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Functional and regulatory network analysis of Pitx3 in *aphakia* – a mouse model for microphthalmia and Parkinson's disease

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In the name of Allah

the most merciful and compassionate

The most gracious and beneficient

Dedicated to my parents

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Abbreviations

Alk 3/6	activin receptor-like kinase 3/6
AMH	anti-Müllerian hormone
Ap-2α	activating enhancer binding protein 2 alpha
Apc	adenomatosis polyposis coli
Bmp4 / 7	Bone morphogenetic protein 4 / 7
BrdU	5-bromo-2'-deoxyuridine
Cdc42	cell division cycle 42 homolog
Cdh1	Cadherin-1
Cdh2	Cadherin-2
Cdkn1b	Cyclin-dependent kinase inhibitor 1B
Cdkn1c	Cyclin-dependent kinase inhibitor 1C
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
CHX10	ceh-10 homeo domain containing homolog
c-Maf	avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog
CNS	Central nervous system
CRBA4	Crystallin, beta A4
Crx	Cone-rod homeobox protein
Cryaa	Crystallin, alpha A
Cryab	Crystallin, alpha B
DA	Dopamine
DAT	Dopamine active transporter
DGFs	Growth and differentiation factors
DIG	Digoxigenin
DMEM	Dulbecco's Modification of Eagles Medium
DMF	di-Methyl formamide
DNA	Deoxy ribose nucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
E4f1	E4F transcription factor 1
ECR	Evolutionary Conservation of Genomes
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag

FC	Fiber cell
FGF	Fibroblast growth factor
FGFR1/3	Fibroblast growth factor receptor 1/3
Foxe3	forkhead box E3
GDFs	Growth and differentiating factors
GDFs	Growth and differentiation factors
GFP	Green fluorescent protein
HEK	Human embryonic kidney
Hes1 / 5	Hairy and enhancer of split 1 / 5
ICN	Intracellular domain of Notch
IPTG	Isopropyl-β-D-thiogalactopyranoside
Jag1	jagged 1
L-DOPA	L-3,4-dihydroxyphenylalanine
Lrp 5/6	Low-density lipoprotein receptor-related protein 5/6
LRRK2	Leucine-rich repeat kinase 2
LV	Lens vesicle
meDA	Meso-diencephalic encephalic dopaminergic neurons
MEF	Mouse Embryonic Fibroblast
MGI	Mouse Genome Investigation
MIP	Major intrinsic protein
Mitf	microphthalmia-associated transcription factor
NCBI	National Center for Biotechnology Information
NR	Neuro-retina
NTE	Nitrium, Tris, EDTA
Nurr1	Nuclear receptor related 1 protein
OAR	otp, aristaless, and rax
Otx2	Orthodenticle homeobox 2
PARK2	Parkinson protein 2, E3 ubiquitin protein ligase (parkin)
PARK7	Parkinson disease (autosomal recessive, early onset) 7
PD	Parkinson's disease
Pax6	Paired box gene 6
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Pfu	*Pyrococcus furiosus*

PINK1	*phosphatase and tensin homolog (PTEN)-induced putative kinase 1*
Pitx1-3	Pituitary homeobox 1-3
Pitx1-3	paired-like homeodomain transcription factor 1-3
POMC	Pro-opiomelanocortin
Prox1	prospero homeobox 1
Rac	Ras-related protein
RBP-Jk	Rrecombination signal binding protein for immunoglobulin kappa J region
Rho	Ras homolog
RNA	Ribonecleic acid
RNase	Riboneclease
RPE	Retinal pigment epithelium
rpm	Revolutions per minute
RT-qPCR	Reverse transcription-quantitative PCR
SDS	Sodium dodecyl sulfate
Six3	Sine oculis homeobox homolog 3
SN	Substantia nigra
SNCA	Synuclein, alpha
SNP	Single necleotide polymorphism
SNpc	Substantia nigra pars compacta
Sox2	SRY (sex determining region Y)-box 2
SPA	Stimulus presentation apparatus
SSC	saline-sodium citrate
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TBS	Tris-buffered-saline
TBST	Tris-Buffered Saline and Tween 20
TE	Tris-EDTA
TESS	Transcription Element Search Software
TGFβ	Transforming growth factor, beta
Th	Tyrosine hydroxylase
Tube1	Tubulin, epsilon 1
UCHL1	Ubiquitin carboxy-terminal hydrolase L1
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
Wnt1	Wingless-type MMTV integration site family, member 1

Summary

Pitx3 is a paired-like transcription factor critical for the development of meso-diencephalic dopaminergic neurons (meDA) and the ocular lens. In humans, polymorphisms in *PITX3* are associated with Parkinson's disease and mutations are responsible for cataracts and varying degree of anterior segment abnormalities. In *aphakia* mice, two deletions in the promoter region of *Pitx3* cause abnormal lens development resulting in the loss of lens in adult. However, the molecular changes in this mutant are not yet revealed. In this study, I investigated the role of Pitx3 in lens development and its molecular targets responsible for abnormalities in *aphakia*. I have shown that lack of Pitx3 in *aphakia* results in reduced proliferation in the lens epithelium and aberrant fiber cell differentiation. This is demonstrated by the loss of Foxe3 expression, complete absence of Prox1 and earlier expression of γ-crystallins in the developing lens. By using luciferase reporter assay, I have shown that Pitx3 can bind to its evolutionary conserved putative binding sites on the 5'-upstream region of *Prox1* and *Foxe3* and directly regulate their expression. Remnants of the lens stalk, which is an important feature of *aphakia* lens seems to be caused by the reduced expression of *Foxe3* and *Tcfap2a* (*Ap-2α)*, which has also found to be directly regulated by Pitx3 at the phase of lens vesicle separation.

Another feature of the *aphakia* lens is that the lens lumen is filled with cells, which is attributed to the loss of cell-cell contact as a result of reduced expression of E-cadherin. Additionally, loss of ε-tubulin which has been detected as a novel target of Pitx3 causes malformation of the lens vesicle as a result of miss-orientation of the mitotic apparatus. Interestingly, defects in *aphakia* during eye development are not confined to the lens only but also found in the retinal pigment epithelium (RPE), where altered expression of Otx2 has been reported for the very first time. Furthermore, I also explored the genetic and molecular interaction between *Pitx3* and *Pax6*, the master controlling gene of lens development, and found that Pitx3 has an inhibitory action on the expression of *Pax6*.

Thus, this study has shown that Pitx3 is regulating various downstream target genes to influence the development of lens including; proliferation, maintenance and cell survival. Identification of Pitx3 targets has helped us to understand the molecular and pathological events in the *aphakia* lens and to develop the regulatory cascade during lens development. These target genes can also be extrapolated to other expression domains of Pitx3, like midbrain and skeletal muscles to decipher its role in these organs.

Zusammenfassung

Pitx3 ist ein *paired-like*-Transkriptionsfaktor, der für die Entwicklung von meso-diencephalische dopaminerge Neurone (mdDA) und der Augenlinse notwendig ist. Beim Menschen sind PITX3-Polymorphismen mit der Parkinsonkrankheit assoziiert und PITX3-Mutationen sind verantwortlich für Kataraktentstehung und unterschiedlich stark ausgeprägte Erkrankungen des vorderen Augenabschnittes. In der *aphakia*-Mausmutante verursachen zwei Deletionen in der Promotorregion von *Pitx3* Störungen der Linsenentwicklung, die zu einem Verlust der Linse in adulten Tieren führt. Allerdings sind die molekularen und morphologischen Veränderungen in dieser Mutante bisher noch nicht aufgeklärt worden. In der vorliegenden Arbeit wurde die Rolle von Pitx3 bei der Linsenentwicklung und die für die Veränderungen in der *aphakia*-Mausmutante verantwortlichen Zielgene von Pitx3 untersucht. Es konnte gezeigt werden, dass der Verlust von Pitx3 in der *aphakia*-Mausmutante zu reduzierter Proliferation im Linsenepithel und fehlerhafter Differenzierung der Linsenfaserzellen führt. Dies ist mit einem Verlust der Foxe3 und Prox1-Expression und verfrühter γ-Kristallin-Expression in der sich entwickelnden Linse verbunden.

