variable anterior fenestration adjacent to the spheno-occipital synchondrosis. The basisphenoid was broader medio-laterally in the mutant and also had fenestration along its posterior border with the spheno-occipital synchondrosis. The presphenoid bone was severely disrupted in the mutant, being hypoplastic, lacking most of the body and whilst the latero-posterior processes were present, there were no defined medial processes.

Figure 4.4 A, B; E, F).
4.3.2 Histological analysis

All Vax1 mutant mice exhibited craniofacial malformations. These included defects in the basal telencephalon as well as cleft palate. Most of these defects occurred in regions where Vax1 is normally expressed -except in the palate (Hallonet et al., 1998; Hallonet et al., 1999; Soria et al., 2004). At earlier stages, it was seen that there was a defect in the septal area tissue at the region of the optic recess (Figure 4.6A, B). A second, rostrally-positioned, pituitary was present with associated thickening of the oral epithelium and a deficient area of mesenchyme (Bharti et al., 2011). At later stages (E13.5) there was a variable deficiency in the growth of the MGE with complete loss of the septal tissue and associated structures such as the preoptic area (Figure 4.6C, D).

Figure 4.6 Deficiencies in the growth of the anterior ventral midline during early and mid gestation

H and E sections. Panels A, B sagittal sections at E11.5 demonstrating presence of a second, more rostrally placed pituitary gland. There is associated thickening of the oral epithelium and concomitant reduction in the size of the mesenchyme. Panels C and D frontal sections at E13.5 demonstrating deficient growth of the mutant ventral forebrain in the septum (sep) and the medial ganglionic eminence (mge). hyp, ventromedial hypothalamic nucleus; lge, lateral ganglionic eminence, mc, median eminence; mge, medial ganglionic eminence; mtb, molar tooth bud; oc, oral cavity; or, optic recess; pit, pituitary; poa, preoptic area; ps, palatal shelf; sp, second (ectopic) pituitary; sep, septal area. Red asterisk indicates cleft palate. Scalbars, Panel B=100μm, Panel D= 500 μm.
At later stages, this midline defect was again identifiable ventrally at the level of the ectopic, second pituitary and continued posteriorly to the missing optic midline structures, such as the optic chiasma (Hallonet et al., 1999) and septal area was associated with variable deficiencies of structures of the cranial base such as the basisphenoid (Figure 4.7A-F). The septum is part of the limbic system and is a telencephalic structure located under the corpus callosum, above the anterior commissure and between the medial walls of the lateral ventricles. Vax1 mutants did not form the septum, in the case of MGE and LGE we observed a lack of separation between these two structures (Taglialatela et al., 2004).

**Figure 4.7** Disruption of the anterior ventral midline at later embryonic stages.

H and E sections (Panels A-D) and mutant skull preparations (E and F). Panel B demonstrates the most anterior point of the septal defect of the midline with an ectopic anterior and inferiorly placed adenohypophysis (ep) and cleft palate. Panel F demonstrates the complete presentation of the midline defects at E17.5 (skull preparations), the pre-maxillary midline fenestration (red arrows) and hypoplasia of the palatal processes (violet arrows), revealing a severe disruption and increasing hypoplasia of the palatal processes of the pre-maxilla (orange arrows) and maxilla. In addition, the body of the palatine bone was also hypoplastic and lacked palatal processes, revealing a severe disruption and increasing hypoplasia associated with the pre-sphenoid (violet arrows) absence of vomer and of the nasal septum, cleft palate and fenestrations in the region of the sphenoid bone synchondrosis (yellow arrow) were also identifiable in the mutant when compared to heterozygous and wild type mice (note that the squamosal and frontal bones have been partially removed in the mutant). as, alisphenoid; bs, basisphenoid; dc, dorsal cortex pmxi, premaxillary incisors; pmx, premaxilla; pmxp, premaxillary processes; pppl, palatal process of palatine; ptg, pterygoid; px, palatine; vm, vomer. Single asterisk indicates the septal defect. Double asterisk indicates cleft palate. Scale bar 250μm.
4. The craniofacial phenotype of Vax1\(^{-/-}\) mice

At stage E14.5 Vax1\(^{-/-}\) mice exhibited defects in the palate (Figure 4.8A-I). All mutants displayed a cleft palate. The palatal shelves appeared to elevate normally (ant, med) but appeared hypoplastic and truncated (Figure 4.8G-I). The apparent medio-lateral widening in the mutant sections may have contributed to the inability of the shelves, which apposite normally, to fuse. The large midface defect was located in the nasal septal area (Figure 4.8G, see also Figure 4.6 and Figure 4.7) and was continuous with the third ventricle and involves the cranial base, the septal area and midline optic structures. Gross examination of the tongue demonstrated no abnormalities, excluding any tongue deformity as a secondary reason for cleft palate formation.

![Figure 4.8](image)

**Figure 4.8** Histological staining of Vax1\(^{-/-}\) and Vax1\(^{+/+}\) palatal sections

The palatal shelves of the Vax1 mutant appear to elevate normally (ant, med) but appear hypoplastic and truncated. Mc, Meckel’s cartilage; mes, mesoepithelial seam; mtb, Molar tooth bud; nc, Nasal cavity; ns, Nasal septum; oc, Optic capsule; ps, Palatal shelf; t, tongue. Scale bar = 250\(\mu\)m.

The Vax1 mutation also affected development of the maxillary incisors which were abnormal or fused (Figure 4.9A, B). The telencephalic phenotype of Vax1 mutants ranged from
4. The craniofacial phenotype of Vax1<sup>−/−</sup> mice

Total absence of growth of medioventral defects such as the septum and preoptic area (Figure 4.6). Figure 4.7).

![Figure 4.9](image)

**Vax1<sup>−/−</sup> single or fused maxillary incisor**

Panel B: red arrow indicating position of single maxillary incisor. Scale bar = 250μm

Whilst some aspects of the craniofacial phenotype such as the septal defect and cleft palate were 100% penetrant, others such as the single incisor ranged from 33%-75%. Other features include a unilateral VNO (Table 1).

<table>
<thead>
<tr>
<th>Embryonic Stage</th>
<th>E12.5</th>
<th>E13.5</th>
<th>E14.5</th>
<th>E15.5</th>
<th>E16.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutants (n)</td>
<td>3</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Cleft secondary palate</td>
<td>N/A</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Fused incisor</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Single VNO</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Septal defect</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 4.1 List of craniofacial defects documented in Vax1 mutant embryos and numbers used

Histological examination also confirmed the presence of lobar HPE in the Vax1 mutant mouse. The Vax1 mutation affected the development of the basal forebrain. A distinct inter-hemispheric
fissure was present; however, some midline continuity of the cingulate gyrus persists (Figure 4.10A, B)

![Image](4.10.jpg)

**Figure 4.10**  
CNS defects in the Vax1 mutant mouse

*Vax1* mutant mouse and wild-type litter mate E15.5. ci, cingulate cortex; cc, corpus callosum; Sa, septal area; tv, Ventricular and subventricular zones, lateral ventricle Scale bar = 250\mu m.

The olfactory bulbs were either absent or hypoplastic in *Vax1* mutant ventral forebrain formation (Figure 4.11A-D).

![Image](4.11.jpg)

**Figure 4.11**  
Hypoplasia of the olfactory bulbs

Sagittal sections through the developing CNS at E15.5 illustrating the absence of an olfactory bulb in the mutant CNS (Panels A-D, arrow denote presence of normally developed olfactory bulb in the wild type brain. Scale bar = 250\mu m.)
4.4 Discussion

*Vax1* plays a poorly-understood role in ventralizing the murine forebrain. *Vax1* is expressed in the neural plate and in the anterior neural ridge during early development and is later in the ventral forebrain, oral epithelium and the MNPs. The skeletal phenotype of *Vax1*−/− mice demonstrated midline defects consistent with lobar HPE- such as fused or single incisors, premaxillary and maxillary hypoplasia, as well as a complete penetrance of cleft secondary palate and anomalies in the pituitary and surrounding cranial base. Gross morphology of the *Vax1*−/− mice demonstrate craniofacial defects from early mid gestation which affect the medio-lateral widths of the midace. Additionally, a spectrum of defects were noted in the development of the ventral forebrain consistent with the HPE phenotype.

Characterization of the craniofacial phenotype has revealed a complete penetrance of cleft secondary palate in the mutant. Furthermore, there was no cleft lip phenotype. Histological examination of *Vax1* mutants during palatogenesis shows apparently normal elevation of the palatal shelves with no gross tongue abnormalities. However, *Vax1* is not expressed in the palatal region and therefore, the cleft phenotype is likely to be secondary to the significant changes seen in the craniofacial skeleton of these mutants. In particular, there is some widening of the basisphenoid and variable abnormalities associated with the presphenoid bones within the cranial base; whilst the nasal septum is absent and there is hypoplasia of the vomer within the nasal cavity. All of these associated structural changes within the craniofacial skeleton may contribute to overall widening of the embryo at the level of the palatal shelves which may lead to clefting within the secondary palate. Interestingly, hypoplasia of the pre-maxillary and maxillary processes and palatal processes of the palatine bones is also seen, which may also be occurring in part due to the change in physical shape and dimensions of this part of developing head rather than just directly due to loss of *Vax1* in these areas.

Furthermore, the absence of a cleft lip phenotype is interesting as, in humans, it has been shown that a *VAX1* mutation is associated with cleft lip (Slavotinek *et al.*, 2012; de Aquino *et al.*, 2013). Lip and palate development depend on the coordination of growth and fusion of distinct facial processes during embryogenesis. Perturbation of this developmental sequence can lead to facial cleft phenotypes (Cobourne, 2004). It is well known that, during facial process fusion, epithelial cells at the tip of each process are eliminated by several mechanisms, including cell
death both in lip and secondary palate fusion and epithelial mesenchymal transition during secondary palate fusion (Cobourne, 2004; Jiang et al., 2006). It has recently been postulated that Vax1 plays a role in murine lip formation (Kurosaka et al., 2014). It was demonstrated that altered Shh signaling contributes to the etiology and pathogenesis of cleft lip through antagonistic interactions with other gene regulatory networks, including the canonical WNT and p63/IRF6 signaling pathways. The mechanisms behind the pathogenesis of cleft lip resulting from altered Shh signaling are still poorly understood as discussed in the introduction. It has been suggested that Vax1, which is expressed normally in the MNP, acts downstream of Shh in this region to help regulate the normal Wnt signaling in this region thus facilitating normal removal of the epithelial seam between the MNP and LNP. The role of Vax1 as a downstream effector of Shh signaling will be discussed in chapter 5. The normal formation of the upper lip in Vax1−/− mice suggests that the absence of Vax1 does not result in cleft lip in mice and in the disruption of the necessary signaling cascades required for epithelial seam breakdown in order to allow formation of a rigid mesenchymal connection in the developing lip and primary palate (Kurosaka et al., 2014).

It also appears that role of Vax1 in craniofacial development is not fundamental to normal development of the CNS, because, unlike Shh−/− mice which demonstrate severe HPE, the forebrain does develop in Vax1 mutant mice, albeit with lobar (rather than alobar) HPE (Hallonet et al., 1999). The mutant embryos showed variable deficient growth of structures expressing Vax1 (Hallonet et al., 1999). Consistent with the skeletal phenotype, there was a wide spectrum of presentation in the Vax1−/− embryo phenotype and a loss of Vax1 did not always result in the same degree of disruption to the midline structures. More specifically, histological examination and analysis showed variable deficient growth of structures expressing Vax1 or the lacZ reporter (Hallonet et al., 1999) in the anterior forebrain, specifically, the MGE, preoptic area, and septum. Medially located structures were more affected than lateral ones. The optic chiasm and preoptic area were systematically absent. The telencephalic phenotype of Vax1 homozygous mutants varied from a total absence of growth of medioventral structures to a growth recovery of dorsolateral structures fusing medially. The deficiencies of the neurocranial midline, which give rise to the base of the skull and derives from the prechordal mesoderm and neural crest (Rossant and Tam, 2004) showed prominent deformities which were highly variable in their presentation.
Previous studies in the eye hypothesized that \( Vax1 \) may have a role downstream of \( Shh \) (Takeuchi et al., 2003; Hallonet et al., 1999). \( Vax1 \) mutant mice demonstrate a range of midline structure anomalies and deficiencies of the developing CNS, including lobar HPE. The wide phenotypic spectrum of presentation in the \( Vax1 \) mutant further suggests a role for \( Vax1 \) as a modifier gene in the spectrum of HPE, as a downstream component of the Shh pathway. Reduced Shh activity as demonstrated in animals (Chiang et al., 1996; Hu and Helms 1999; Cordero et al., 2004; Marcucio et al., 2005) as well as in humans (Belloni, 1995; Roessler et al., 1997; Taniguchi et al., 2012; Allen et al., 2011; Ohkubo et al., 2002) have shown to be associated with a loss of midline tissue that is responsible for a number of characteristic features of HPE.

Few examples of lobar HPE exist in the mouse; however, one such model is the \( Gas1^{-/-}; Boc1^{-/-} \) mutant, which has lobar HPE associated with CL/P, and arrested maxillary incisor development, secondary to reduced Shh transduction in the central nervous system and face. \( Gas1 \) and \( Boc \) encode Shh co-receptors, with \( Gas1 \) acting as a modifier for HPE in its own right, genetically interacting with \( Shh \) by potentiating signaling in the developing face (Seppala et al., 2006). Although \( Gas1 \) mutants only have microform HPE, with an intact central nervous system, combined loss of \( Boc \) produces lobar HPE (Seppala et al., 2013). These mice demonstrate the complex and varying requirements for components of the Shh signaling pathway during early craniofacial development.