Durch Verwendung eines Luciferase-Reporterassays konnte gezeigt werden, dass Pitx3 an seine evolutionär konservierten, vermutlichen Bindestellen in der 5`-stromaufwärts gelegenen Region von *Prox1* und *Foxe3* binden kann und ihre Expression direkt reguliert. Überreste des Linsenstiels, die ein wesentliches Merkmal der *aphakia*-Linse darstellen, scheinen durch die verringerte Expression von Foxe3 und Ap-2α verursacht zu werden. Ap-2α wird in dieser Phase der Linsenvesikeltrennung ebenfalls direkt durch Pitx3 reguliert.

Eine weitere Eigenschaft der *aphakia*-Linse ist, dass das Linsenlumen mit Zellen gefüllt ist. Dies ist auf den Verlust von Zell-Zell-Kontakten als Folge der reduzierten E-Cadherin-Expression zurückzuführen. Zusätzlich verursacht der Verlust von ε-Tubulin, welches als neues Zielgen von Pitx3 ermittelt wurde, eine Fehlbildung des Linsenvesikels als Folge der Missorientierung des mitotischen Apparates. Interessanterweise beschränken sich bei der *aphakia*-Mutante die Defekte während der Augenentwicklung nicht ausschließlich auf die Linse, sondern sich auch auf das retinale Pigmentepithel, für das erstmals eine veränderte Otx2-Expression beschrieben wurde. Des Weiteren wurden die genetischen und molekularen Interaktionen zwischen *Pitx3* und *Pax6*, einem Hauptkontrollgen der Linsenentwicklung, untersucht. Es konnte festgestellt werden, dass Pitx3 eine hemmende Wirkung auf die *Pax6*-Expression aufweist.

Somit konnte diese Arbeit zeigen, dass Pitx3 verschiedene stromabwärts gelegene Zielgene reguliert, um die Linsenentwicklung, bezüglich Proliferation, Erhaltung und Überleben der Zellen zu beeinflussen. Die Identifizierung molekularer Pitx3-Zielgene trägt dazu bei, die

molekularen und pathologischen Vorgänge in der *aphakia*-Linse zu verstehen und die molekulare Signalkaskade während der Linsenentwicklung zu identifizieren. Diese Zielegene können auch auf andere Expressionsdomänen von Pitx3 übertragen werden, wie das Mittelhirn und die Skelettmuskulatur, um die Rolle von Pitx3 in diesen Organen aufzuklären.

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1 Introduction

Pitx3 is a paired-like homeodomain transcription factor; it belongs to the *RIEG / Pitx3* homeobox gene family. This gene is mapped to chromosome 19 in the mouse and is a homolog of human *PITX3*. Sequence analysis revealed that 99% amino acids are identical to human while the homeodomain is fully conserved [1]. The mouse *Pitx3* gene comprises of four exons and encodes a protein of 302 amino acids, consisting of a 60aa chain of DNA-binding homeobox domain and an OAR motif of 14aa (Fig. 1.1). This OAR domain, named for the first three members (orthopedia, aristaless, and rx) of the paired–typed homeobox factors containing this domain, acts as an intra-molecular switch for the activity of these transcription factors [2,3]. The homeodomain box is identical in the Pitx protein family and contains an important lysine residue. This lysine residue is critical for the recognition of the TAA(T/G)CC motif [4,5] that is present in the promoter region of most of their target genes [6,7].

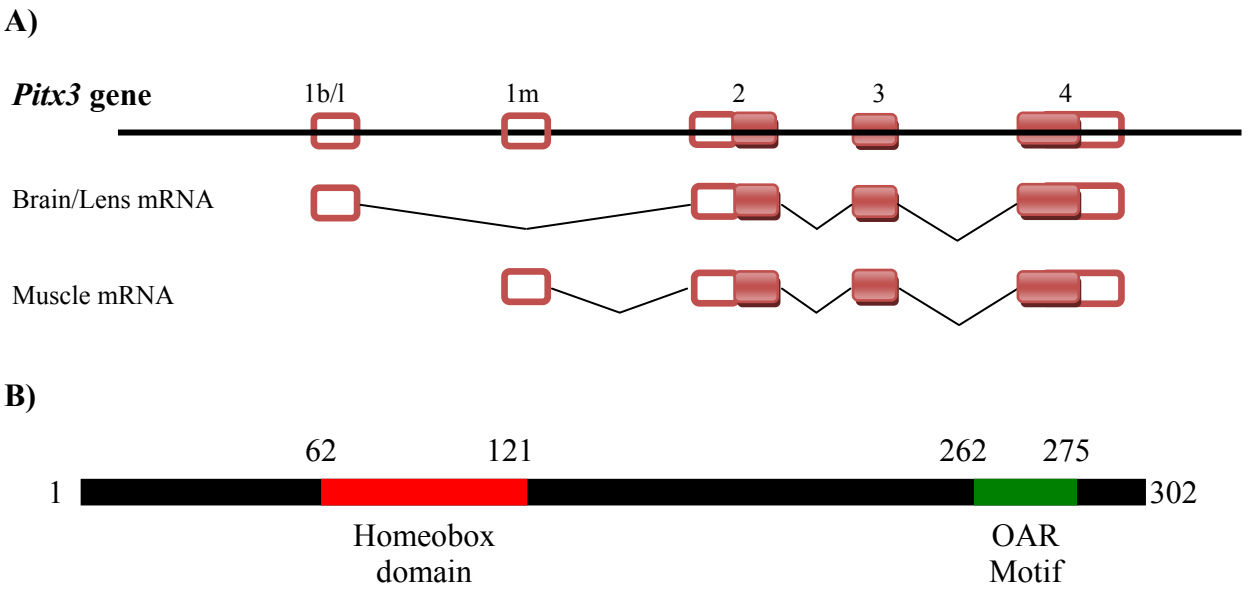


Figure 1.1: Map of mouse *Pitx3* gene with its transcript variants and protein structure. Exons are shown in pink coloured boxes with their numbers mentioned on the top (A). The empty and filled pink coloured boxes represent non-coding and coding exons, respectively. Two transcript variants differ in their 1st exon, the one present in the brain and lens is represented as 'b/l' and the other present in the muscle is shown as 'm' (modified from Coulon et al. [8]). The transcript encodes a protein of 302aa that contains a DNA binding homeodomain and an OAR domain (B) (http://www.uniprot.org).

Expression analysis has shown that *Pitx3* is expressed in the midbrain dopaminergic neurons [9], ocular lens [10] and skeletal muscle cells [8]. The muscle transcript is a variant of the midbrain and eye transcript with 1st exon from the mid of 1st intron of the latter transcript. However, the coding transcript is homologous with all the domains conserved in these expression areas. Due to very specific expression pattern its association has been studied in many ocular and neuronal disorders relevant to dopaminergic neurons but did not gained much attention in muscular

disorder. These disorders are hampered by the mutations or single nucleotide polymorphisms (SNPs) within *PITX3* gene itself or in its promoter region. Various epidemiological studies have found an association of *PITX3* promoter SNP (rs3758549) with Parkinson [11-14]; a disease with loss of dopaminergic neurons (described in section 1.3). In addition to this, SNPs in the coding (rs2281983) as well in the intronic region (rs4919621) have also been identified as risk factors for Parkinson [15]. So far, no SNP in this gene has been linked to the ocular disorders. However, three mutations in human *PITX3* have been reported in different studies that cause varying degree of ocular phenotype. These mutations affect either N- or C-terminal region of Pitx3. The only N-terminal mutation is a single nucleotide substitution (*S13N*), identified in a family with an autosomal-dominant congenital cataract [1]. The other two mutations are; a single nucleotide deletion (650delG) [16,17] and the most frequently observed 17 bp duplication (657-673dup17) [1,16,18]. These latter two mutations affect the OAR domain of *PITX3*, thus affecting its interactions with other proteins and hindering its functionality that results in various defects. Although, posterior polar cataract is the major feature of these mutations yet a number of patients also show anterior segment mesenchymal dsygenesis (ASMD) [1]. However, the only *Pitx3* homozygous mutation (650delG) reported so far in humans [17] exhibits more severe phenotype with microphthalmia and neurological deficits like, mental retardation, weak reflexes, increased muscle tone and body disequilibrium. In addition, mutations in this gene have also been reported in mice and sheep that show severe microphthalmia and are discussed later in more detail (see section 1.2 & 1.4).

1.1 Microphthalmia

Microphthalmia, as the name indicates (micro = small; ophthalmos = eye) is a developmental disorder characterized by a small eye. It can be unilateral, affecting one eye, or bilateral, affecting both eyes. Human epidemiological studies have shown that 30 per 100,000 childrens are affected with congenital microphthalmia, and its prevalence in blind childrens is 3-11% [19]. The disorder is congenital, involving majorly genetic factors with varying degree of severity and in most severe cases can result in anophthalmia [19,20], a condition where eyes are completely missing. Genetic analysis in humans has revealed some regulatory factors implicated in this disease and are critical for eye development. Some of the important genes identified so far in anophthalmia and microphthalmia include, *PAX6*, *SOX2*, *FOXE3*, *OTX2*, *MITF*, *CHX10, CRYBA4* and *PITX3*. Additionally, a missence mutation in *PITX3* has also been linked with this disease in Texel sheep [21]. Pathogenesis of this disease is not clear so far; however, from morphological studies it is evident that defects in lens development is a major cause of microphthalmia and anophthalmia [22].

1.2 Lens development

Lens development is a complex process involving various factors including many signalling cascades. During lens development series of events take place in the optic vesicle. Formation of optic vesicle is the earliest stage of eye development and induces lens development. It develops from either side of forebrain as a diverticulum that expand laterally into the mesoderm of head. As this process proceeds, thickening of surface ectoderm takes place forming a lens placode (Fig. 1.2 A). The lens placode then starts invaginating and forms the lens vesicle which is then separated from the surface epithelium (Fig. 1.2 B). Meanwhile, the cells from the posterior side of the lens start elongating in the lens cavity and form primary fiber cells (Fig. 1.2 C). But the cells from the anterior side of the lens keep on dividing and give rise to secondary fiber cells (Fig. 1.2 D). These fiber cells express large amount of crystallins and become transparent forming the complete lens [23].

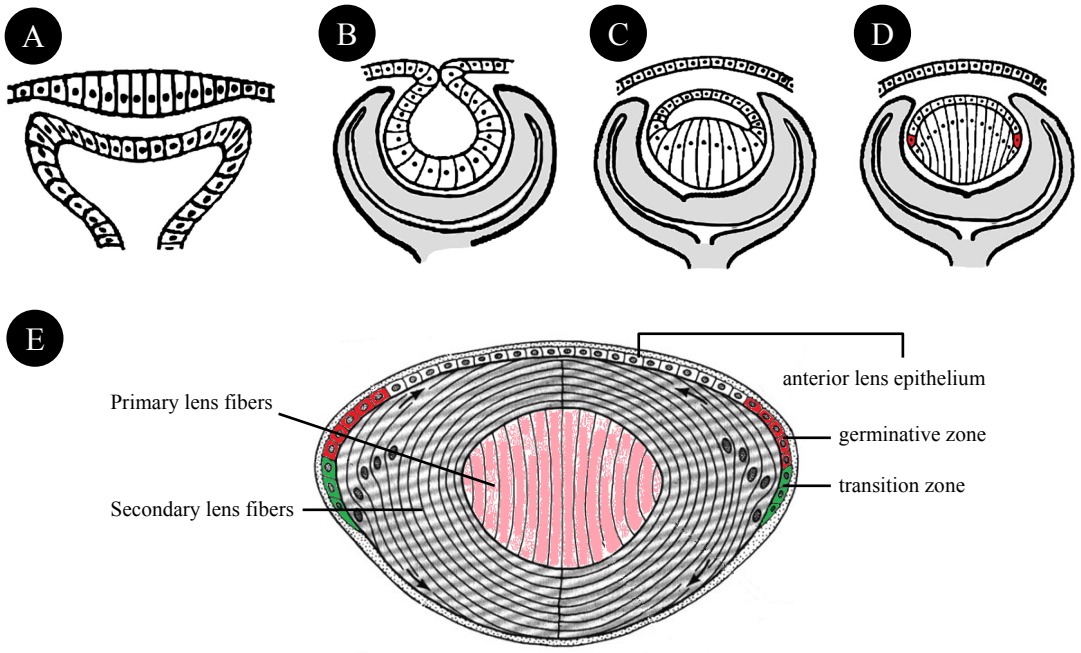


Figure 1.2: Different stages of lens development. Lens placode formation is the 1st step in lens developemt (A), followed by the formation of lens vesicle (B). As the lens vesicle formed cells form the posterior side start elongating and form the primary lens fibers (C). Anterior lens epithelial cells keep on diving at the equatorial region (shown in red color) and differentiate into secondary fiber cells (D) that organize in crescentric form around the primary lens fibers (E). These fiber cells express crystallins and become transparent forming the complete lens (E). (modified from Lang [24], Paton & Craig [25] and http://www.mc.vanderbilt.edu/)

Lens development is a complex processes involving cascade of events from pluripotent cells to mature lens fiber cells. These events are orchestrated by the input of various transcription factors, signalling cascades, and structural and functional proteins. Some of the important factors are discussed below.

1.2.1 Transcription factors in lens development

a) Pax6, a key regulator of eye development

Pax6 is a paired homeodomain transcription factor mapped to mouse chromosome 2. This gene is transcribed by three promoters: P1, P0 and Pα (Fig. 1.3A) regulated mainly by a variety of tissue specific enhancers (P0 by lens, cornea, surface ectoderm and pancrease; P1 by forbrain, hindbrain, spinal cord; Pα by neurons, neuroretina, retinal pigment epithelium and Iris) [26]. In addition to the usage of different promoter *Pax6* has many isoforms; however, two isoforms are studied extensively: Pax6-1 and Pax6-5a. The 5a isoform has an insertion within the paired domain (Fig. 1.3) resulting in an altered DNA binding [27]. Both of these Pax6 isoforms are expressed in the lens and present in equal amount in human lens [28] but in mice, Pax6-1 is the predominant from [29]. *Pax6* is positioned at the top of hierarchy of the factors determining the ocular morphogenesis [30,31], as its ectopic expression in *Drosophila* (eyeless, a homolog of Pax6) [32] and *Xenopus laevis* [33] results in the formation of ectopic eyes and lens respectively. Pax6 is widely expressed during the neural plate stage regulating embryogenesis and is critical for the eye development [30,34]. Its expression is detected from E8.5 in the surface ectoderm of the presumptive eye region [35,36]. However, as the development proceeds its expression is downregulated in the surface ectoderm and lens fiber cells and restricted to the lens epithelial cells [37].

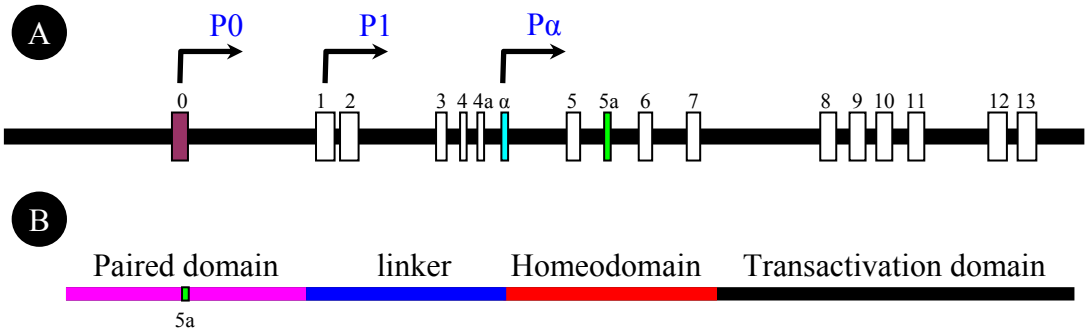


Figure 1.3: Map of the mouse *Pax6* gene with its transcripts. This gene has 13 exons and various isoforms as a result of different splicing events and use of different promoters mentioned in blue color (A). The protein has different domains shown in different colors. They are conserved among species (B). The paired domain has an insertion of exon 5a in the *Pax6*-5a isoform. [38-40].

Mutations in the *Pax6* gene cause various abnormalities. Heterozygous mutation in this gene results in aniridia in human, a severe congenital abnormality, and micropthalmia in mice ($Sey^{+/-}$) and rat, while homozygous mutations give rise to anophthalmia in both human and mice. $Pax6^{-/-}$ homozygous mice exhibit severe facial and head abnormalities and die perinatally, as they can not breath due to the lack of nose [41]. Moreover, overexpression of Pax6 also results in micropthalmia in mice. Further experiments using a conditional knockout approach in mice have

shown arrested development of lens. These studies suggest that Pax6 is crucial for lens placode formation and has a cell autonomous role in lens proliferation and differentiation [42,43].

b) Sox2 *(SRY-box2)*

Sox2 also known as SRY (sex determining region Y)-box2 belongs to a super-family of transcription factors. This family has 20 members in human and mice and share at least 50% homology with HMG (high mobility group)-box, a DNA binding domain highly conserved in eukaryotes [44]. This family has several sub-families depending on the similarity of their structural motifs. It has been observed that Sox proteins need some partner factors to activate transcription of their target gene to regulate developmental processes [45-47].