Skeletal analysis of \( Vax1^{-/-} \) mice showed for the first time a detailed examination of the association between \( Vax1 \) and morphogenesis of the craniofacial complex. Complete penetrance of the cleft palate can be confirmed to be the cause for the high mortality rate of these mice. \( Vax1^{-/-} \) mice presented a number of serious anomalies that can affect humans and recent studies have confirmed the link between \( VAX1 \) and HPE, non syndromic CL/P and eye related defects such as microphthalmia and colomboma (Slavotinek et al., 2012; de Aquino et al., 2013; Williamson and FitzPatrick, 2014). Phenotypic analysis of \( Vax1^{-/-} \) mice in combination with recent data from human studies has confirmed \( Vax1 \) as a putative candidate gene for a number of human craniofacial anomalies such as solitary median maxillary central incisor (SMMCI), cleft palate, lobar HPE and duplicated pituitary formation.
5 The early craniofacial phenotype of Vax1<sup>−/−</sup> mice

5.1 Introduction

The skeletal analysis had clearly shown that a loss of Vax1 results in significant craniofacial anomalies, which correlate with the observed expression pattern in the craniofacial region. In particular, the defects affecting midline development were suggestive of a reduction in Shh signaling in Vax1<sup>−/−</sup> mice. These findings taken together with previous evidence demonstrating a physical interaction between Shh and Vax1 in the developing eye (Kim and Lemke, 2006; Takeuchi et al., 2003) led us to investigate Shh pathway gene expression during early facial development.

5.2 Results

Comparison of the gross and histological phenotype of Vax1<sup>−/−</sup> embryos with wild type littermates from E10.5 demonstrated variable deficient growth of structures expressing Vax1. This included the ventral anterior forebrain, MGE, preoptic area and septum (Hallonet et al., 1999). Medially located structures were more affected than lateral ones. The optic chiasm and preoptic area were systematically absent.

5.2.1 Normal expression of Vax1 and Shh in the craniofacial midline

Analysis of Shh and Vax1 expression in the craniofacial region demonstrated similar, partially overlapping areas of expression in the forebrain and developing facial processes at E11.5. Shh transcripts were present in the ventral neuroectoderm of the diencephalon; specifically, the median eminence, optic recess and in the dorsal half of the MGE. Shh was also evident in oral ectoderm of the maxillary and mandibular processes. Vax1 is also expressed in the oral ectoderm at this stage. In the developing nasal cavity, Shh is expressed proximally, at the area of fusion of the MNP and LNP. Vax1 showed similar areas of expression in the ventral forebrain; however, expression extended ventrally to the septal area and across the outer cell layers of the entire MGE. Extensive expression was evident around the optic area in both the neuroectoderm and underlying mesenchyme. In the nasal cavity, there was also expression around the developing VNO.
5. Normal expression of Vax1 during early craniofacial development

Figure 5.1  
Expression of *Vax1* and *Shh* in the early mouse embryo (E11.5 sectional Dig in situ hybridization)

Sagittal sections E11.5 wild-type *Vax1* (Panels F-J) and *Shh* (Panels K-O) expression in ventral region of the forebrain and nasal cavity. cp, cerebral peduncle; hn, hypothalamic neuroepithelium; if, interpeduncular fossa; ir, infundibular recess; dn, dorsomedial nucleus; me, median eminence mge, medial ganglionic eminence ventricular zone; or, optic recess; pit, pituitary (anterior lobe); rp, Rathke's pouch; rn, reticulotegmental nucleus; Sep, septal area Scale bar 250μm.
5.2.2 Normal expression of Vax1, Shh and Ptc1 in the craniofacial midline

We also investigated expression of the Shh receptor and downstream signaling target, Ptc1 (Roessler et al., 2003) and compared these domains to those of Shh and Vax1. Ptc1 is expressed in overlapping areas of both Shh and Vax1 during midgestation in the developing forebrain with a decreasing gradient of transcriptional activity in the field of responding cells. Specifically, Ptc1 is continuously expressed throughout the developing ventral forebrain and surrounding mesenchyme rostral to the developing forebrain and adjacent to the developing facial structures as well as the developing visual and olfactory systems (Figure 5.2).

![Figure 5.2](image)

**Figure 5.2** Expression of Vax1, Shh, and Ptc1 in the early mouse embryo (E10.5 wholemount digoxigenin in situ hybridization)

Whole-mount digoxigenin-labelled antisense *in situ* hybridization of Vax1 expression in the early mouse embryos. Sagittal views show Shh pathway gene expression in the craniofacial region. Distinct areas of Shh expression are evident in the developing ventral forebrain and optic recess which partially overlap Vax1 expression in the ventral NE whereas Ptc1 is ubiquitously co-expressed ventrally with both Shh and Vax1 as well as in the underlying mesenchyme in the facial midline and oral ectoderm. fp, frontal process; MGE, medial ganglionic eminence; or, optic recess; sep, septal area. Scale bar =250μm.

It has previously been demonstrated that a loss of Shh signaling in the developing forebrain results in a wide range of craniofacial defects which is dependent on both time of loss and by degree of loss of signaling- i.e. the spatiotemporal impact of this loss (Chiang et al., 1996; Helms et al., 1997). Furthermore, subtle reductions in signaling results in lesser defects
5. The early craniofacial phenotype of Vax1^{−/−} mice

which are in turn further affected by the timing of these reductions (Helms et al., 1997; Cordero et al., 2005). Therefore it can be postulated that Vax1 may have a role as a mediator of Shh signaling during mid gestational development of the forebrain.

5.2.3 Craniofacial dimensions of Vax1^{−/−} mutant embryos

Skeletal characterisation of Vax1^{−/−} mice revealed a range of craniofacial defects. Gross anatomical observations displayed a significant reduction in size of Vax1^{−/−} embryonic heads. In particular, the anteroposterior dimensions appeared shortened with a noticeable widening and truncation of the midface. As it has been previously reported, craniofacial defects are first observed at E10.5 in the Vax1^{−/−} mouse (Hallonet et al., 1999). This period coincides with large morphological changes in the developing prosencephalon; the forebrain and facial area. Vax1 and Shh are expressed in these areas throughout this period and it has been well documented that aberrations in Shh signaling at this stage are responsible for a wide range of craniofacial defects (Helms et al., 1997; Hu and Marcucio, 2009). Further investigation of these dimensional changes were thus necessary and were carried out beginning at E10.5 to assess the degree of change at this early stage and the relative size reductions within the developing Vax1^{−/−} prenatal skull.

In order to quantify the relative changes in sizes of the Vax1^{−/−} mutant heads, we began by measuring a range of distances between clearly defined gross anatomical points on the mouse skulls at E10.5, the stage at which craniofacial defects were first observed and at which, Vax1 and Shh are clearly expressed This was carried out by measuring a series of distances between frontal and sagittal anatomical landmarks in mutant (n=3) and wild type (n=6). Statistical analysis was carried out using comparison of mean distances and utilising Student's t-test, which demonstrated a significant reduction in size of the Vax1^{−/−} embryos (Figure 5.3).
5. The early craniofacial phenotype of Vax1°/° mice

Further measurements were then carried out on histological midline sagittal sections of both Vax1°/° and Vax1°/+ heads at E10.5 (mutant n=3, wildtype n=6) and at E11.5 (mutant n=4, wildtype n=6) (Figure 5.4). The measurements were taken between clearly defined anatomic landmarks (Schambra, 2008). Four measurements were taken, one corresponding to the anteroposterior length of the developing skull, whilst the other three measured a range of distances within the developing forebrain and midface. For each sample, two adjacent sections were measured. Statistical analysis was carried out plotting mean distances utilising Student’s t-test. Results demonstrated a clear reduction in size of the Vax1°/° embryonic forebrain at both of these stages more specifically, in the ventral forebrain and in the facial processes. This confirmed...
the dimensional changes observed in the skeletal analysis and demonstrated a marked difference in size at this early stage with a particular emphasis on the reduction in size of the diencephalonic forebrain and facial processes.

Figure 5.4  Craniofacial measurements of Vax1−/− and wild-type heads at E10.5 (histological measurements).

The panels on the top show E11.5 embryonic mouse skull histological slide sections of both wild type (A) and Vax1−/− (B) at the craniofacial midline. Four lines were constructed and measurements were taken in millimetres. Lines were constructed from most anterior point of the isthmus [1] to the hypothalamus [2]; from [1] to the most anterior point of the septum [3]; from [3] to the optic recess [4] and from [4] to the most anterior-superior point at the fronto-nasal region [5]. For each measurement a Student’s t-test was performed. The diagrams demonstrate the results of the statistical analysis for each line between the mutant embryo and the wildtype at both E10.5. The y-axes represent the distances in millimetres error bars represent standard deviation, one asterisk indicates significant finding (P<0.05).

Following this, in order to investigate any dimensional change around the secondary palate, further anatomical points were measured via midline sagittal sections of both Vax1−/− and Vax1+/+ skulls at E13.5 (mutant n=3, wildtype n=3). The measurements were taken between clearly defined anatomic landmarks of the mouse skull utilizing the widest lateral points at the level of the palatal shelves to determine overall width of the embryo at this point and measured
in millimetres (Schambra, 2008). The palatal shelves were also measured. The lateral extent of the palate shelf was determined by drawing a perpendicular line from the “hinge” region to the opposite palatal surface. The medial aspect of the palate shelf was defined as the level of the medial edge epithelium or the fusion plane of the palate. To account for palate orientation, all measurements were performed with the palate in a horizontal position, by rotating the unelevated palate shelf images to a horizontal plane. (Goudy et al., 2000.) For each sample, two adjacent sections were measured. Statistical analysis was carried out utilising Student's t-test. Results demonstrated a relative increase in width of the Vax1−/−embryonic skull at the plane of the palatal shelves at E13.5. There was no significant difference in sizes in the palatal shelves between the Vax1−/−samples and control. This confirmed the dimensional changes observed in the skeletal analysis. The palatal shelves rise normally and are not obstructed by the tongue. The shelves appear normal but the overall anatomical width of the Vax1−/−embryonic skull at the plane of the palatal shelves at this stage is wider which would indicate that the lack of fusion and therefore the reason behind the cleft palate would be due to the increased distance between the opposing palatal shelves preventing apposition and subsequent fusion.

Figure 5.5  Craniofacial measurements of Vax1−/− and wild-type skulls at E13.5 (histological measurements)
The panels on the left show E10.5 embryonic mouse skull histological slide sections of both Vax1−/−and wildtype at the craniofacial midline at E13.5 at the coronal level of the palatal shelves. Mutant embryos n=3, wild-type embryos n=3. Measurements in mm. For each measurement a Student's t-test was performed. The graphs below demonstrate the results of the statistical analysis for each line (A,B,C, and D) between the mutant embryo and the wildtype at E13.5. The y-axes represent the distances in millimetres error bars represent standard deviation, one asterisk indicates significant finding (P<0.05). Scale bar =500 μm.
5. The early craniofacial phenotype of Vax1\(^{-}\) mice

5.2.4 Proliferation analysis in the developing palate of Vax1\(^{-}\) mice

Reduced mitogenic activity within the palatal shelves is one of the commonest reasons for failure of the palatal shelves to approximate in the midline during development. Proliferative levels within these regions were therefore investigated by performing BrDU assay prior to elevation of the palatal shelves at E13.5. Three wildtype and three Vax1\(^{-}\) mice were included in the study and BrDU stained cells were counted out of the total number of cells separately for mesenchyme and epithelium of both the apex region of the palatal shelves and the bend region between the palatal shelves. As heterogeneity in the molecular mechanisms involved in anterior and posterior palatal development has been previously described (Hilliard et al. 2005) anterior, medial and posterior regions of the palatal shelves were analyzed using Student's T-test and a finding with \(P<0.05\) was considered statistically significant. There were no significant differences in epithelial or mesenchymal proliferation indices between wildtype and Vax1\(^{-}\) at E13.5 (Figure 5.6).

![Figure 5.6](image-url)  
**(A–B)** BrDU analysis of E13.5 (A) WT and (B) Vax1\(^{-}\) palatal shelves. BrDU-positive cells were counted in the mesenchyme and epithelium of the middle palatal shelf. For mesenchyme, counting was undertaken within an ocular scale grid (black box) orientated at the apex (lower box: 0.03 mm\(^2\)) and bend (upper box: 0.015 mm\(^2\)) regions. For epithelium, counting was undertaken within 263 μm and 165 μm lengths of epithelium at the palatal apex (lower dotted line) and bend (upper dotted line), respectively. (C, D) Percentage BrDU incorporation for (C) epithelium and (D) mesenchyme. A total of 3 WT and 3 Vax1\(^{-}\) mice were analysed. Student’s \(t\) tests were performed to assess whether the means between groups were statistically different from each other. Data is represented as mean and standard deviation. \(P\) values of less than 0.05 were considered statistically significant. Proliferation rates were not statistically different for either region. Scale bar 100μm.
5.2.5 *Proliferation analysis in the anterior forebrain and facial mesenchyme of Vax1<sup>−/−</sup> mice*

In order to further elucidate the basis of this reduction of ventral forebrain size during early development in the *Vax1<sup>−/−</sup>* mouse, the levels of proliferation in this region were investigated by performing a BrdU assay during E10.5 to ascertain mitogenic activity within the developing forebrain.

Three control and three *Vax1<sup>−/−</sup>* mice from both stages were included in the study and BrdU-stained cells were counted out of the total cells in the neuroectoderm at four anatomically distinct sites in the forebrain-neuroectoderm, which normally demonstrate *Shh* and *Vax1* expression (Schambra, 2008). Comparison of E10.5 in control and *Vax1<sup>−/−</sup>* forebrain areas showed very significantly reduced levels of proliferation in the neuroectoderm (Figure 5.7).
5. The early craniofacial phenotype of Vax1<sup>−/−</sup> mice

5.2.6 Levels of cell death in the anterior forebrain of the Vax1<sup>−/−</sup> mouse

The ventral forebrain phenotype of Vax1<sup>−/−</sup> mutant mice was investigated further by analyzing cell apoptosis in the neuroepithelial tissue of the developing forebrain in the same areas measured in the cell proliferation assay at both E10.5 and E11.5 using TUNEL staining. Low levels of cell death took place in both mutant and wild type mice. However, there were no significant differences in the levels of cell death within the forebrain areas when comparison was made between control and Vax1<sup>−/−</sup> mice (Figure 5.8).
5. The early craniofacial phenotype of Vax1<sup>+/+</sup> mice

Figure 5.8  Cell death in the developing forebrain of the Vax1<sup>+/+</sup> and Vax1<sup>-/-</sup> mice at E10.5 and E11.5
Panel A demonstrates the area of expression of Vax1 in a sagittal section of a wild-type sample. Panel B demonstrates the areas of expression of Shh in a sagittal section of a wild-type sample. Numbers 1-4 illustrate the four areas analysed for cell death. 1, septal area; 2, MGE area; 3, Median Eminence; 4, Hypothalamic area. Panel C and D show TUNEL stained samples of both Vax1<sup>+/+</sup> and Vax1<sup>-/-</sup> mice at E10.5 and E11.5. No significant differences were demonstrated between mutant and control samples at both stages (n=4 for both mutant and for wildtype).