Sox2 belongs to the sub-family 'B' and is encoded by an intronless gene, mapped to human chromosome 3q26.3-q27. In the mouse, it is located on chromosome 3 at 34.84 Mb and has 3 variants, encoding similar proteins (Fig. 1.4).

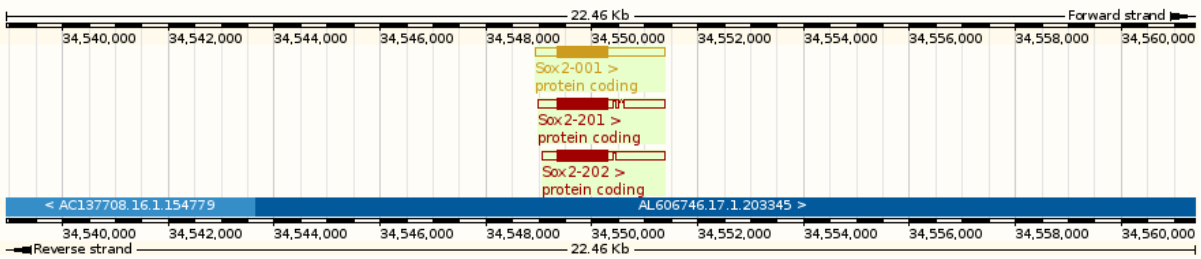


Figure 1.4: Map of the *Sox2* gene with its transcript variants. Different transcript variants are shown in red color. Exons are shown as boxes and the introns as lines. Filled boxes indicate the coding region while empty boxes represent non-coding exons (http://www.ensembl.org).

Sox2 is expressed from the early embryonic stages in the CNS overlapping with its other sub-family members, *Sox1* and *Sox3* from the early embryonic stages [48]. In the lens, expression of Sox2 starts before the lens placode formation along with Sox3. However, as the development proceeds its expression is restricted to the lens epithelium while reduced in the lens vesicle. This reduction in Sox2 level is accompanied by the initiation of *Sox1* expression that persists throughout lens morphogenesis with higher expression in the lens fiber cells [49]. Sox2 is involved in the regulation of different genes during lens development depending on the presence of different co-factors [50-52].

Importance of *Sox2* in lens development is evident from many human studies where various mutations in this gene have been linked to microphthalmia and anophthalmia [53-59]. Mice lacking *Sox2* die *in utero* and do not develop eyes [60].

c) *Tcfap2a* (Transcription factor activating protein-2α)

Ap-2α belongs to a family of retinoic acid responsive transcription factors. The family has five members, Ap-2α, AP-2β, Ap-2γ, Ap-2δ, and Ap-2ε, which are encoded by *Tcfap2a*, *Tcfap2b*, *Tcfap2c*, *Tcfap2d* and *Tcfap2e*, respectively. All these genes have distinct expression patterns and are crucial for many developmental processes.

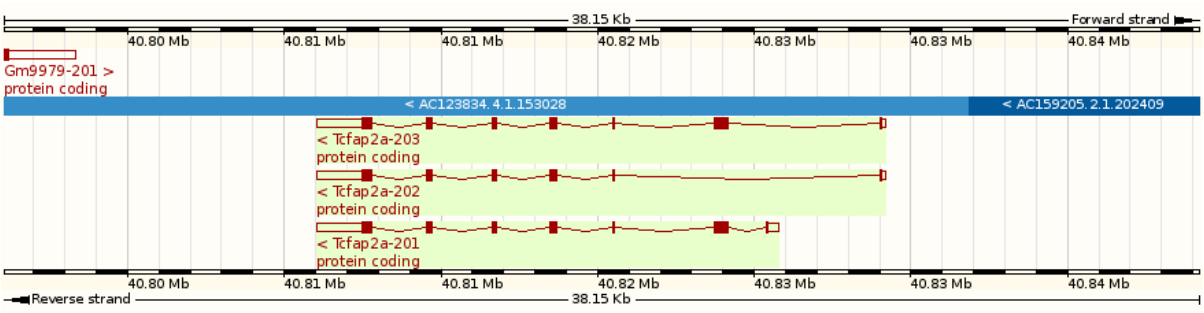
Tcfap2a is the first member of this family that was cloned and mapped to the mouse chromosome 13A5-B1 [61,62]. The human homolog is located on chromosome 6p24 [62,63].

The main Ap-2α isoform consists of 437 amino acids and has a molecular weight of 52 kDa. TFAP2A proteins contain a unique, highly conserved helix-span-helix dimerization motif at the C-terminal half of the protein, a central basic region and a less conserved proline- and glutamine-rich domain at the amino terminus. The helix-span-helix motif and the basic region mediate DNA binding and dimerization while the proline- and glutamine-rich region is responsible for transcriptional transactivation (Fig. 1.5).

Ap-2α is expressed in the developing eye including surface ectoderm and neural plate cells and their derivatives [64,65]. Deletion in the gene causes microphthalmia, corneal clouding and other congenital anomalies [66]. Experiments done using gene targeted approach to explore the role of this gene in different developmental processes have shown that *Ap-2α* is required for the formation of eye, face, body-wall, neural plate, fore-limbs and cardiovascular system [67-71] in line with its expression pattern.

Tcfap2a null mice exhibit craniofacial and eye abnormalities including anophthalmia starting from post coitum day 9.5, and die perinatally. However, detailed ocular analysis have shown that they possess persistent lens stalk, a condition where the ocular lens remains attached with the surface ectoderm, which was confirmed by using lens specific conditional knockout approach [72]. But the molecular mechanisms that lead to these defects are still not fully revealed. However, it is believed that these defects could be mediated partially through the regulation of cell adhesion molecules, including cadherins. In the ocular lens, E-cadherin is expressed in the lens epithelium while N-cadherin is expressed in the fiber cells as well [73]. *Ap-2α* has putative binding sites in the 5'-upstream region of these cadherins and other epithelial genes (like keratin 14) and can regulate the expression of these genes *in vitro* [72,74-79].

A)



B)

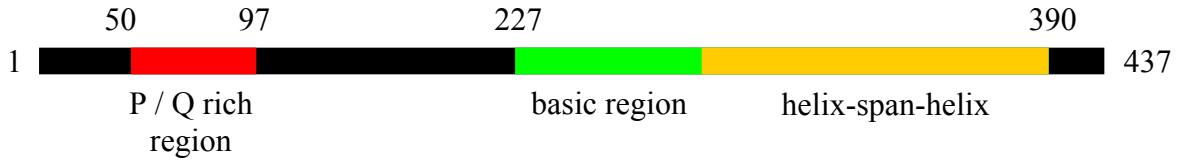


Figure 1.5: Map of the *Tcfap2a* gene with its transcript variants and main Ap-2α isoform. Different transcript variants are shown in red color (A). Exons are shown as boxes and the introns as lines. Filled boxes indicate the coding region while empty boxes represent non-coding exons (http://www.ensembl.org). *Ap-2α* has different domains for its activity which are shown here with respect to the main variant (C) (Modified from Williams and Tjian, 1991).

d) Foxe3

Foxe3 is a member of forkhead transcription factors and was first described by Larsson et al. [80]. The characteristic feature of these factors is the presence of 80 to 100 amino acids forming a DNA binding motif [81], thus influencing the expression of genes involved in cell growth, proliferation, differentiation and survival [82,83] either as activators or repressors.

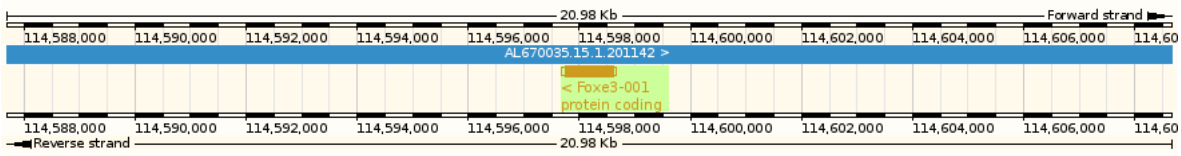


Figure 1.6: Map of the *Foxe3* gene with its transcript. This gene is an intronless with coding region shown in yellow filled box and the noncoding as an empty box (http://www.ensembl.org).

The *Foxe3* gene is located on chromosome 4 in the mouse, while its human homolog is present on chromosome 1p32 transcribing an intronless single frame mRNA [81,84] (Fig. 1.6). Murine *Foxe3* expression is detectable from post coitum day 9.5 in the lens placode and is limited to the anterior proliferating lens epithelium from E14.5 and persists in the adult lens. *Foxe3* mutants, *dyl* (symbol for dysgenetic lens) appeared spontaneously in the Balb/C strain and exhibit many congenital defects and have small eyes. In these mice, lens vesicle fails to detach from the overlaying epidermis [85,86]. Mutations in this gene in humans cause congenital primary *aphakia* [87]

e) Prox1 (Prospero homeobox protein 1)

Prox1 is a homeodomain transcription factor, it was first cloned as a homologue of the *Drosophila* gene *prospero*. Murine *Prox1* is mapped to chromosome 1 at position 106.3cM [88] (Fig. 1.7) and its human homolog is mapped at position 1q32.2-1q32.3 [89]. Both the homologues encode a protein of 737 amino acids, which are 98% identical [88].

Prox1 is highly expressed in the endothelial cells of the lymphatic system [89] and is considered a marker for these cells [90]. However, its expression has also been observed in mouse CNS, skeletal muscles, liver, pancreas and lens during different developmental stages [91].

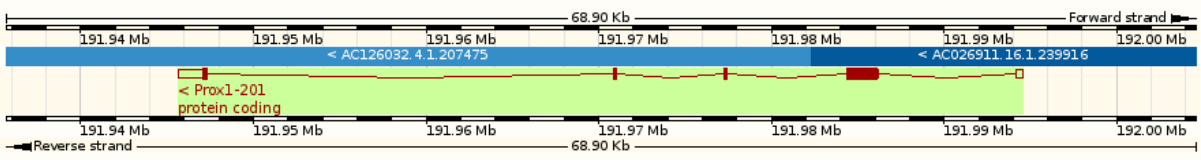


Figure 1.7: Map of *Prox1* gene with its transcript. Transcript is shown in red color with its exons shown as boxes and the introns as lines. Filled boxes indicate the coding region while empty boxes represent non-coding exons (http://www.ensembl.org).

In the lens, expression of *Prox1* is first detected in the lens placode at post coitum day 9.5 [92] and continues at later stages, becoming more intense at E11.5 and E12.5 in the posterior portion of the lens vesicle [93], a region of fiber cell elongation. Mouse homozygous for Prox1-null protein die at the mid-gestation due to various developmental defects [92]. Targeted inactivation of *Prox1* by inserting β-galactosidase gene has revealed severe defects in lens fiber formation. These mice exhibit deficits in cell cycle withdrawal from the posterior part of the lens as a result of down-regulation of cell cycle inhibitors *Cdkn1b* and *Cdkn1c* and show inappropriate apoptosis. Additionally, these mutant lenses retain the expression of E-cadherin, which is lost during fiber cell differentiation under normal circumstances providing evidence that Prox1 activity is necessary for the terminal fiber cell differentiation and elongation [92].

f) Pitx3

Structure of Pitx3 gene has already been discussed in the previous section. In the lens, its expression starts around the developmental day E9.5 [94,95] in the lens placode and is expressed in all lens vesicle cells. However, at latter stages and in the adult lens its expression is restricted to the anterior lens epithelium. The role of *Pitx3* in lens development is not yet clear but it is important for the normal development of lens, as evident from the mutant mice studies. The first spontenous mouse mutant, *aphakia* (explained latter) show small eyes that lack lenses [96]. Experiments using GFP tagged Pitx3 have shown that it is crucial to maintain the characteristic progenitor lens epithelial [97]. Recently, appearance of another *Pitx3* spontaneous mutant mouse

(eyeless) in the C3H strain provides additional evidence for the role of this gene in lens development. These mice have a point mutation in the 4th exon [98] hindering the functional impact of this gene. *Eyeless* (*eyl*) mice show a similar phenotype as *aphakia* but more details are known because of its comprehensive screening in the German Mouse Clinic (GMC), Helmholtz Center Munich.

g) Other transcription factors important for lens development

Otx2 is a homeobox gene related to the *Drosophila* gene *orthodenticle* (*otd*) expressed in the developing head [99-101]. In the mouse eye, it is expressed in the optic vesicle but latter it is restricted to the retinal pigment epithelium (RPE) and neuro-retina (NR). *Otx2* null mice die because of severe malformations of the head region; however, analysis of the *Otx2* heterozygous mice show wide range of ocular abnormalities including microphthalmia and anophthalmia [102-104].

Six3 is a homeobox gene, essential for the murine eye development [105]. In mice, its expression is observed in the developing lens at the lens placode stage and is restricted to the lens epithelium as the development proceeds. Misexpression of murine *Six3* results in ectopic lens formation in otic vesicle in Medaka [106]. Additionally, ectopic *Six3* expression in mice promotes the formation of ectopic optic vesicle-like structures in the hindbrain-midbrain region of developing embryo [107]. Although the exact role of this gene in lens development is not clear; however, it may play an important role in maintaining the pluoripotency of lens epithelium by suppressing γ-crystallin expression [108].

Retinoic acid receptors (RARs) are ligand-inducible transcription factors and required for lens development. Overexpression of RAR receptors in the lens lead to cataract in mice [109], as they are required for the induction of the γ- and α-crystallin expression [110-112].

1.2.2 Signalling molecules in lens development

Lens development is a complex process involving the input of many signalling pathways. Important signalling cascades playing their role in lens induction and differentiation are: transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP) families, fibroblast growth factors (FGF), Wnt and Notch.

h) BMP/TGF-β signalling

The TGF-β superfamily of ligands include: Bone morphogenetic proteins (BMPs), Growth and differentiation factors (GDFs), Anti-müllerian hormone (AMH), Activin, Nodal and TGFβ's. A number of these ligands and their receptors are expressed in the lens and surrounding tissues that

regulate the multiple stages of lens development [113,114] through the activation of Smad signalling pathway. Inhibition of TGF-β signalling in mice results in impaired expression of fiber specific proteins in the lens including MIP, filensin and phakinin. Additionally, *in vitro* analysis of these lenses revealed defects in cytoskeleton organization and cell migration; demonstrating the role of this signalling in the terminal differentiation of fiber cells [114]. These lines of evidences were further supported by studies on chick [115] and mice [113], where disruption of BMP signalling by treating the lens epithelial explants with noggin (a BMP antagonist) in an *in vitro* explant system results in the suppression of primary fiber cells. This supression can be recovered by adding exogenous BMPs (2, 4 and 7).

BMP4 and BMP7 are the most important members of TGF-β superfamily involved in lens development. *Bmp4* is expressed in the optic vesicle and is essential for lens induction as indicated by the lack of Sox2 in *Bmp4*-null mice [116]. Lens induction also does not take place in *Bmp7* mutant mice; however, this is due to the loss of Pax6 expression [117] contrary to the *Bmp4* mutants, where *Pax6* expression is normal [116]. Additionally, lens specific elevated expression of *Bmp7* in transgenic mice causes delay in lens fiber differentiation and degradation of neural retina (NR) as a result of apoptosis [118]. Moreover, inactivation and overexpression of BMP-receptor type 1a (*Alk3*) and 1b (*Alk6*) respectively results in defects in primary lens fiber differentiation [115,119].

i) FGF signalling

Fgf is a large family of growth factors involved in regulating cell proliferation, mobility and differentiation. There are 23 members of FGF in mammals, many of them are expressed in the eye and influence its development. Role of Fgf signalling in lens development is evident form various experiments. Expression of Fgf1 and Fgf3 in the lens induces the premature differentiation of lens epithelial cells with the expression of fiber cell specific proteins, MIP and β-crystallin followed by the degeneration of the entire lens [120,121]. Additionally, overexpression of FGF4, 7, 8 and 9 causes the lens epithelial cells to exit the cell cycle prematurely resulting in the formation of cataract [122].