5.2.7 Comparison of Shh signaling activity in wild type and Vax1<sup>-/-</sup> mice
Shh signaling activity was investigated in both the developing ventral forebrain and facial processes by analyzing expression of Shh and Ptch1. Shh produced by the prechordal mesendoderm (PCM) is necessary to initiate the midline of the forebrain and midface to develop (Rubenstein and Beachy, 1998; Muenke and Cohen, 2000; Kiecker and Niehrs, 2001). This occurs by a progressive mechanism where Shh produced by one midline structure induces Shh expression in a successive midline structure. As the embryo develops, Shh produced by the forebrain induces Shh and Ptch1 expression in the ectoderm of the FNP and MXP. The Shh produced by these structures then pattern neural-crest-derived facial primordia to produce
specific bones and cartilage of the craniofacial midline (Hu and Helms 1999; Hu et al., 2003; Helms et al., 2005; Marcucio et al., 2005).

At E10 and E10.5 in the developing midline, *Shh* is expressed in the hypothalamic neuroepithelium and in the developing ventral telencephalon around the developing optic area and MGE. Mild localised reduction in expression were noted rostrally to the optic area around the caudal area of the MGE this was confirmed by radioactive *in situ* hybridisation (Figure 5.9).

![Figure 5.9](image)

Expression of *Shh* in the Vax1 early mouse embryo (E10.5 sectional radioactive in situ hybridization)

Comparative sagittal sections E10.5. Panels A, B show strong *Shh* expression in the telencephalon with reduced expression more ventrally around the optic recess, medial ganglionic eminence (black arrow) Scale bar 250μm..

Further to this, the expression of *Shh* and *Ptch1* was examined in the developing forebrain at the same stages (E10.0 and E10.5) which confirmed both the previous analysis as well as demonstrating clear reduction in *Ptch1* expression (Figure 5.10).
5. The early craniofacial phenotype of Vax1<sup>−/−</sup> mice

Figure 5.10  Expression of Shh in the Vax1 early mouse embryo (E10.0 sectional digoxigenin in situ hybridization)
Sagittal sections E10.0 Panels A,B show strong Shh expression in the telencephalon with reduced expression more ventrally around the optic recess, MGE (black arrow) Scale bar 250μm.

Figure 5.11  Expression of Ptch1 in the Vax1 early mouse embryo (E10.0 sectional Dioxigenin in situ hybridization)
Sagittal sections E10.0 Panels A-D show Ptch1 expression in the telencephalon, around the optic recess, and in underlying menchyme which shows a marked reduction in expression in the Vax1<sup>−/−</sup> embryo Scale bar 250μm.

This was repeated in several embryos and confirmed in separate in situ experiments demonstrating a clear localised reduction in expression of Shh in the ventral forebrain as well as a generalised reduction in Ptch1 expression throughout the ventral telencephalon ectoderm and mesenchyme (Figure 5.10, Figure 5.11 and Figure 5.12).
5. The early craniofacial phenotype of Vax1<sup>−/−</sup> mice

Figure 5.12  Comparison of Shh and Ptc1 expression in Vax1<sup>−/−</sup> and Vax1<sup>+/+</sup> forebrain and nasal processes (E10.5 sectional digoxigenin in situ hybridization)

Sagittal serial sections E10.5 further demonstrating both Shh and Ptc1 expression in the telencephalon, with reduced Shh expression in the MGE (black arrow) and a generalised reduction of Ptc1 expression in the ventral forebrain and developing facial processes in the Vax1<sup>−/−</sup> mouse. Scale bar 250μm.e.

This pattern of expression continued to later stages at E11.5, with marked reduction of Shh expression in the MGE and septal area. Ptc1 expression is also markedly reduced later at E11.5 around the neuroectoderm and underlying mesenchyme of the diencephalon, adenohypophysis and midface (Figure 5.15).
5. The early craniofacial phenotype of Vax1<sup>+/−</sup> mice

Figure 5.13 Comparison of Shh expression in Vax1<sup>+/−</sup> and Vax1<sup>+/+</sup> forebrain (E11.5 sectional digoxigenin in situ hybridization)
Sagittal serial sections of Vax1<sup>+/−</sup> (panels A and B) and Vax1<sup>+/+</sup> (Panels C and D) at stage E11.5. Comparison of expression demonstrates ongoing reduction in localized Shh expression in the ventral forebrain. MGE; medial ganglionic eminence; Sep, septal area Scale bar 250μm.

These findings indicate down regulation of Shh signaling during mid gestation- which would result in defective formation of the anterior ventral forebrain, facial processes and facial sensory organs such as the eye and nose.

Figure 5.14 Comparison of Shh and Ptc1 expression in Vax1<sup>+/−</sup> and Vax1<sup>+/+</sup> forebrain (E12.5 sectional digoxigenin in situ hybridization)
Frontal sections E12.5 demonstrating reduced expression in both Shh and Ptc1 expression in the ventral forebrain, in the Vax1<sup>+/−</sup> mouse compared to Vax1<sup>+/+</sup>. LGE; lateral ganglionic eminence, MGE; medial ganglionic eminence; POA; preoptic area; sp, second pituitary Scale bar 250μm.
5. The early craniofacial phenotype of Vax1<sup>−/−</sup> mice

The early craniofacial phenotype of Vax1<sup>−/−</sup> mice is characterized by a generalised reduction of Ptch1 expression in the anterior forebrain and midface. This reduction is observed in sagittal sections of Vax1<sup>+/+</sup> and Vax1<sup>−/−</sup> forebrain at stage E11.5. Black arrows indicate areas of marked reduction in Ptch1 expression around median eminence, optic area, adeno-hypophysis, and ectopic adeno-hypophysis of the Vax1<sup>−/−</sup> mice. Scale bar 250 μm.

5.2.8 Ectopic Pituitary formation in Vax1<sup>−/−</sup> mice

The pituitary gland is an endocrine organ that is developmentally derived from a fold in the oral ectoderm and a juxtaposed fold in the neural ectoderm. In Vax1<sup>−/−</sup> mice, the rostral oral ectoderm forms an ectopic fold that eventually develops into a separate second pituitary with all the pituitary cell types and neuronal fibers characteristic of the normal pituitary (Bharti et al., 2011). The induction of the second pituitary is associated with a localized ectopic expression of Fgf10, a growth factor known to recruit oral ectodermal cells into the pituitary. Moreover, Shh, which normally is excluded from the invaginating primary pouch, was also excluded from the second pouch (Figure 5.13.D) but was present in the remainder of the rostral oral ectoderm, with reduced expression of Ptch1 in the adjacent mesenchyme (Figure 5.15). Interestingly, in the aforementioned study, it was believed that Vax1 was not expressed in the oral ectoderm. However, from the Vax1 expression study and in other studies (Hallonet et al., 1998), we know Vax1 to be expressed in the oral ectoderm during formation of Rathke's pouch and it may be suggested that Vax1 has a more direct role during its' formation.
5.2.9 *Incisor Fusion in Vax1<sup>−/−</sup> mice*

Coronal sections at E12.5 in *Vax1<sup>−/−</sup>* embryos showed a narrowing and truncation of developing face as confirmed previously (Figure 5.11). There was also some accompanying disorganization of the nasal tissue and septum. Early thickenings of oral ectoderm, giving rise to dental placodes are demarcated by well restricted expression of *Shh* at E12.5. In the FNP of control embryos, the dental laminae of the premaxillary incisors were separated by a field of non-*Shh* expressing epithelium (Figure 5.16). In contrast, in the *Vax<sup>−/−</sup>* mutants, *Shh* expression revealed a single enlarged expression domain, demonstrating fusion of the incisor placodes. Furthermore, *Shh* expression was reduced in the nasal capsule. *Ptc<sub>1</sub>* expression was also reduced in the mesenchyme and did not extend to the nasal capsule as seen in the *Vax1<sup>+/+</sup>*. As a result, this reduced Shh signaling has resulted in a loss of midline tissue with failed separation of the incisor tooth buds.

![Image](image_url)

**Figure 5.16** Comparison of *Vax1<sup>+/+</sup>* and *Vax1<sup>−/−</sup>* mouse midline development at E12.5

Frontal sections demonstrating expression of *Shh* and *Ptc<sub>1</sub>* in the frontonasal process (C, D, G, and H). At this early stage, loss of midline tissue was evident in the mutant and resulted in narrowing and disruption of the nasal septum. Shh expression marks the two dental lamina giving rise to the premaxillary incisors in the wildtype (D). In contrast, a single *Shh* expression domain expanding over the midline was evident in the *Vax1<sup>−/−</sup>* mutant resulting in a single or fused incisor placode (dli) (F). At the same stage *Ptc<sub>1</sub>* transcripts did not extend as far dorsally and were confined to the proximity of the *Shh* expression domain (G). Furthermore, there was an absence of both *Shh* and *Ptc<sub>1</sub>* expression in the base of the developing nasal cavity (G, H). dli, dental lamina of the premaxillary incisors; fnp, frontonasal process; fvb, follicle of the vibrissae; nc, nasal cavity; ns, nasal septum. Scale bar = 250μm.
5. The early craniofacial phenotype of Vax1\(^{-/-}\) mice

5.2.10 mRNA levels of Shh and Ptch1 in Vax1\(^{-/-}\) embryonic heads

In order to confirm reduced Shh signaling in Vax1\(^{-/-}\), we performed qPCR analysis of the levels of mRNA expression in Vax1\(^{-/-}\) skulls at E10.5 (Livak and Schmittgen, 2001). Results demonstrate significant reductions in levels of Shh and Ptch1 at this stage confirming the results of our in situ data.

![Figure 5.17 mRNA analysis of Shh and Ptch1 expression in Vax1\(^{+/+}\) and Vax1\(^{-/-}\) embryonic heads at E10.5](image)

RT-PCR (qPCR) was performed (A). Shh expression. (B) Ptch1 expression. Mutant embryos n=3, wild-type embryos n=3. Samples were analysed as outlined by Livak and Schmittgen (2001). Statistical significance was ascertained by Student's t-test. The y-axes represent the fold change relative to the housekeeping gene β-actin. Error bars represent standard deviation, one asterisk indicates significant finding (P<0.05).

5.2.11 Fgf8 and Nkx2.1 expression in the facial midline

Recent studies have revealed the principles that regulate the development of the anterior forebrain at early stages of embryogenesis. There is a highly coordinate interplay of signaling centers during normal morphogenesis of the embryonic head that guards the development of the anterior forebrain and the face (Aoto et al., 2002; Ohkubo et al., 2002). Furthermore, Fgf8 and Shh direct and dictate dorsoventral patterning, cell proliferation and death in the developing forebrain (Fernandes and Hébert 2008; Ohkubo et al., 2002; Wilson and Houart 2004). As the embryo develops, Shh produced by the forebrain induces Shh in the ectoderm of the FNP and MXP of the developing face. In the developing forebrain, Shh induces the expression of Nkx2.1 (Shimamura and Rubenstein, 1997) whose function is required for the formation of the ventral forebrain (Sussel et al., 1999).
5. The early craniofacial phenotype of Vax1<sup>−/−</sup> mice

![Figure 5.18 Comparison of expression of Shh, Fgf8, and Nkx2.1 in the Vax1 early mouse embryo (E10.5 sectional radioactive in situ hybridization)](image)

Sagittal sections E10.5. Panels A and D show strong Shh expression in the telencephalon with reduced expression more ventrally around the optic recess, medial ganglionic eminence as previously demonstrated. Panels B and E demonstrate reduced Fgf8 expression caudally in the diencephalic neuroectoderm and more rostrally in the septal areas. Panels C and F display a similar mild reduction in expression to Shh in the medial ganglionic eminence. Scale bar 250μm.

Comparison of early Fgf8 expression in Vax1<sup>+/+</sup> and Vax1<sup>−/−</sup> developing forebrain and facial processes demonstrate a reduction in expression in the developing ventral forebrain (Figure 5.18). Similarly, the reduction of the expression of Nkx2.1 in absence of functional Vax1 during ventral forebrain development suggests that Vax1 could affect the regulation of Nkx2.1 expression. (Figure 5.18 and Figure 5.19). These alterations in gene expression in the Vax1<sup>−/−</sup> ventral forebrain midline and facial primordia demonstrate the effect that loss of Vax1 has on the Shh signaling pathway and related forebrain regulatory genes at midgestation.
5. The early craniofacial phenotype of Vax1<sup>−/−</sup> mice

Figure 5.19  
Comparison of expression of Shh, Fgf8, Ptc1 and Nkx2.1 in the Vax1 early mouse embryo (E10.5 sectional Dioxygenin in situ hybridization)

Sagittal serial sections E10.5. Upper montage of each gene expression montage illustrates expression of said gene in a Vax1<sup>+/+</sup> embryonic head in the midline, the lower montage shows the Vax1<sup>−/−</sup> equivalent. As previously demonstrated, Shh is expressed in the telencephalon with reduced expression more ventrally around the optic recess, medial ganglionic eminence. Fgf8 expression is reduced caudally in the diencephalic neuroectoderm and more rostrally in the septal areas. Ptc1 is clearly downregulated throughout the developing forebrain and face. Nkx2.1 display a similar mild reduction expression to Shh in the medial ganglionic eminence Scale bar 100μm.
5.3 Discussion

*Vax1* is expressed in the neural plate and in the anterior neural ridge during early development and is later confined to the ventral forebrain; however, this early role is not fundamental to normal development of the CNS, as the forebrain does develop in *Vax1* mutant mice, albeit with lobar (rather than the more severe alobar phenotype) HPE (Hallonet *et al.*, 1999). These mutants also exhibit a range of midline and craniofacial abnormalities, including cleft palate, malformed maxillary incisors and duplication of the developing pituitary gland, but the basis of the *Vax1* craniofacial phenotype has not been formally investigated.