Furthermore, alterations in FGF receptors also result in various effects on the lens development. Defects in lens placode formation has been observed by overexpressing the dominant-negative form of *Fgfr1* accompanied by the diminished expression of *Pax6*, *Sox2* and *Foxe3* [113]. Transgenic mice with a secreted version of ocular FGFR3 result in expansion of anterior proliferating lens epithelium to posterior with changed expression pattern of *cMaf*, *Prox1* and $p57^{kip2}$ (*Cdkn1c*) [123], indicating a gradient requirement of FGF in lens development. This notion is further supported by experiments on rats, where it has been shown to play a role in

determining lens polarity and growth in concentration dependent manner; requiring low for proliferation and high for differentiation [122].

j) Wnt signalling

The Wnt signalling pathway involves a series of events including large number of proteins that regulate many developmental and physiological processes. In mammals, there are 19 members of Wnt protein and 10 types of Frizzled receptors which require co-repressors, Lrp5/6 for their action [124]. Wnt signalling can act through the canonical pathway (Wnt/β-catenin) involving interaction with the transcription factor LCF/TCF or through the non-canonical pathway, involving interactions with GTPases, Rho, Rac and Cdc42 [125,126]. These enzymes participate in cytoskeletal rearrangements and may be involved in the lens fiber cell elongation. The impact of the Wnt pathway in lens development came from the studies on *Lrp6* mutant mice that show disrupted lens epithelium [127]. Further conditional mutations of β-catenin and *Apc* result in decreased lens epithelial cell proliferation [128,129], suggesting the role of canonical Wnt signalling in maintaining the lens progenitor cells.

k) Notch signalling

Notch signalling is a highly conserved pathway important for cell-cell communication. It is one of the major pathways involved in the maintenance of proliferation in different progenitor cells [130,131]. Four different Notch receptors (Notch 1-4) and five different ligands (Jagged 1-2 and Delta1, 2 and 4) have been characterized in mammals. Activation of Notch by its ligand results in the release of the intracellular domain of Notch (ICN) [132-134] that forms a complex with DNA binding protein RBP-J^k (in mouse) [135] and activates the transcription of downstream target genes. Most important target genes of Notch pathway are, *Hes1* and *Hes5* (mammalian *hairy* and *enhancer-of-split* homologues 1 and 5) [136,137].

Notch signalling plays an important role in lens development, as demonstrated by various studies. Mice having disruption in *Hes1* exhibit defects in early lens development of varied severity including microphthalmia [138,139]. In line with these, conditional deletion of RBP-Jk result in smaller lenses with premature cell cycle exit of lens epithelium and fiber cell differentiation accompanied by upregulation of $p57^{Kip2}$ expression [140,141]. Additionally, *Jag1* mouse mutants show abnormal proliferation and secondary fiber cell differentiation due to the loss of anterior lens epithelium [142,143].

1.2.3 Crystallins in lens development

Crystallins are water soluble proteins of the lens responsible for its transparency (reviewed by Graw during the last decade [20,144,145]). They are grouped into three main types; α, β and γ based on the order of chromatographic fractions and are encoded by genes present on different chromosomes. In reptiles and birds γ-crystallins are replaced by δ-crystallins [146,147].

α-crystallins are mainly composed of two proteins, αA- and αB-crystallin, encoded by *CRYAA* and *CRYAB* respectively in humans and *Cryaa* and *Cryab* in mouse. They are highly expressed in the mature lens staring during development from post coitum day 10 in the mouse with the αA-crystallin starting a day earlier. Mutations in *CRYAA* and *CRYAB* gene are responsible for cataractous lenses in human. These studies are supported by the evidence of *Cryaa*-knockout mice that also develop cataracts. However, *Cryab*-knockout mice do not develop cataracts and have transparent lenses. This anomaly could be because of additional complexity of interaction between various factors in human.

β- and γ-crystallins share many similarities in sequence and structure and thus grouped into a βγ-crystallins superfamily. β-crystallins are encoded by two groups of genes, *Cryba* and *Crybb* based on their acidic or basic properties respectively, comprises of four (*Cryba1-4*) and three members (*Crybb1-3*) respectively. Although, expression of β-crystallins start during early development but it increases after birth. Mutations in these genes result in the formation of cataract in mouse as well as in humans.

γ-Crystallins (Cryg) are monomeric proteins encoded by 8 genes (*CrygA-F*, *S*, *N*). Cryg has been detected in the murine lens starting from developmental day E12.5 in the differentiating lens fiber cells [148,149]. Its expression increased around the time of birth but decreased after birth [150]. Mutations in the genes coding various forms of γ-crystallins cause cataract in human and mouse like other crystallins.

1.3 Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder that progresses with age; first described in 1817 by an English surgeon James Parkinson. The disease usually starts around the age of 60 years, with an estimated prevalence of 1% in the population at the age of 65 years and increased to 4 – 5% at the age of 85 years [151]. The hallmark of the disease is reduced amount of the neurotransmitter dopamine in the midbrain (especially, substantia nigra) [152]. It is believed that loss of 60–80% of dopaminergic neurons causes enough reduction in the amount of dopamine to trigger the symptoms of Parkinson [153,154]. The major symptoms include [155-159],

- Motor;
    - Tremor
    - Rigidity
    - Bradykinesia
    - Postural instability
- Non-motor;
    - Neuropsychiatric problems; cognitive, mood, behaviour
    - Autonomic dysfunction
    - Sensory and sleep disturbances

Among the motor symptoms, slowness of the movement (bradykinesia) in association with the initiation and execution of the movement is the most important clinical feature of PD. Tremor is observed in 70% of the patients at the onset of disease; however, rigidity is among the initial symptoms of the disease and may be associated with joint pain. Cognitive impairment is the most common neuropsychiatric problem in PD and is present in 80% of the patients. The risk of dementia is six folds higher in these patients. Other important non-motor symptoms include the impaired sense of smell and pain with a 70% patients having problem in smell identification [160-162]. It is among the earliest signs of the disease [163,164] and appears even before any other clinical symptoms [165], putting this parameter an important factor to include in the battery of test for the identification of people at risk of developing PD [166].

Although PD is considered an idiopathic syndrome, there are growing evidences that it is orchestrated by environmental and genetic factors. Some important genes associated so far with this disease are *SNCA*, *PARK2*, *PARK7*, *UCHL1*, *PINK1*, *LRRK2*, *NURR1* and *PITX3*. Human epidemiological studies have shown association of various polymorphisms in the *PITX3* gene in relation to its role in the development of dopaminergic neurons [11-13]. Although many genetic mouse models for the above genes are available to explore the mechanisms of dopaminergic loss and pathogenesis of PD, but none of them represent the true model of Parkinson. *Aphakia*, the first spontaneous mouse mutant affecting Pitx3 shows specifically the loss of dopaminergic neurons in substantia nigra.

1.4 Aphakia mouse

The word *aphakia* comes from ancient Greek, which means ‘no lens’, given to a spontaneous mutant appeared in 129/Sv-S1J strain that lack lenses [96]. *Aphakia* phenotype is recessive in this mouse mutant characterized by small eyes.

Molecular analysis has revealed two deletions in the promoter region of *Pitx3*. Proximal or major deletion is 1423 bp [167] while the distal or minor deletion is 652 bp [94], however, going through the current genomic databases distal deletion is confirmed as 765 bp (Fig. 1.8).

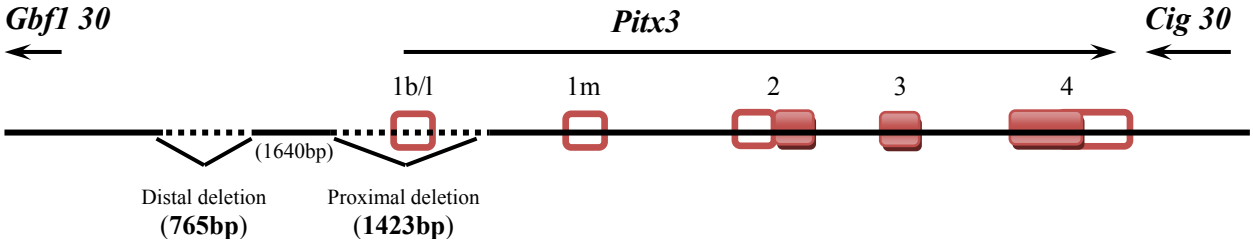


Figure 1.8: Two deletions identified in the 5'-upstream region of the *Pitx3* in *aphakia* mice. Proximal and distal deletions are with respect to the transcription start site and are represented as dotted lines. The empty and filled pink coloured boxes represent non-coding and coding exons respectively. Number of exon is mentioned on the top of the box. 'b/l' and 'm' represent the 1st exon of brain/lens and muscle transcript respectively (see also Fig1.1).