Shh signaling is a major factor in HPE development. Model organisms such as mouse and chick demonstrate that *Shh* produced by the PCM is necessary to initiate the midline of the forebrain and midface to develop (Rubenstein and Beachy, 1998; Muenke and Cohen, 2000; Kiecker and Niehrs, 2001). This occurs by a progressive mechanism where *Shh* produced by one midline structure induces *Shh* expression in a successive midline structure. *Shh* produced by the PCM induces expression of pathway target genes in the rostral diencephalon ventral midline of the developing forebrain, including *Ptc1, Gli1* and *Shh* (Rubenstein and Beachy, 1998; McMahon *et al.*, 2003; Cordero *et al.*, 2004; Marcucio *et al.*, 2005; Aoto *et al.*, 2008; Geng and Oliver, 2009). The relationship between *Vax1* and Shh signaling may provide an important insight into the role of *Vax1* in lobar HPE and its role within craniofacial development.

Characterization of the craniofacial phenotype has revealed a generalized reduction in the overall dimensions of the *Vax1<sup>−/−</sup>* head from E10.5 which is most significant at the ventral forebrain and midface. This period of development marks a significant point in the development of the facial primordia and midline structures. Furthermore, cell proliferation is markedly reduced in the same regions of the ventral forebrain.

*VAX1* is a candidate CLP gene (Slavotinek *et al.*, 2012; Butali *et al.*, 2013; de Aquino *et al.*, 2013; Zawislak *et al.*, 2014; de Araujo *et al.*, 2016; Gowans *et al.*, 2016; Peng *et al.*, 2016; Wang *et al.*, 2016; Wen *et al.*, 2016;) and loss-of-function mutation has been associated with microphthalmia, optic nerve hypoplasia and absence of the corpus callosum (Slavotinek *et al.*, 2012). There was no evidence of *Vax1* expression in the developing palate and no difference in the gross anatomy or levels of proliferation in the palatal shelves between wildtype and mutant. There is a complete penetrance of secondary cleft palate in the mutant. Histological examination
of Vax1 mutants during palatogenesis shows apparently normal elevation of the palatal shelves (which are of typical length) with no gross tongue abnormalities. Significantly, the observed CP appears to be a consequence of the associated gross craniofacial defects, rather than a direct effect of Vax1 function. It is most likely that a large midline cavity within the CNS, extending from the floor of the hypothalamus through the nasal cavity to the roof of the oral cavity, was responsible for the failure of the palatal shelves to approximate in the midline. This structure has been described previously and ascribed to the presence of CP, but is most likely the cause of this defect (Bertuzzi et al. 1999).

It has been demonstrated that Vax1 and Shh have adjacent and partially overlapping domains of expression in the normally developing forebrain and facial primordia. Given the evidence that Vax1 is a downstream target of Shh in the developing eye and face it is likely that Vax1 may play a role as a modifier gene in the spectrum of HPE, as a downstream component of the Shh pathway (Hallonet et al., 1999; Ohsaki et al., 1999; Takeuchi et al., 2003). It has been demonstrated in the Vax1 mutant forebrain at E10.5 and E11.5 that there is highly localised down regulation of Shh (which, in the forebrain and FNP, is induced and maintained by different regions of Shh expression) in the developing forebrain with a concomitant generalised down regulation of Ptch1 throughout the developing midline in both the neural ectoderm and underlying mesenchyme. Adjacent and overlapping areas of expression of Vax1 in the ventral diencephalon, and later telencephalon, with Shh suggest a role for Vax1 in mediating Shh signaling at different stages of development of the ventral forebrain and later during midline development of the FNP. As the embryo develops, Shh produced by the forebrain induces Shh in the ectoderm of the FNP and MXP of the developing face. Loss of Vax1 in the ventral forebrain and rostral oral ectoderm results in a loss of midline tissue and in fused central incisors. Quantitative analysis of Shh signaling in the Vax1 mutant forebrain utilizing RT-PCR (qPCR) confirms the reduction of mRNA levels of Shh and Ptch1 at E10.5.

Down regulation of Fgf8 in the ventral forebrain has been demonstrated at E10.5. Shh produced by the rostral diencephalon ventral midline is required for the maintenance of Fgf8 expression in the rostral commisural plate (Aoto et al., 2002; Ohkubo et al., 2002). Fgf8 and Shh together with dorsally derived bone morphogenetic proteins (BMPs), direct and dictate
The early phenotypic and expression studies demonstrated that changes in Shh signaling underlie various craniofacial defects evident in the Vax1−/− embryos resulting in lobar HPE. Few examples of lobar HPE exist in the mouse; however, one such model is the Gas1−/−; Boc1−/− compound mutant, which demonstrates lobar HPE associated with CL/P, and arrested maxillary incisor development, secondary to reduced Shh transduction in the central nervous system and face. Interestingly, Gas1 and Boc encode Shh co-receptors, with Gas1 acting as as a modifier for HPE in its own right, genetically interacting with Shh by potentiating signaling in the developing face (Seppala et al., 2006). Although Gas1 mutants only have microform HPE, with an intact central nervous system, combined loss of Boc produces lobar HPE (Seppala et al., 2013) It has been observed through previous mouse studies that thresholds of SHH signaling may be a factor in phenotypic outcome in patients. It has been further suggested that individuals with more severe forms of HPE have a second genetic alteration that modifies the primary mutation to produce a more severe phenotype (Ming and Muenke, 2002). Vax1 mice serve to demonstrate the complex and varying requirements for components of the Shh signaling pathway for transcription factors such as Vax1 and their role in gene expression during early craniofacial development.
6 Development of the vomeronasal organ in Vax1\textsuperscript{\textminus} mice

6.1 Introduction

Vax1 is expressed in the nasal placode and in the developing vomeronasal organ (VNO) from E10-13.5. Initial histological and skeletal analysis of the Vax1\textsuperscript{\textminus} demonstrated anomalies in the nasal capsule and midface with absence or hypoplasia of the AOB. Furthermore, histological examination of later stage embryos revealed disruption of the nasal capsule and septum, with the presence of a single VNO in some cases. Deletion of Vax1\textsuperscript{\textminus} therefore resulted in significant disruption of the AOS, which allows for odorant detection, an important function in mammals (Eckler et al., 2011).

Vax1\textsuperscript{\textminus} pups die soon after birth but Vax1\textsuperscript{+/-} mice are healthy, viable and born at Mendelian ratios (Hallonet et al., 1999). It has been shown that both male and females are subfertile, with the subfertility originating in the hypothalamus as a result of significantly reduced numbers of GnRH neurons. IHH and its anosmic counterpart, Kallmann syndrome, are two rare genetic disorders, which can lead to various degrees of subfertility, including complete infertility. It has previously been reported in IHH studies that heterozygous deletion of Vax1 in mice causes subfertility (Hoffmann et al., 2014). Mutations in genes involved in ventral forebrain development are often associated with IHH, as GnRH neurons arise in the olfactory placode and then migrate through the cribriform plate to the hypothalamus.

Given the olfactory phenotype characteristics in the Vax1 mice, in combination with reduced fertility in Vax1\textsuperscript{+/-} mice, as well as Vax1 expression in the developing VNO (Hallonet et al., 1998; Hoffmann et al., 2014), we began to analyze the developing nasal capsule, and in particular, the AOS in the Vax1\textsuperscript{\textminus} mouse.
6. Development of the vomeronasal organ in Vax1\textsuperscript{+/–} mice

Figure 6.1  \textit{Vax1} expression in the developing VNO
Radioactive \textit{in situ} frontal sections. Panel A and B; demonstrate expression in the medial wall of the developing nasal process at E10 and E10.5 respectively. Panels C and D show ongoing expression at E11.5 in the early VNO and in the ventral forebrain Scale bar 250\mu m.

6.2 Materials and methods

The aim of this study was to examine the VNO in Vax1\textsuperscript{+/–} mice. This commenced by examining development of the VNO in wild type mice at midgestation by establishing a correlation between embryonic day, embryonic weight and detailed early morphological development of the VNO. We subsequently analyzed early development of the VNO utilising 3D reconstructions of the developing nasal capsule in wild type mice and then in the Vax1\textsuperscript{+/–} mouse. Following this, we then examined cell identity in the developing nasal capsule and VNO in the Vax1\textsuperscript{+/–} mouse through expression analysis for the zinc-finger transcription factors \textit{Fezf1} and \textit{Fezf2}. \textit{Fezf1} regulates the maturation and identity of main olfactory epithelial (MOE) sensory neurons whereas \textit{Fezf2} is responsible for the survival and proliferation of VNO progenitors (Eckler \textit{et al.}, 2011).

Initially, the aim of the investigation was to establish a parameter to correlate the prenatal developmental staging of the VNO according to embryonic weight. This was carried out in order to establish a reliable and specific method for identifying VNO developmental stage related samples. The morpho- and histo-differentiation of the early VNO were compared in embryos.
6. Development of the vomeronasal organ in Vax1<sup>−</sup> mice

classified according to embryonic day of development specified by wet body weight. The embryos of CD1 mice were harvested at several hourly intervals from E10.0 until E11.5, weighed, fixed, and processed histologically. The staging was determined according to developmental progress of the VNO, evaluated based on morphological criteria (Cuschieri and Bannister, 1975).

Chronological staging, as determined in terms of days of prenatal development, is generally acknowledged as an insufficient indicator for detailed embryonic staging. Significant intra- and inter-litter variation of morphological stages has been observed in mouse embryos of the same chronological age as determined by embryonic days (Miyake et al., 1996). Additional morphological criteria have been proposed for more detailed staging by a number of authors. In early stages, the number of somites is often used (Goedbloed and Smits-van Prooije, 1986). In later stages, crown–rump length can be utilized, and a general table of prenatal staging in mouse has been created based on the developmental progress of external features and internal organs and tissues (Theiler, 1972). Kaufman’s atlas of mouse development complements this with comprehensive histological pictures. (Kaufman, 1994). For more detailed age specification, developmental staging of the frontonasal area and vibrissae (Miyake et al., 1996.) or limbs (Wanek et al., 1989) has been used. Štěrba’s method is based on external morphological criteria that allow the developmental stages of embryos of different species to be correlated (Štěrba, 1995).

The individual wet body weight of prenatal mice has been employed as a very useful parameter for specifying chronological age during horizontalization of palatal shelves and primary palate closure and in digit formation in limb buds (Watanabe and Endo, 1988), early stages of tooth development (Peterka et al., 2002) and the course of craniofacial morphogenesis in mouse fetuses affected by cleft lip and palate (Nonaka et al., 1997).
6.3 Results

6.3.1 Development of the VNO at mid-gestation specified by body weight of mouse embryos

Between E10.5-E11.5, we can categorize early development of the VNO into 4 distinct stages from a thickening of the oral epithelium on the lateral nasal placode to the budding-off of the early morphologically distinct organ from the nasal capsule. A corresponding relationship between embryonic weight and developmental stage of the VNO was established.

[Graph illustrating the stage of development of the mouse embryos harvested between stages E10.5 and E11.5 according to prenatal stages of histodifferentiation. Panel A, Lateral schematic of a E10.5 embryo with the area of the nasal capsule and VNO highlighted in red; B, preanlage stage; C, anlage stage; D, budding stage; E, budded-off stage. The mean weight of the embryos were categorized into different morphological stages of development of the VNO. These mean figures are represented along with the first standard deviation. Each embryo was sectioned (7μm) in a frontal orientation and subsequently H and E stained. The degree of development of the VNO in each embryo was determined by the most developmentally advanced section of the VNO. N=65 (Cuschieri and Bannister, 1975).]

6.3.2 Three-dimensional reconstruction of the developing VNO

We subsequently identified wild-type embryos at approximate stages for each group by weight. They were fixed, processed and paraffin wax embedded. They were then mounted on glass slides and stained for Haematoxylin and Eosin. Using a 3D software, Deltaview (University of Osaka), we then created reconstructions of the developing nasal process and VNO at anlage and budded-off stages (Figure 6.4).
6. Development of the vomeronasal organ in Vax1−/− mice

Figure 6.3 Reconstruction of nasal capsule and developing VNO at E10.5 (Anlage stage)

Panel A: Medio-lateral view of the right nasal capsule (dark purple) and VNO (light purple), Panel B; superior view of paired nasal capsules (developing, nasal septum not shown), Panel C; Medio-lateral view of the left nasal capsule (dark purple) and VNO (light purple). Histological diagrams underneath panel represent corresponding histological section at the corresponding cross-section of the 3D reconstruction. Ant; anterior; L, left; nc, nasal capsule, post; posterior, R; right, VNO, vomeronasal organ.

The 3D reconstructions displayed the anatomical position of the developing VNO within the nasal capsule at mid-gestation.

Figure 6.4 Reconstruction of nasal capsule and developing VNO at E10.5 (Budded-off stage)

Panel A: Medio-lateral view of the right nasal capsule (dark purple) and VNO (light purple), Panel B; superior view of paired nasal capsules (developing, nasal septum not shown), Panel C; Medio-lateral view of the left nasal capsule (dark purple) and VNO (light purple). Histological diagrams underneath panel represent corresponding histological section at the corresponding cross-section of the 3D reconstruction. Ant; anterior; L, left; nc, nasal capsule, post; posterior, R; right, VNO, vomeronasal organ.