1.4.1 *Aphakia* as a model of microphthalmia

Aphakia homozygous mice are characterized by small eyes that lack lenses, representing a mouse model to study the pathological mechanisms and the role of Pitx3 in the development of lens. Investigation of lens development in this mutant revealed that lens formation is induced as normal but arrested at the lens developmental stage around post coitum day 10.5-11 [96,168]. The a*phakia* lens remained attached with the overlaying epithelium and shows a persistent lens stalk lacking the formation of anterior chamber. They are degraded latter during the development resulting in eyes that lack lenses (Fig. 1.9).

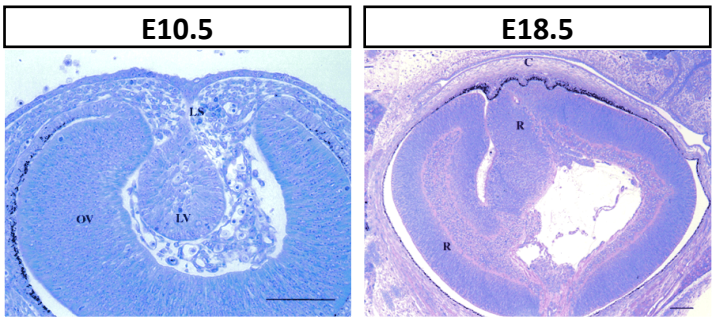
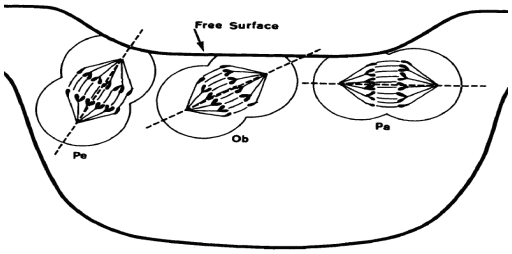


Figure 1.9: Histological sections of *ak/ak* mouse during development. At E10.5 the lens vesicle is attached to the surface ectoderm and show persistent stalk. The lens latter disappears and only retina is visible in the eye. LS; lens stalk, LV; lens vesicle, OV; optic vesicle, R; retina. (Semina et al. [94]).

Another important feature of the *aphakia* lenses is that their lumen is filled with cells but how the lens cells aggregate in the lumen of lens vesicle is not known. However, disturbance in the mitotic spindle orientation observed in *aphakia* lenses decades before [169] could be responsible for this phenotypic feature.

Mitotic spindle orientation can be of three different types with respective to the surface; parallel, perpendicular and oblique (Fig. 1.10). All three types of spindle orientation exist in the developing lens; however, parallel orientation is more frequent during the development of normal lens. Zwaan et al., [169] have found that in the *aphakia* lenses oblique and perpendicular orientations are more frequent and the paprallel orientation is decreased compared to the control lenses. This change in mitotic apparatus orientation may result in the abnormal localization of post mitotic cells that accumulate and fill the *aphakia* lens vesicle.

A



B

	Parallel	Perpendicular	Oblique
Wild-type	79	28	33
ak / *ak*	38	6	14

Figure 1.10: Different orientation of the mitotic apparatus in *aphakia*. During lens development three different forms of orientation of mitotic apparatus is shown. Pa; parallel, Ob; oblique, Pe; perpendicular (A). In *aphakia* lens perpendicular and oblique orientations are more frequent and parallel orientation is decreased (B) (Zwaan et al [169]).

Since the identification of *aphakia*, various studies have been done to explore the molecular mechanism that result in the abnormal lens development in these mutants. Grimm et al [168], investigated the expression of the lens key regulator, *Pax6* by *in situ* hybridization and have shown that it is expressed in the rudimentary lens including lens stalk. They also claimed that *Six3* is expressed in the *aphakia* lens. Expression of other transcription factors involved in lens development have shown that these mice show impaired expression of Foxe3 and Prox1 [95,97]. In additions to these factors, expression of crystallins has also been explored in *aphakia* lenses. Earlier studies did not observed the expression of crystallins in the mutant lenses [170]; however, latter studies have identified low expression of α-crystallin in the *aphakia* lenses at E14 but no expression of β- and γ-crystallin was observed [171]. Recent studies have also detected small amount of βA3- and γ-crystallin in the mutant lenses at E12.5 using *in situ* hydridization [95]. Although these studies provided better understanding of the *aphakia* phenotype with the improvement of the technology and methodology but further studies are necessary to fully understand the mechanism of pathogenesis that results in microphthalmia.

1.4.2 *Aphakia* as a model of Parkinson's disease

Pitx3 deficient *aphakia* mice show specifically loss of dopaminergic neurons in the midbrain region; characteristic of Parkinson's disease. These mice show greater than 90% loss of dopaminergic neurons in substantia nigra (SN) while those in the ventral tegmental area (VTA) are less affected [172-174], representing 1st mutant model showing the specific loss of dopaminergic neurons. Neurons form the VTA region project to the ventral striatum e.g., *nucleus accumbans* and olfactory tubercle forming the mesolimbic pathway. Contrary to these, neurons from the *substantia nigra pars compacta* (SNpc) project to the dorsal striatum e.g., caudate and putamen forming the nigrostriatal pathway that is involved in the voluntary movements and thus are responsible for motor symptoms in Parkinson's disease.

The neurons from VTA region and substantia nigra also release substance P, a neurotransmitter associated with pain. Reduction in the mRNA level of substance P has been observed in *aphakia* mice [175], which may result in enhanced pain. However, no behavioural analysis has been done in *aphakia* so far to test for nociception but such changes has been observed in another recently identified *Pitx3* mutant mouse, *eyeless* (*eyl*) [98].

Behavioural analysis of the *aphakia* mice revealed motor impairments [174], which can be reversed by L-DOPA [176]; a precursor of neurotransmitters including dopamine and used to treat PD. In addition to the motor symptoms these mice also show deficits in learning striatum-dependent cognitive tasks [177].

1.5 Objectives of this study

The transcription factor, *Pitx3* plays a pivotal role in the development of the ocular lens and dopaminergic neurons. However, the exact role of *Pitx3* in these diverse processes has not yet been established due to the limited knowledge of its downstream targets especially in the lens. So far, most of the research groups focused on the role of Pitx3 in the regulation of genes important for the development and maintenance of dopaminergic neurons. In this context, Lebel et al. [178] provided evidences that Pitx3 can directly bind to the promoter and regulate the expression of tyrosine hydroxylase (*Th*), an enzyme expressed specifically in the dopaminergic neurons and responsible for the conversion of the amino acid L-tyrosine to L-DOPA (precursor of dopamine). Further studies using chromatin immunoprecipitation (ChIP) has shown that VMAT2 (vesicular monoamine transporter 2) and DAT (dopamine transporter) are direct downstream targets of Pitx3 [179].

To identify the role of Pitx3 in lens development first effort was made in our research group by Doris Muenster [180]. She did microarrays using mRNA from different tissues of the *aphakia*

mice at different developmental stages to find out the alteration in gene expression and possibly the downstream targets of Pitx3 (Fig. 1.11). Changes in the expression levels of a variety of genes has been observed including those involved in the blood system, enzymes, eye development and ESTs.

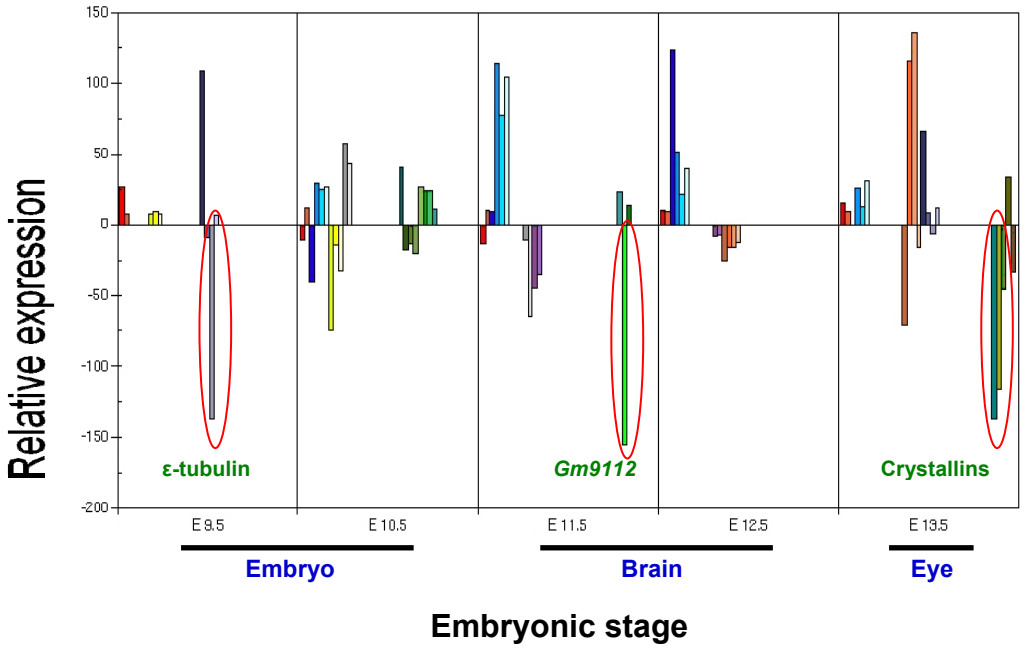


Figure 1.11: Differential expression of genes in *ak/ak* mouse. Microarray analysis using RNA form different tissues at different developmental stages has shown the differential regulation of many genes. The genes that are highly down-regualted are (shown in red lined boxes), *Tube1*, Gm9112; an EST, and crystallins, which also served as controls as the lens is absent in *aphakia* mice (Muenster, 2005)