The 3D morphology of a single VNO in the Vax1−/− was then examined and compared to a wild-type littermate. Although the gross morphology of the developing VNO appeared normal, it was truncated and shorter than its wild-type counterpart.
6. Development of the vomeronasal organ in Vax1−/− mice

Figure 6.5
Reconstruction of a developing VNO at E13.5 (Vax1+/+ and Vax1−/− littermates)

Panel A: Medio-lateral view of the wild-type VNO (purple). Panel B: superior view of paired both wild-type and mutant VNO (developing, nasal septum not shown). Panel C: Medio-lateral view of the mutant VNO (purple). Ant; anterior; L, left; post; posterior, R; right, VNO, vomeronasal organ.

6.3.3 Expression of zinc-finger transcription factors, Fezf1 and Fezf2, in the Vax−/− nasal capsule and VNO

Histological examination of Vax1 mutant embryos at E13.5 revealed the presence of a large anterior epithelial-lined nasal septal defect situated in the midline between the paired VNOs. In an attempt to identify the cell lineage of this defect—(nasal cavity or vomeronasal), in situ hybridization was performed using molecular riboprobes for Fezf1 and Fezf2.

During development, the MOE and VNO both originate from the olfactory pit; however, the mechanisms regulating development of these anatomically distinct organs from a common olfactory primordium are unknown. These two closely related zinc-finger transcription factors, Fezf1 and Fezf2, regulate the identity of MOE sensory neurons and are essential for the survival of VNO neurons respectively. Fezf1 is predominantly expressed in the MOE while Fezf2 expression is restricted to the VNO.
6. Development of the vomeronasal organ in Vax1<sup>−/−</sup> mice

Both transcription factors appear to be normally expressed in the Vax1 mutant with no Fez2 expression in the midline defect (Asterisk) confirming its neuroepithelial origin.

Zinc-finger transcription factor genes *Fezl1* and *Fez2* in Vax1<sup>+/+</sup> and Vax1<sup>−/−</sup> nasal capsules at E13.5 are expressed with reduced levels of transcripts in both genes. The midline defect does not express Fez2 confirming its non-vomeronasal origin.

Both transcription factors appear to be normally expressed in the Vax1 mutant with midline defect (Asterisk) Scale bar 250μm.
6. Development of the vomeronasal organ in Vax1−/− mice

Zinc-finger transcription factor genes Fezf1 and Fezf2 in Vax1+/+ and Vax1−/− nasal capsules at E13.5 confirm reduced levels of expression in the mutant. The midline defect does not express Fezf2 confirming its non-vomeronasal origin (Figure 6.7).

6.4 Discussion

Vax1 expression analysis led this study to investigate further the development of the VNO and its role within the olfactory system. It has previously been demonstrated that Vax1 heterozygous mice are subfertile. It is believed the subfertility originated in the hypothalamus where GnRH transcripts were altered, along with a substantial reduction of GnRH neuron numbers (Hoffmann et al., 2014). GnRH neurons originate in the olfactory placode at E11 in the mouse, migrating through the cribriform plate, reaching their final location in the hypothalamus. GnRH neuron migration and final localization is restricted to the ventral forebrain (Hoffmann et al., 2016). The MNP is the site of origin of the VNO and of GnRH neurons that leave the placode from E11 onward, migrate out and settle in the septal and preoptic areas (Schwanzel-Fukuda et al., 1996; Parhar et al., 1996; Hallonet et al., 1998). It has this been suggested that Vax1 could function in early developing progenitors present in the medial olfactory placode before they migrate to the basal forebrain. In general, it may be proposed that Vax1 could thus function in the early steps of the neuronal differentiation (Hallonet et al., 1998; Taglialatela et al., 2004; Hoffmann et al., 2016).

During development, the VNO originates from the olfactory pit and the zinc-finger transcription factors, Fezf1 and Fezf2, regulate the identity of MOE sensory neurons and are essential for the survival of VNO neurons respectively. Fezf2 expression is restricted to the VNO and expression data in Vax1+/+ and Vax1−/− nasal capsules at E13.5 confirm expression (albeit reduced) in the mutant indicating that the development of the VNO is unaffected at this stage in the Vax1−/− mouse.

It has been demonstrated that the individual wet body weight of prenatal mice can be employed as a very useful parameter for specifying early VNO developmental staging than with only embryonic day. As a result, we can categorize early development of the VNO into 4 distinct stages from a thickening of the oral epithelium on the lateral nasal placode to the budding-off of the early morphologically distinct organ from the nasal capsule during mid-gestation in murine development.
6. Development of the vomeronasal organ in Vax1−/− mice

Vax1 expression is important for normal development of the nasal passages and olfactory systems as demonstrated by histological and skeletal analysis of the Vax1−/− mouse (Hoffmann et al., 2014; Hoffmann et al., 2016). Vax1 expression corresponds to spatiotemporal development of the ventral forebrain, the hypothalamus, olfactory bulb and midface including the MNP, which gives rise to the VNO and olfactory neural circuitry as well as the presumptive oral ectoderm.

Specifically, Vax1 was expressed in the ectoderm in a transverse band between the two olfactory placodes, medially contacting the neural tube. Vax1 was also expressed strongly in the ventral forebrain and in a medially-restricted transverse band of ectoderm situated adjacent to the forebrain (Hallonet et al., 1998). At this stage in the Vax1−/− mouse, craniofacial defects are observed including within the olfactory system, the midface is noticeably shorter furthermore, in situ and RT-PCR data demonstrates significant downregulation of Shh signaling during normal development, fusion between the nasal processes occur initially at the posterior part of the nasal pits and proceeds anteriorly (Gaare and Langman, 1977). These morphogenetic changes gradually convert the nasal pits to nose chambers and to nasal ducts as the fusion process between the medial and lateral nasal processes complete. The choanal membranes at the dorsal ends of the capsule perforate to connect the nostrils to the posterior oral cavity. The nostrils then proceed to transform to small slits and their lower edge are remodelled by the fusion between the medial nasal and primary palate maxillary processes during the last stages of upper lip formation. The Vax1−/− mouse has a cleft palate but lip fusion occurs normally. Vax1 transcripts are not normally expressed in the developing palate but are expressed earlier, in the oral ectoderm, and in the MNP; however, Vax1 is not expressed around the site of fusion of the nasal processess but caudally around the area of the putative VNO. Recent genome wide association studies (GWAS) show significant genetic associations have been identified for VAX1 with nonsyndromic cleft lip with or without cleft palate (CL(P)) (Butali et al., 2013) implying a role in normal lip development.

Further to the formation of the lip, Vax1 continues to express throughout the AOS. It is during this developmental time point that medialization of the nose chamber and the filling of the medial groove (the groove between the medial nasal processes) occurs followed by outgrowth of the intermaxillary segment into the oral cavity to form the anterior palate (Cobourne, 2004). Loss of Vax1 in the mutant mouse results in disruption of the facial midline,
within the premaxillary incisal region, palatal region, nasal septum, olfactory and optic systems, and in the anterior forebrain. as well as demonstrating a marked reduction in the width of the skull laterally with concomitant relative widening of the midface at the level of the palatal shelves.

These new data, in combination with previous studies, indicates an important role for Vax1 in the normal development of the olfactory systems as part of the midline structures of the face and anterior CNS (Hallonet et al., 1999; Goetz 2012; Hoffmann et al., 2014; 2016). Whilst Vax1 deletion in the mouse results in lobar HPE, a milder form of HPE than seen in Shh mutants, the role of Vax1 as an effector of Shh signaling in the forebrain and face can be demonstrated by the impact on the development of the embryonic head size and facial midline as well as through the defects in sensory structures such as the eye and nose.

The forebrain is the foundation upon which the face is built- as the forebrain develops, and undergoes its own morphogenesis, the developing face and developing sensory organs are shaped in response (Marcucio et al., 2015). Both the pattern and rate of growth of the brain and sensory systems effect and influence facial morphogenesis. The olfactory system, like the ventral forebrain and the eye, is an ancient sensory organ that is essential for the survival and reproduction of a species. The organization of this system displays many evolutionarily conserved features in vertebrates, including molecular mechanisms and complex migratory pathways (Huilgol and Tole, 2016). Development of sensory structures are closely entwined with that of the developing facial structures. This is evident, particularly in the mouse which, like other mammals has a heightened dependence on the sensory input of the nose for communication and survival, when compared to humans. In the Vax1−/− mouse, there has been a significant impact in the developing forebrain and developing midline structures of the ventral CNS and face as well as in the olfactory and visual systems. It has been demonstrated that the olfactory system in the Vax1−/− mouse has been effected by phenotypic aberrations in the nose structure, the olfactory neuronal circuitry development and within the CNS and in the olfactory bulb (Hallonet et al., 1999; Taglialatela et al., 2004; Hoffmann et al., 2014; Hoffmann et al., 2016).
7. The craniofacial phenotype of Gas1<sup>−/−</sup>;Vax1<sup>−/−</sup> mice

7.1 Introduction

Previously, it has been demonstrated that Shh expression is reduced in the craniofacial region of Vax1<sup>−/−</sup> mice. These findings correspond with data from studies in the eye where it has been demonstrated that Vax1 lies downstream of Shh signaling (Hallonet et al., 1999). In reciprocal gain-of-function experiments, the injection of Shh into early Xenopus embryos expands the territory of vax1 expression. In addition, a loss of shh leads to downregulation of vax1/2 expression and coloboma in zebrafish (Takeuchi et al., 2003). Inactivation of both vax genes results in severe coloboma supporting the model that Vax1 acts upstream of Shh in eye development. Also, it has been shown that Hh signals acting through Smoothened act downstream of the Nodal pathway to promote vax gene expression (Takeuchi et al., 2003) in the eye. However, in the absence of both Nodal and Hh signals, vax genes are expressed, revealing that other signals including Fibroblast Growth Factors (Fgf) contribute to their regulation (Takeuchi et al., 2003). Targeted mutation of Vax1 in mice also causes a fully penetrant cleft of the secondary palate as well as disruptions to midline structures (Hallonet et al., 1999).

In the absence of Shh, Vax1 and Vax2 are not expressed in the murine eye (Wilson and Houart 2004). The study of Vax1 homozygous mutants has shown that Vax1 and Pax2 expression in the optic stalk requires midline signals, such as Shh (Hallonet et al., 1999). Also, Shh over-expression leads to dorsal expansion of the Vax2 expression domain (Sasagawa et al., 2002). Vax2 has been thought to play an important role in eye development from analysis of its' expression patterns and functional studies carried out in frog and chicken. In zebrafish it has been demonstrated, in the developing eye, that Hh signals acting through Smoothened act downstream of the Nodal pathway to promote Vax1 gene expression pathways. Over-expression of Hh induces vax1 and vax2 and conversely, expression of vax1 and vax2 is lost in the Hh pathway (Takeuchi et al., 2003).

The presence of lobar HPE in the Vax1<sup>−/−</sup> mouse (Hallonet et al., 1999) and decreased Shh signaling in the anterior forebrain as well as in the frontonasal processes was strongly indicative of a positive interaction between Shh and Vax1. This was confirmed by significant relative reductions in Shh mRNA transcripts in our qPCR analysis.
Gas1−/− mice demonstrate the presence of a number of features associated with microform HPE, including midfacial hypoplasia, premaxillary incisor fusion, and cleft palate, in addition to severe ear defects; each with varying levels of penetrance. However, gross integrity of the forebrain remained intact (Seppala et al., 2007). It has previously been shown that there is a positive interaction between Shh and Gas1 as demonstrated by decreased Shh signaling activity in the FNP and palatal shelves of Gas1−/− mice. Indeed, compound Shh+/−; Gas1−/− mice have been generated in the past which demonstrated an exacerbated midline phenotype and provided evidence that Gas1 positively regulates Shh signaling (Seppala et al., 2007).

Compound mutant mice have been a powerful method to investigate genetic interactions and combinatorial functions between two specific genes. Shh expression is reduced in both Gas1−/− and Vax1−/− mice. In order to determine whether the interaction of Gas1 and Vax1, both modifiers of HPE, has any importance in vivo, the craniofacial phenotype of Gas1; Vax1 compound mutant mice was analyzed.

7.2 Materials and methods

Gas1; Vax1 compound mutant mice were generated by crossing heterozygous Gas1 and Vax1 mice. This strategy was pursued as the majority of both Gas1−/− and Vax1−/− mice die soon after birth, usually within three to seven days and only a few survive up to three weeks (Hallonet et al., 1999; Martinelli and Fan, 2007). Matings produced litters containing all expected genotypes at Mendelian ratios. Mice were mated overnight and the embryonic age it again was estimated 0.5 upon observing a plug. The genotype of individual embryos derived from Gas1+/−;Vax1+/− double heterozygous crosses was determined by PCR, as described below. Embryos collected were washed in tissue culture grade 1X PBS at least two times at room temperature (RT) and have been described in chapter two. Embryonic tissue was immediately obtained from tails for genomic DNA (gDNA) extraction. In previous studies, two sets of primers were used for Gas1 genotyping (Martinelli and Fan 2007; Seppala et al., 2007). The first set amplifies the LacZ gene present in the mutant allele. The second set amplifies a 350-bp fragment of the wild-type Gas1 allele that is deleted in the mutant. The sequences of the LacZ primers were as follows: (Forward) CTCGGCAGGAGCAAGGTGAGATG, (reverse) GTCAAGACCGACCTGTCCGGTGC. The Gas1 wild-type allele primers were: (forward) ATCTCGGCCTTATCCAGGTCCTCAAC (reverse) CATCGCACACGCAGTCGGTGTGCA.
Vax1 mutant mice have previously been described. These mice were originally generated by a targeted mutation of Vax1 using homologous recombination in embryonic stem (ES) cells (Figure 7.1). The mutation replaces the amino terminus of the Vax1 protein including the exon coding for the two first helices and part of the third helix of the Vax1 homeobox, with the β-galactosidase reporter (Le Mouellic et al., 1990).

As both the Vax1 and Gas1 mutant alleles contained the LacZ reporter gene (see Figure 4.1), a novel genotyping strategy was required (Hallonet et al., 1999; Martinelli and Fan 2007). The forward primer was therefore constructed to align against the promoter region of Gas1 and the reverse primer aligned against the tau-LacZ cassette. Tau-Lacz was originally constructed as an axon-targeted beta-gal reporter by fusing cDNA encoding the bovine microtubule-binding protein (tau) to LacZ, the E. coli gene encoding beta-gal (Callahan and Thomas 1994). This reporter labels cell bodies and axons when expressed by developing and adult Drosophila neurons. It also reveals the entire cellular extent of nonneuronal cells such as muscle fibers and glia to generate neuronal markers for studies of Drosophila neural development (Callahan and Thomas 1994). The new primers were then tested using known Gas1 mutant DNA samples as well as using known Vax1 mutant samples to aid confirmation of specificity. The products were then cut from the agarose gel and DNA extracted from the gel following the Qiagen® QIAquick Spin protocol.

The resultant PCR product was sequenced and shown to be 392-bp aligning against the promoter region of Gas1 on chromosome 13, with the reverse primer aligning against the tau-
LacZ cassette. The primers were also tested twice with two separate Gas1 positive controls, Vax1 positive controls and negative controls.

![PCR gel Gas 1 mutant allele](image)

Figure 7.2  PCR gel Gas 1 mutant allele

PCR utilising the new Gas1 mutant allele primers which are positive for Gas1 mutant allele in a Gas1 positive control and negative in a Vax1 positive control.

**Genomic DNA isolation**

Isolation of high molecular weight DNA from mouse embryonic tissue and mouse adult tails was performed using the GenElute Mammalian Genomic DNA Purification Kit (Sigma) according to the manufacturers' instructions. Two small pieces (0.5-0.6cm) were cut from embryonic tails or adult ears and transferred to a microcentrifuge tube with 180µl lysis solution and 20µl proteinase K (10mg/ml). Samples were then incubated at 55°C ON in a rocking oven. After full digestion 200µl lysis solution and 200µl ethanol (100%) were added into the mixture and transferred to binding column. Mixture was centrifuged at 6500g for 1 minute. In order to remove any contaminants, column was transferred to a new collection tube, 500µl wash solution were added to column and centrifuged at 6500g for 1 minute. Another 500µl wash solution was added to column and centrifuged at 12000g for 3 minutes allowing column to dry. Then, the column was transferred to a new collection tube, 200µl elution solution was added and centrifuged at 6500g for 1 minute. Genomic DNA was finally stored at -20°C for further use.
Polymerase chain reaction: Gas1; Vax1 mice

Selective amplification of DNA fragments was performed by polymerase chain reaction (PCR). PCR reaction was performed in the PTC-200 Peltier Thermal Cycle (MJ Research). Two new primers (Sigma) were used for the Gas1 mutant allele. The forward primer contained the following sequence CCGGAGAGTGGAGAAAGGAG and the reverse sequence was CCCCTGAGCATGATCTTCCA.

A duplex reaction was performed which was designed to detect the targeted allele (this reaction involves both new primers). a second reaction is performed that only detects the wild type allele. The second set amplifies a 350-bp fragment of the wild-type Gas1 allele that is deleted in the mutant. The sequences of the primers are as follows: (forward) ATCTCGGCGCTTATCCAGCTCAAC and (reverse) CATCGCACACGCAGTCGTTGAGCA.

After PCR, aliquots of the mixture were loaded onto a 1.4% agarose gel and electrophoresed (180 Volts for 45 minutes) to detect amplified product. In order to identify the size of product on the gel a 100 base pairs DNA ladder (Fermentas) was loaded alongside the DNA sample. Visualization took place under UV light.

7.3 Results

7.3.1 Gross morphology of Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup>, Gas1<sup>+/−</sup>; Vax1<sup>+/−</sup> with Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup> and wild-type mice at E17.5

At E17.5, gross morphology of the Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup> mouse showed a further reduction in body size when compared with single mutants, with notable further narrowing and truncation of the midface (Figure 7.3A-J). The phenotypic craniofacial characteristics of the Gas1<sup>−/−</sup> mouse were apparent in the compound mutant; which included truncation of the nasal and maxillary structures as well as defects associated with the eye and external ear. The microphthalmic eye ingressed markedly from the epithelial surface, whilst the pinna of the ear was small, malformed and in a ventral position (Lee et al., 2001; Seppala et al., 2007). All phenotypes displayed a broadly normal facial midline with both external nares and a delineation of the philtrum present.
7. The craniofacial phenotype of Gas1<sup>−/−</sup>;Vax1<sup>−/−</sup> mice

Figure 7.3  
**Gross morphology of Gas1<sup>++</sup>;Vax1<sup>++</sup>, Gas1<sup>−/−</sup>;Vax1<sup>++</sup> with Gas1<sup>−/−</sup>, Vax1<sup>−/−</sup> and wild-type at E17.5**

Comparison of the Gas<sup>−/−</sup>Vax1<sup>−/−</sup> with Gas1<sup>−/−</sup> and Vax1<sup>−/−</sup> littermates show a similar but smaller phenotype with further narrowing and truncation of the mid-face. The compound mutant embryonic craniofacial phenotype (E,F) show characteristic features to that of Gas1<sup>−/−</sup> including microphthalmia (red arrow in F) and severe malformation of the pinna of the ear (red arrow) ee, eye; en, external nares; md, mandible; pe, pinna of the ear; ph, philtrum; vb, vibrissae. Scale bar = 500μm.
7.3.2 Comparison of the gross morphology and histological midline phenotype of Gas1+/−;Vax1+/− and Gas1+/−;Vax1+/- with Gas1+/-; Vax1+/- and wild-type mice at E15.5

Examination at an earlier stage demonstrated a microstomic phenotype in the Gas1+/−;Vax1+/− mouse in conjunction with the further relative mid-face truncation. As in stage E17.5, the external nares and philtrum appeared normal. Histological analysis at the same developmental stage demonstrated a worsening of the phenotype in the compound mutant, as demonstrated by a complete absence of the premaxillary incisors and loss of the septal cartilage.

Figure 7.4. Comparison of the gross morphology and histological midline phenotype of Gas1+/-Vax1+/-, Gas1+/-;Vax1+/- with Gas1-, Vax1-, and wild-type at E15.5

Frontal views of wild-type (Panels A,B), Gas+/-;Vax1+/- (C, D) and Gas+/-;Vax1+/- (E,F) with Gas1+/- (G, H) and Vax1+/- (I,J) litter mates at E15.5 showing the gross morphology of the midline at the earlier stage E15.5 and Hand E stained coronal sections demonstrating the premaxillary incisor phenotype at the same stage. The compound mutant (F) demonstrated a microstomic phenotype in conjunction with the truncation of the midface. In general, both external nares are evident in all phenotypes as is a demarcated philtrum. Coronal views of H and E stained sections at the same age showed normal premaxillary formation in Gas+/-;Vax1+/- (C) and Gas1+/- (G). However, in Vax1+/- (J) there is fusion of the premaxillary incisors and there is a loss of premaxillary incisors in Gas+/-;Vax1+/- mice (Red asterisk) (F). Also in the compound mutant, note the loss of the septal cartilage. en, external nares; md, mandible; nc, nasal capsule; ns, nasal septum; oc, oral cavity; pe, pinna of the ear; ph, philtrum; pmxi, premaxillary incisor; vb, vibrissae. Scale bar = 250μm.
7.3.3 **Comparison of the gross morphology and histological midline phenotype of \( \text{Gas}1^{-/-};\text{Vax}1^{-/-} \) and \( \text{Gas}1^{+/-};\text{Vax}1^{+/-} \) with \( \text{Gas}1^{-/-}, \text{Vax}1^{-/-} \) and wild-type mice at E17.5**

The overall appearance of the skeletal analysis of the \( \text{Gas}1^{-/-};\text{Vax}1^{-/-} \) skull at E17.5 reveal a microstomic phenotype in conjunction with the truncation of the midface. In general both external nares are evident in all phenotypes as is a demarcated philtrum. Further analysis \( \text{Gas}1^{-/-};\text{Vax}1^{-/-} \) skull at E17.5 showed a similar pattern of craniofacial anomalies as described in \( \text{Vax}1^{-/-} \), and in \( \text{Gas}1^{-/-} \) mice (Seppala et al., 2007), with defects affecting the skeletal and cartilaginous elements of the neurocranium, splanchnocranium and dematocranium (Figure 7.5).

The \( \text{Gas}1^{-/-};\text{Vax}1^{-/-} \) skull at E17.5 demonstrated a synostic premaxilla and loss of the premaxillary incisors as well as a cleft in the primary palate. Within the nasal cavity of the mutant, the nasal septum was either hypoplastic or absent, with accompanying hypoplasia of the paraseptal cartilages and vomer. There was associated hypoplasia of the palatine and maxillary bones, which resulted in a continuous cleft extending from the primary to secondary palate. Within the region of the sphenoccipital synchondrosis there were also fenestrations associated with the basisphenoid and basioccipital bones. The trabecular basal plate, which extends as a cartilaginous plate from the nasal septum through the ethmoid, presphenoid and basisphenoid bones was more noticeably dismorphic (and perforated) in the double mutant compared to the \( \text{Gas}1^{-/-} \) mouse.

Further skull deformities were also evident in the calvarial bones. The coronal suture between the frontal and parietal bones displayed craniosynostosis (Figure 7.5F) and the nasal bones did not show a patent midline suture.
7. The craniofacial phenotype of Gas1⁻/⁻;Vax1⁻/⁻ mice

Figure 7.5  Comparison of Gas1⁺⁺;Vax1⁺⁺ and Gas1⁻/-;Vax1⁺⁺ with Gas1⁺⁺;Vax1⁻/- and wild-type at E17.5

Differential bone (red) and cartilage (blue) staining of Gas¹⁺⁺;Vax¹⁺⁺, Gas¹⁻⁻;Vax¹⁺⁺, Gas¹⁺⁺;Vax¹⁻⁻, Gas¹⁺⁺⁺;Vax¹⁻⁻ and Gas¹⁻⁻;Vax¹⁻⁻ at E17.5 (Panels A-J) The ventral view of the Gas¹⁻⁻;Vax¹⁻⁻ skull reveals a synostic maxilla with absent incisors (red arrow in E), widened midline clefting involving the maxilla, palatine and basisphenoid bones (blue arrow in E) and an absence of the vomer and presphenoid. Severely disrupted development of the neurocranial base is also marked by a dysmorphic and perforated trabecular basal plate (yellow arrow in E). The lateral view reveals truncation of the midface when compared to the Gas¹⁺⁺;Vax¹⁺⁺ more closely resembling the midface phenotype of the Gas¹⁺⁺⁺;Vax¹⁻⁻. The lateral view (F-J) of the calvarial bones demonstrated craniosynostosis between the frontal and parietal bones (green arrow in J). bo, basioccipital; bs, basisphenoid; cni, cupola nasi anterior of nasal capsule; etm, ectotympanic process; fn, frontal bone; md, mandible; mx, maxilla; na, nasal bone; pa, parietal bone; pCa, pars canalicularis; pCo, pars cochlearis; pppl, palatal process of palatine; pmx, premaxilla; pmxi, premaxillary incisors; ptg, pterygoid; rtp, retrotympanic process; sq, squamosal; zmx, zygomatic process of maxilla.
7. The craniofacial phenotype of Gas1\(^{-/-}\);Vax1\(^{-/-}\) mice

7.3.4 Craniofacial midline phenotype of Gas1\(^{-/-}\);Vax1\(^{-/-}\) and Gas1\(^{+/+}\);Vax1\(^{+/+}\) with Gas1\(^{-/-}\);Vax1\(^{-/-}\) and wild-type at E15.5

Skeletal preparations and histological studies have revealed that both Vax1\(^{-/-}\) and Gas1\(^{-/-}\) mice display a cleft secondary palate. Penetrance of this cleft is 100% in Vax1\(^{-/-}\). However, penetrance of the cleft in the Gas1\(^{-/-}\) was incomplete with 40% of mice demonstrating essentially normal palatal development (Seppala et al., 2007). Both Vax1\(^{-/-}\) and Gas1\(^{-/-}\) have cleft secondary palate phenotypes. However, the Vax1\(^{-/-}\) mouse phenotype is 100% penetrant demonstrating midline deficiencies from the anterior CNS through the nasal cavity with standard histological analysis suggesting that the initially vertical positioned palatal shelves elevating above the tongue to apposite horizontally. In affected mice, no fusion of shelves took place.

Gas1\(^{-/-}\); Vax1\(^{-/-}\) mice demonstrated a similar midline phenotype with more extensive abnormalities in the developing nose, such as an absent nasal septum and malformed bilateral VNOs. There was an incomplete cleft with evidence of further dysmorphic arrangement of a secondary more rostrally positioned ectopic pituitary similar to that reported in Vax1\(^{-/-}\) (Bharti et al., 2011). Gas\(^{+/+}\); Vax1\(^{+/+}\) also demonstrated disruption in the posterior secondary palatal shelf fusion with persistence of the MES. Gross examination of the tongue revealed no abnormality.
7. The craniofacial phenotype of Gas1−/−;Vax1−/− mice

Figure 7.6  Histological analysis of Gas1+/−;Vax1+− and Gas1+/−;Vax1−/− with Gas1−/−;Vax1−/− and wild-type mice at E15.5

Coronal sections through the developing craniofacial region of Gas1+/−;Vax1+−, Gas1+/−;Vax1−/−, Gas1−/−;Vax1+−, Gas1−/−;Vax1−/− and Gas1−/−;Vax1−/− at E15.5 (Panels A-O) The compound mutant demonstrates worsening of the midline phenotype with absent nasal septum and dysmorphic vomeronasal organs (red arrow D) There is an incomplete cleft anteriorly. The septal cartilage is also absent in the Vax1−/− (blue arrow, N) which also shows an ectopic adenohypophysis in communication with the oral cavity (N, yellow arrow). The Gas1−/−;Vax1+/− also demonstrates some disruption in the posterior secondary palatal shelf fusion with persistence of the mesenchymal epithelial seam (MES), McMeckel's cartilage; mes. midline epithelial seam; mtb. Molar tooth bud; nc. Nasal cavity; ns. Nasal septum; oc. Optic capsule; ps. Palatal shelf; t. tongue; vno, vomeronasal organ Scale bar = 500μm.
7.3.5 The histological analysis of the forebrain phenotype of Gas1\(^{-/-}\); Vax1\(^{-/-}\) and Gas1\(^{+/-}\); Vax1\(^{+/-}\) with Gas1\(^{-/-}\); Vax1\(^{-/-}\) and wild-type mice at E15.5

At E15.5, the forebrain has subdivided into three vesicles; left and right telencephalic hemispheres and the more posterior single diencephalon. Gas1\(^{-/-}\) embryos have all three clearly defined structures, including the choroid plexus, internal capsules and pars intermedia (Seppala et al., 2007). The Vax1\(^{-/-}\) basal forebrain is severely disrupted (Hallonet et al., 1999), with the olfactory bulbs either absent or hypoplastic. There is a distinct inter-hemispheric fissure with midline continuity of the cingulate gyrus present in some cases and variable deficiencies in growth of the medial ganglionic eminence, preoptic area and septum. Structures located medially were more affected with the optic chiasm and preoptic area being systematically absent.

The telencephalic phenotype of Vax1\(^{-/-}\) ranged from a total absence of growth of medioventral structures to some dorsolateral structures fusing medially. The medioventral defects typically included a defective cleavage of the dorsal forebrain into bilateral vesicles resulting in a Lobar holoprosencephalic phenotype (Hallonet et al., 1999; Taglialatela, et al., 2004). The Gas1\(^{-/-}\); Vax1\(^{-/-}\) forebrain demonstrated further disorganisation of the cortical structure (neopallial cortex and cingulum cortex) with an indistinct interhemispheric fissure indistinct or missing septal tissue and of the choroid plexus demonstrating a further exacerbation of the HPE phenotype (Figure 7.8).
Figure 7.7  Histological comparison of the forebrain phenotype of Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup> and Gas1<sup>+/−</sup>; Vax1<sup>+/−</sup> with Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup> and wild-type litter mates at E15.5.

H and E stained histological sections of the Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup> and Gas1<sup>+/−</sup>; Vax1<sup>+/−</sup> with Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup> and wild-type litter mates at E15.5 (Panels A-L). Coronal sections show normal gross morphology of the forebrain (A-C) Gas1<sup>−/−</sup>; Vax1<sup>−/−</sup> demonstrate further disorganisation of the cortical layers of the forebrain (D,E) such as the cingulum cortex (red arrow) and septal area (blue arrow) to a total absence of medioventral sturtures (F) when compared to the Vax1<sup>−/−</sup> forebrain. cc, cingulum cortex; cp, choroid plexus; dth, diencephalon (thalamus); ita, interthalamicadhesion (massa intermedia); lic, left internal capsule; mb, molar tooth bud; nc, nasal capsule; ncc, neopallial cortex; poa, preoptic area; sep, septal area, slv, superior horn of the right lateral ventricle; tv, third ventricle. Scale bar 500μm.
7.4 Discussion

The Gas1; Vax1 mutant mouse shows that the combined loss of two putative HPE modifiers associated with Shh signaling can have a deleterious effect on craniofacial development in the mouse. This was exemplified by some exacerbation of the craniofacial phenotypic characteristics associated with individual Gas1 and Vax1 mutant skulls, which included an absence of the maxillary incisors and further disruptions associated with development of the anterior forebrain. Therefore Gas1−/−; Vax1−/− mice provide a novel mouse model for investigating the role of modifying genes associated with Shh in the pathogenesis of HPE.

Gas1−/− mice demonstrate a number of features associated with microform HPE, including midfacial hypoplasia, premaxillary incisor fusion, and cleft palate; in addition to severe ear defects; each with varying levels of penetrance. However, gross integrity of the forebrain remained intact (Seppala et al., 2007). The human homolog of murine Gas1 maps to chromosome 9q21.3–q22, a locus previously associated with a number of human disorders. Recent studies suggest that GAS1 could be considered a candidate locus for one of the types of human HPE (Ribeiro et al., 2010).

HPE (OMIM#236100) is the most common forebrain birth defect in humans (Solomon et al., 2013). Eighty percent of cases of HPE are associated with facial abnormalities. The range of presentation can vary from cyclopia and complete absence of lobar division to more subtle defects such as median cleft lip, absent olfactory bulb, agenesis of the corpus callosum and a single central maxillary incisor (Geng and Oliver, 2009). HPE is a complex multifactorial disorder which displays a wide spectrum of severity. The heterogeneity in the HPE phenotype reflects the complex spatiotemporal interplay that occurs between multiple signaling pathways during early morphogenesis of the forebrain and facial region. Indeed, there is evidence from some pedigrees that phenotype is influenced not only by the type of mutation but also the number, with multigenetic inheritance being identified in some cases (Ming and Muenke, 2002).

The association between Hh signaling and HPE provides clear evidence of this complex interplay. The early craniofacial region is sensitive to perturbations in Shh signaling and this changes with time; disruption of the pathway during discrete periods of embryonic development can reproduce the phenotypic spectrum of HPE, with the severity of the phenotype displayed
7. The craniofacial phenotype of Gas1\(^{-/-}\);Vax1\(^{-/-}\) mice

depending on the timing and magnitude of signal loss (Cordero et al., 2004). In addition, modifying loci have also been identified which act in tandem with SHH in the induction of HPE; these include TGIF and ZIC2 (Dubourg et al., 2004). Mutations in SHH, GLI2, DISP1, and PTCH1 have been identified in HPE-affected individuals (McMahon et al., 2003; Ingham and McMahon 2001). Mutations in SHH alone account for 12.7% of HPE cases (Dubourg et al., 2007).

Early phenotypic analysis of both Gas1\(^{-/-}\) (Seppala et al., 2007) and Vax1\(^{-/-}\) mice demonstrated a number of craniofacial defects arising as a consequence of altered Shh activity. Gas1 and Vax1 are expressed in similar regions and have adjacent and partially overlapping domains of expression in the developing forebrain and facial processes with Shh and Ptch1. At mid-gestation in both Gas1 and Vax1 mutant embryonic forebrains, there is a reduction in Shh and Ptch1 expression. Therefore, both Gas1 and Vax1 seem to be important for Shh to achieve its' full range of signaling and help mediate this activity between the forebrain and developing facial primordia. The Gas1\(^{-/-}\);Vax1\(^{-/-}\) embryos demonstrate a range of midline defects that results in worsening of the holoprosencephalic phenotype displayed by either Gas1\(^{-/-}\) or Vax1\(^{-/-}\). This further demonstrates the role of both Gas1 and Vax1 in positively regulating Shh signaling. Similarly, Gas1\(^{-/-}\);Cdo\(^{-/-}\) mice also exhibit exacerbated midline defects in comparison to phenotypes in either Gas1\(^{-/-}\) or Cdo\(^{-/-}\) mice, leading to reduced transduction of Shh signaling (Allen et al., 2007; Zhang et al., 2011; Seppala et al., 2014).

It has previously been suggested that modifying genes such as TGIF and ZIC2 (Dubourg et al., 2004) act in tandem with SHH in the induction of HPE. GAS1 and VAX1 have also been identified as candidate genes in HPE (Ribeiro et al., 2010 and Slavotinek et al., 2012, respectively). The Gas1\(^{-/-}\);Vax1\(^{-/-}\) mice demonstrated a more severe HPE phenotype than that of the microform HPE demonstrated in the Gas1\(^{-/-}\) mouse (Seppala et al., 2007) and the lobar HPE of the Vax1\(^{-/-}\) mouse. Similar studies addressing the roles of other key Hh components will eventually lead to a more complete picture of the genetic basis of midline development and how it relates to human syndromes.
8 General discussion

8.1 The role of Vax1 during craniofacial development

GWAS have reported significant associations between nonsyndromic CL/P and Single Nucleotide Polymorphisms (SNP) in the VAX1 gene (Beaty et al., 2010; Mangold et al., 2010; Butali et al., 2013). These associations were confirmed in different population group studies (Nikopensius et al., 2010; de Aquino et al., 2013). Animal studies have proposed a role for Vax1 in the Hh signaling pathway both in the developing eye, and in dorso-ventral patterning (Takeuchi et al., 2003; Bharti et al., 2011). Phenotypic analysis of Vax genes have demonstrated the role of Vax1 in the development of the CNS and the developing eye (Hallonet et al., 1999; Takeuchi et al., 2003; Soria et al., 2004; Kim et al., 2014); however, its role in craniofacial development has not been described. Vax1 acts downstream of Hh signaling in the eye and in dorso-ventral patterning of the CNS (Takeuchi et al., 2003; Bharti et al., 2011). The early phenotypic and expression studies demonstrated that changes in Shh signaling underlie various craniofacial defects evident in the Vax1−/− embryos resulting in lobar HPE. These mice demonstrate the complex and varying requirements for components of the Shh signaling pathway and related forebrain markers during early craniofacial development. Phenotypic analysis of Vax1−/− mice demonstrates the role of Vax1 in normal midline development (Hallonet et al., 1999). Given the critical role for Shh in midline patterning of structures of the face and brain (Chiang et al., 1996; Belloni et al., 1996; Roessler, 1996; Nanni et al., 1999), this would allow for speculation that Vax1 plays an important role within the Hh signaling pathway during craniofacial development (figure 8.1). Furthermore, it may be suggested that other known, Shh mediated, modifiers of HPE such as Gas1 may act synchronically, albeit indirectly, with Vax1 to exacerbate the resultant HPE phenotype exemplifying the impact of multiple genetic defects on the spectrum of HPE phenotypic presentation.
In early development of the embryonic head, Fgf8 restricts Wnt8b to dorsal midline and regulates Bmp4. (Bmp4 appears necessary to restrict Fgf8 and Shh). By maintaining Fgf8 expression in the commissural plate, Shh regulates development of dorsal midline structure (by antagonizing Gli3) Fgf8 in turn activates Nkx2.1 expression in the ventral telencephalon which in turn induces Shh. Vax1 acts as effector of Shh in the developing midline (Blue background represents alobar HPE, green representing Semilobar and yellow alobar).

Detailed analysis of Vax1 expression showed Vax1 transcripts are first detected at E8 at the rostral level of the medial neural plate including the ANR and surrounding ectoderm (Hallonet et al., 1998). It is then seen that Vax1 was expressed in the ectoderm in a transverse band between the two olfactory placodes, medially contacting the neural tube, in the rostral oral ectoderm and in the rostral and medial regions of the olfactory placodes. the optic disk and stalk, later the optic nerve, the optic chiasm, the suprachiasmatic area, the hypothalamic cell cord, the whole preoptic area, the septum, the entopeduncular area and the basal ganglia. Laterally, there was confinement of expression at the LGE and MGE. This expression pattern was suggestive of roles in patterning and morphogenesis of craniofacial structures. The phenotypic presentation of the Vax1−/− mice were further suggestive, revealing multiple craniofacial anomalies including single incisors, cleft secondary palate, duplicated pituitary glands, disruption in the development of the forebrain as well as defects in the olfactory and optic systems. These craniofacial defects and presence of lobar HPE in the Vax1−/− mice combined with the results of previous studies were strongly suggestive of reduced Shh signaling activity.
Skeletal analysis showed that Vax1−/− mice show a range of cranial dimension changes demonstrating an overall reduction in anteroposterior length with truncation of the midface and widening of the embryonic head. A number of these craniofacial defects were first evident at E10.5 (Hallonet et al., 1999). These were revealed to be a consequence of reduced Shh signaling activity. In the developing forebrain, Vax1 was expressed in partially overlapping regions of expression of Shh in the ventral forebrain between the hypothalamus through the optic recess and ganglionic eminence to the septal area ventrally, and in the developing rostral oral ectoderm with adjacent regions of expression in the nasal processes. This pattern of expression was clearly identifiable at E10.5 and E11.5 as the telencephalon bifurcates dorsally to form the two cranial hemispheres. In Vax1−/− mice during this period, a reduction in Shh and Ptch1 expression throughout corresponding craniofacial structures and in underlying mesenchyme was observed. This reduction in Shh and Ptch1 expression in the developing ventral forebrain and facial structures was confirmed using qPCR techniques. BrdU assay demonstrated proliferative changes in these areas. Therefore Vax1 is important during spatiotemporal development of the ventral forebrain and face in effecting the induction of different regions of Shh expression from the developing ventral forebrain to the midface at these stages.

Furthermore, the observed CP appears to be a consequence of the associated gross craniofacial defects, rather than a direct effect of Vax1 function. There was no evidence of Vax1 expression in the developing palate and no difference in the gross anatomy or levels of proliferation in the palatal shelves between WT and mutant mice. Gross examination of the tongue demonstrated no abnormalities, excluding any tongue deformity as a secondary reason for cleft palate formation. It is most likely that a large midline cavity within the CNS, extending from the floor of the hypothalamus through the nasal cavity to the roof of the oral cavity, was responsible for the failure of the palatal shelves to approximate in the midline. This structure has been described previously and ascribed to the presence of CP, but is most likely the cause of this defect (Bertuzzi et al. 1999). Investigation of the role of Vax1 demonstraet reduced Shh signaling in the ventral forebrain associated with reduced levels of proliferation, facial truncation and lobar HPE. Despite expression of Vax1 in ectoderm of the medial nasal processes the upper lip forms normally in Vax1 mutant mice. Moreover, the CP would seem to be the result of disrupted craniofacial development rather than a specific function of Vax1. In addition, Vax1; Gas1 mutant mice had a complete absence of the maxillary incisors but upper lip formation remained normal.
Despite strong evidence for VAX1 as a candidate gene for CLP in human populations, this gene is seemingly dispensable for normal upper lip development and plays an indirect role in palatogenesis in the mouse.

In addition to HPE, which produces a combination of CNS and facial defects, other structural defects of the face may result when interactions of the brain and face are affected during craniofacial development. Non syndromic cleft lip and palate is a particularly interesting area of exploration. These forms of clefting often have no known underlying genetic cause and occur in isolation from other facial malformations. It appears that changes or defects in the rate of brain growth could potentially lead to clefting malformations due to the influence of the brain on the facial primordia during growth (Diewert et al., 1993; Diewert and Lozanoff 1993a; Diewert and Lozanoff 1993b; Jiang et al., 2006; Petryk et al., 2015). The developing facial processes demonstrate a highly coordinated growth pattern that brings them in apposition in order to fuse and form the primary and secondary palates. Failure of normal temporo-spatial development may result in failure of processes to fuse and result in clefting.

The pituitary gland is an endocrine organ that is developmentally derived from a fold in the oral ectoderm and a juxtaposed fold in the neural ectoderm. It has been observed that in Vax1−/− mice, the rostral oral ectoderm forms an ectopic fold that eventually develops into a separate second pituitary with all the pituitary cell types and neuronal fibers characteristic of the normal pituitary (Bharti et al., 2011). The induction of the second pituitary was associated with a localized ectopic expression of Fgf10, a growth factor known to recruit oral ectodermal cells into the pituitary. Interestingly, in the aforementioned study, it was claimed that Vax1 was not expressed in the oral ectoderm. However, from our expression studies and in other studies (Hallonet et al., 1998), we know Vax1 to be expressed in the rostral oral ectoderm during formation of Rathke's pouch and it may be therefore suggested that Vax1 has a more direct role during the early development of the pituitary gland. Furthermore, it has been noted that pituitary defects have been observed in HPE as well as in other HPE modifier genes such as Gas1 (Seppala et al., 2007).

It has been demonstrated that the olfactory system in the Vax1−/− mouse has been effected by phenotypic aberrations in the nose structure, the olfactory neuronal circuitry development and within the CNS and in the olfactory bulb (Hallonet et al., 1999; Taglialatela et al., 2004; Hoffmann et al., 2014; Hoffmann et al., 2016). It has this been suggested that Vax1 could
function in early developing progenitors present in the MNP before they migrate to the basal forebrain. In general, it may be proposed that Vax1 could thus function in the early steps of the neuronal differentiation (Hallonet et al., 1998; Tagliatala et al., 2004; Hoffmann et al., 2016).

Another example of a lobar HPE murine model is the $\text{Gas}1^{-/-};\text{Boc}1^{-/-}$ compound mutant, which demonstrates lobar HPE associated with CL/P, and arrested maxillary incisor development, secondary to reduced Shh transduction in the central nervous system and face. $\text{Gas}1$ and $\text{Boc}$ encode Shh co-receptors, with $\text{Gas}1$ acting as as a modifier for HPE in its own right, genetically interacting with Shh by potentiating signaling in the developing face (Seppala et al., 2007). Although $\text{Gas}1$ mutants only have microform HPE, with an intact central nervous system, combined loss of $\text{Boc}$ produces lobar HPE (Seppala et al., 2013). Generation of $\text{Gas}1;\text{Vax}1$ mutant mice provided further evidence of the nature of the role of Shh mediated modifiers in the aetiology of HPE. Further reduction in the availability of Shh in $\text{Gas}1;\text{Vax}1$ mutant mice results in truncation and exacerbated facial midline defects such as an absence of the maxillary incisors and further disruptions associated with development of the anterior forebrain as well as an absent nasal septum and malformed bilateral VNOs. There was an incomplete cleft with evidence of further dysmorphic arrangement of a secondary more rostrally positioned ectopic pituitary similar to that reported in $\text{Vax}1^{-/-}$ mice (Bharti et al., 2011).

The phenotypic investigations utilizing $\text{Vax}1$ and $\text{Gas}1;\text{Vax}1$ mouse models suggest important roles for $\text{Vax}1$ during craniofacial development. In the mouse, $\text{Vax}1$ is expressed in ectoderm of the medial nasal processes and persists as these structures fuse with the lateral nasal processes at the lambdoid junction during upper lip formation. Moreover, $\text{Vax}1$ acts as a downstream target of Shh signaling in the medial nasal process, potentially acting to restrict Wnt pathway activity, promote cell cycle exit and epithelial fusion as lip continuity is established (Kurosaka et al. 2014). It is therefore surprising that $\text{Vax}1^{-/-}$ mice do not display a cleft lip phenotype. One possible explanation is that other factors may compensate for Vax1 function in the early face, particularly during the restriction of Wnt signaling activity in the nasal processes (Kurosaka et al. 2014). Alternatively, thresholds of activity and genetic background may influence phenotypic outcome in the mouse embryo. $\text{Vax}2$ gene expression is restricted to the developing eye and targeted disruption of both genes results in conversion of the optic nerve to retina, whilst the upper lip remains intact (Mui et al. 2005). Similarly, gross development of the
VNO was normal in *Vax1* mutants, despite transcripts being strongly expressed in this organ throughout its development. Interestingly, by reducing the levels of Shh signal activity in the mid-facial region by generating abrogating *Vax1* on a *Gas1* mutant background did not result in CLP, although the maxillary incisor phenotype did worsen, with agenesis as opposed to SMMCI.

Cleft palate, fused incisors, pituitary defects, optic and olfactory defects as well as ventral forebrain anomalies are frequently seen in humans as isolated anomalies or as a feature or as features of a particular syndrome such as HPE (Cohen 1989a; Cohen 1989b; Cohen and Sulik 1992; Roessler, 1996b; Barr and Cohen 1999; Ming *et al.*, 2002; Solomon *et al.*, 2013). SHH is the most frequently mutated locus amongst HPE patients; however identical mutations can result in a whole spectrum in phenotypic presentation even amongst members of the same pedigree. How this spectrum occurs has been a subject of detailed investigation and scrutiny for several years. Work in mice suggests that threshold of SHH signaling may be an important factor in this. However, a major obstacle remains; why does haploinsufficiency in people cause the wide range of phenotypes that are seen in HPE patients? One possibility lies in the 'second-hit' model where a second genetic impact modifies the initial one to produce a more severe impact (Ming and Muenke, 2002). This has been supported in mice both in this study and others (Tenzen *et al.*, 2006; Seppala *et al.*, 2007; 2014). Secondly, mutations can sensitize animals to teratogenic insult (Hong and Krauss, 2012). An alternative model to help explain this wide range of phenotypic presentation has been suggested involving dose responsive function of signaling pathways. Large variation in cell responsiveness to concentrations of SHH in avian embryos were observed (Young *et al.*, 2010). This indicates that for small signal changes, large facial changes may result. *Vax1*−/− mice present with lobar HPE demonstrating a number of serious anomalies that can affect humans and recent studies have confirmed the link between *VAX1* and HPE, non syndromic CL/P and eye related defects such as microphthalmia and coloboma (Slavotinek *et al.*, 2012, de Aquino *et al.*, 2013; Williamson and FitzPatrick, 2014). The phenotypic analysis of *Vax1*−/− mice has confirmed *Vax1* as a putative candidate gene for a number of human craniofacial anomalies such as SMMCI, cleft palate, lobar HPE and duplicated pituitary formation.
8.2 Future considerations

Vax1−/− mice revealed various craniofacial defects that are associated with changes in Shh signaling activity. Altered Shh activity can result in a wide spectrum of phenotypic presentation of a number of defects; these mice provide an excellent model for lobar HPE as well as for further investigation of the role of Vax1 in the regulation of Shh signaling.

During craniofacial development, there is a complex, highly orchestrated morphogenetic process involving TGF-β, BMP, Fgf, Wnt as well as Hh signaling pathways. These pathways all respond to extracellular factors and environmental cues (Gou et al., 2015). Both Vax1 and Gas1; Vax1 mouse models demonstrate variations of lobar HPE with Vax1−/− demonstrating a range of defects of the developing CNS, secondary palate, olfactory and optic systems. Further reductions in Shh availability in Gas1−/−;Vax1−/− mice result in absence of maxillary incisors as well as other exacerbations in defects of the midline most notably the nasal capsule. Other mouse models of HPE exist which are based on these major signaling pathways (Hayhurst and McConnell 2003; Geng and Oliver 2009). It has previously been demonstrated using a mouse model that different strains of mice may demonstrate a phenotype if they have a mutation in a second gene—demonstrating an example of the 'second-hit' model (Ming and Muenke 2002; Tenzen et al., 2006; Zhang et al., 2011). Furthermore, it has been shown that administration of varying doses of teratogens in phenotypically normal mutant mice may produce a range of phenotypes reflecting the spectrum of HPE seen in patients, suggesting that a sensitizing mutation synergizes with a teratogenic or subteratogenic environment to produce HPE (Hong and Krauss 2012). Therefore the Vax1, and indeed, the Gas1;Vax1, mouse serves as an interesting tool to examine further the aetiology of HPE.

It has previously been demonstrated in animal studies that Vax genes are involved in the control of DV patterning of the ventral forebrain and its derivatives, including the corpus callosum, optic chiasm, optic nerve and retina, and that they drive the differentiation of these ventral structures by inhibiting the differentiation of dorsal structures (Takeuchi et al., 2003; Vacik et al., 2011). It was subsequently hypothesized that Vax genes might function in DV specification of the forebrain and facial structures through their ability to induce antagonists of canonical Wnt signaling (Vacik et al., 2011). Interestingly, it has been shown that disruption of canonical WNT signaling is also associated with CL/P both in mice and humans (Song et al.,
2009; Lipinski et al., 2010; Jin et al., 2012). Mutations in genes that disrupt Shh and Wnt signaling have been identified in both mice and humans with cleft lip. In addition, it has been shown in mice that altered Hh signaling contributes to the etiology and pathogenesis of cleft lip through antagonistic interactions with other gene regulatory networks, including the Wnt signaling pathways mediated, in part, by the Vax1 gene (Kurosaka et al., 2014). Despite various mechanisms or signaling pathways that have been proposed to be the cause of cleft lip, a detailed mechanism that links individual signaling pathways to cleft lip has not been fully realized. A recent study has identified Vax1 as a novel downstream target for FGF signaling in the face (Goetz, 2012). Further investigation into the role of Vax1 in the development of the upper lip could help to elucidate both the role of Vax1 within the context of this highly organized process involving several of the major signaling pathways.

Furthermore, Vax1 has been identified as an important gene in the development of the olfactory system and in regulation of fertility in the mouse (Goetz, 2012; Hoffmann et al., 2014; 2016). All three forms of olfactory epithelia (main, VNO, and respiratory) epithelia and several migratory cell populations- among them Gonadotropin-releasing hormone (GnRH-1) producing neurons - are derived from the olfactory placodes. GnRH-1 neurons originate on the medial nasal process, a highly specific region of Vax1 expresson and are incorporated into the developing VNO. From there, they migrate into the CNS and regulate sexual maturation and fertility via the hypothalamic-pituitary-gonadal axis (HPG). Vax1 is expressed in the anterior hypothalamus adjacent and rostrally to a region of Shh expression in this area of the developing forebrain during midgestation. Vax1−/− mice demonstrate pituitary defects with the formation of an ectopic Rathke's pouch. Vax1 controls fertility in that Vax1 is required for maintenance of Gnrh1 gene expression and deletion of Vax1 from GnRH neurons leads to complete infertility. The Vax1−/− mouse may be further utilized as a mouse model of IHH (idiopathicic hypogonadotropic hypogonadism) (Hoffman et al., 2014) and further investigation of the role of Vax1 in olfaction and in the regulation of sexual maturity may be carried out. Interestingly, the role of Vax1 during development of the pituitary gland was previously believed to be indirect as Vax1 was not reported to have been expressed in the oral ectoderm (Bharti et al., 2011) However, from our expression studies and in other studies (Hallonet et al., 1998), we know Vax1 to be expressed in the rostral oral ectoderm during formation of Rathke's pouch and it may be suggested that Vax1 has a more direct role during the early development of the pituitary gland. Finally, it has been
noted that pituitary defects are phenotypic traits that have been routinely observed in HPE as well as in studies involving other HPE modifier genes such as *Gas1* (Seppala et al., 2007). Shh expression is found surrounding the developing pituitary gland, where it induces proliferation of and expression of *Bmp2* within the developing Rathke's pouch. *Bmp2* induces expression of a number of ventrally expressed genes that take part in determining cell types of the anterior pituitary (Treier et al., 2001; Bharti et al., 2011). Further examination of the role of *Vax1* during the development of the pituitary may be undertaken by both analysis of Shh and Fgf signaling in the area of the Rathke's pouch as well as examining expression of early pituitary gland marker gene expression (such as *Bmp2*, *GATA2*, and *Prop1*) in *Vax1*−/− mice in both the normal and ectopic pituitary at E10.5-11.5.
9 Bibliography


de Aquino, S.N. et al., 2013. Polymorphisms in FGF12, VCL, CX43 and VAX1 in Brazilian patients with nonsyndromic cleft lip with or without cleft palate. *BMC medical genetics*, 14, p.53.


Bibliography


Butali, A. et al., 2013. Replication of genome wide association identified candidate genes


Edison, R.J. & Muenke, M., 2004a. Central nervous system and limb anomalies in case reports


Endoh-Yamagami, S. et al., 2009. *The Mammalian Cos2 Homolog Kif7 Plays an Essential Role in Modulating Hh Signal Transduction during Development*.


Francis-West, P.H., Tatla, T. & Brickell, P.M., 1994. Expression patterns of the bone morphogenetic protein genes Bmp-4 and Bmp-2 in the developing chick face suggest a role


Hong, M. & Krauss, R.S., 2012. Cdon mutation and fetal ethanol exposure synergize to produce


Kim, N. et al., 2014. Regulation of retinal axon growth by secreted Vax1 homeodomain protein.


Mangold, E. et al., 2010. Genome-wide association study identifies two susceptibility loci for


Miyake, T., Cameron, A.M. & Hall, B.K., Detailed staging of inbred C57BL/6 mice between Theiler’s [1972] stages 18 and 21 (11-13 days of gestation) based on craniofacial development. *Journal of craniofacial genetics and developmental biology*, 16(1), pp.1–31.


Sasagawa, S. et al., 2002. Axes establishment during eye morphogenesis in Xenopus by coordinate and antagonistic actions of BMP4, Shh, and RA. Genesis (New York, N.Y. :
Bibliography


Taglialatela, P. et al., 2004. Compromised generation of GABAergic interneurons in the brains