One of the important gene highly down-regulated in *aphakia* is ε-tubulin at E9.5. ε-tubulin is one of the recently identified tubulins involved in centriole duplication and defining the orientation of mitotic spindles [181-184]. As the orientation of the mitotic apparatus has already been reported as disturbed and could be as a result of reduced *Tube1* expression. So, one of the objective of the current study is to verify the microarray result and to analyze *Tube1* as a potential down-stream target of Pitx3.

In addition to this, I want to explore the molecular events taking place during lens development in Pitx3-deficient *aphakia* mice to understand the role of Pitx3 in different processes and eventually its targets genes using *in vitro* approaches. One of the morphological hallmark of *aphakia* mice is the persistent lens stalk, which is also present at some extent in the *Pax6* (*Aey18*$^{+/-}$ and *Aey11*$^{+/-}$) and *Foxe3*$^{-/-}$ mutants; therefore, I also analyzed the expression of different genes in these mouse mutants to understand the molecular mechanisms responsible for this morphological feature and look for the co-operative role of these gene in lens development.

Moreover, as the *aphakia* mice are also considered as a model for Parkinson's disease (discussed previously), they are also evaluated for the presence of deficits in olfaction (an early non-motor symptom of PD).

2 Materials and Methods

2.1 Materials

a) Equipments

Equipment	Product	Manufacturer
Agarose-Gel electrophoresis apparatus	Power supply (Powerpac Basic)	Biorad, Laboratoreis Inc., Herculus, U.S.A.
	Electrophoresis chamber	peQLab Biotechnologie GmbH, Erlangen, Germany
Balance	TE 1502S	Sartorius AG, Goettingen, Germany
	analytical	Sartorius AG, Göttingen, Germany
Camera for light microscope	Axiocam	Zeiss, Oberkochen, Germany
Cell counting chamber	Counting chamber	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Centrifuge	Microfuge: Eppendorf 5415R	Eppendorf, Hamburg, Germany
	Microfuge: Biofuge pico	Heraeus, Osterode, Germany
	Minifuge: SD220	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
	Benchtop: Sigma 3K18	Sigma Zentrifugen GmbH, Osterode am Harz, Germany
	CM1950	Leica Microsystems, Wetzlar, Germany
Fume hood	Variolab Mobilien W 90	Waldner Laboreinrichtungen GmbH & Co. KG, Wangen, Germany
Gel documentation system	Argus X1	biostep GmbH, Jahnsdorf, Germany
Heating plate	Ikamag	Ludwig Empgenzeder, Munich, Germany
Incubators	(CO_2)-Teco 20	Selutec GmbH, Hechingen, Germany
	Celsius 2007	Memmert, Schwabach, Germany
Laminar Flow cabinet	Laminer flow cabinet	Gelaire Pty Ltd., Sydney, Australia
Luminometer	Centro LB 960	Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany
Microscope	Light: Axioplan2	Zeiss, Oberkochen, Germany

	Light: Axiovert 35	Zeiss, Oberkochen, Germany
	Fluorescence: DMI 6000B	Leica Microsystems GmbH, Wetzlar, Germany
		Olympus, Hamburg, Germany Olympus Co., Tokyo, Japan
Microtome	RM2050	Leica Microsystems, Wetzlar, Germany
	Cryotome Leica CM 1950	Leica Microsystems, Wetzlar, Germany
Microwave	M1712N	Samsung Electronics GmbH, Schwalbach, Germany
pH Meter	pH Meter 761 calimetic	Knick, Berlin, Germany
Pipette	Pipetman (2µl, 10µl, 20µl, 200µl, 1ml)	Gilson S:A.S., Villiers-le-Bel, France
Pipette aid	Macro-612-1900	VWR International Ltd Lutterworth, England
Shaker	Plateform: Polymax 1040	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
	Orbital: Rotamax 120	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
	Centromat S	Braun Melsungen AG, Germany
Spectrophotometer	Nanodrop: ND1000	peQLab Biotechnologie GmbH, Erlangen, Germany
	Biophotometer	Eppendorf AG, Hamburg, Germany
Thermal cycler	Step one (Realtime)	Applied Biosystems,Darmstadt, Germany
	MJ research PTC-225	Bio-Rad Laboratories GmbH, Munich, Germany
Thermomixer	7410	Bachofer, Reutlingen, Germany
	5436	Eppendorf, Hamburg, Germany
Vortex	G-560E	Scientific Industries,Boehemia, USA
Water bath	Köttermann	LTF Labortechnik GmbH & Co. KG, Wasserburg, Germany
		Memmert, Schwabach, Germany
Water purification system	MilliQ biocel	Millipore, Schwalbach, Germany

b) **Consumables**

Item	Catalog no.	Manufacturer
48-well optical well adhesive film	437816	ABI, Foster City, U.S.A.
96 well plates for luminometer	Nunc 236105	Thermo Fisher Scientific, Roskilde, Denmark
Cell culture plates: 96 wells	353047	Falcon
Coverslips	H878	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Disposable cuvetes	UVetta	Eppendorf, Hamburg, Germany
Fast 48-well reaction plates	437523	ABI, Foster City, U.S.A.
Glass Slides	Superfrost® Plus	Gerhard Menzel GmbH, Braunschweig, Germany
Parafilm	4621.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Petri disches	633180	Greiner Bio-One GmbH, Frickenhausen, Germany.
Pipette tips	Filter	Biozyme Inc. St. Joseph, U.S.A.
	sterile	Biozyme Inc. St. Joseph, U.S.A.
Sterile filter	Millex-GP	Millipore, Carrigtwonhill, Ireland
Tissue culture flasks	Cell star	Greiner bio-one GmbH, Frickenhausen, Germany
Tubes	0.2ml	Sarstedt, Nuembrecht, Germany
	2ml & 1.5 ml	Eppendorf, Hamburg, Germany
	15 ml&50 ml	Becton Dickinson GmbH, Heidelberg, Germany
Weighing boats	A230	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

c) Commercial kits

Kit	Catalog no.	Company
1 kb ladder	SM0311	Fermentas GmbH, St. Leon-Rot, Germany
100 bp ladder	SM0241	Fermentas GmbH, St. Leon-Rot, Germany
Anti-Digoxigenin-Ap Fab fragments	11093274910	Roche Dignostics GmbH, Mannheim, Germany

Beetle Juice	102511	P.J.K GmbH, Kleinblittersdorf, Germany
BM purple Ap substrate	11442074001	Roche Diagnostics GmbH, Mannheim, Germany
Cell lysis buffer (5x)	E194A	Promega corp. Madison; U.S.A.
DH5α	18265-017	Invitrogen, Darmstadt, Germany
Dig RNA labeling kit	11277073910	Roche Diagnostics, Mannheim, Germany
DNA dephosphorylation and ligation kit	04898117001	Roche Diagnostics GmbH, Mannheim, Germany
DNA Midi Prep	12143	Qiagen GmbH, Hilden, Germany
DNase1 (on column)	79254	Qiagen GmbH, Hilden, Germany
Dual-Luciferase assay kit	E1960	Promega GmbH, Mannheim, Germany
Eva Green Mix	08-24-00001	Solis BioDyneBi,Tartu, Estonia
Nucleospin® Extract II	740609.50	Nucleospin, Macherey-Nagel, Dueren, Germany
Nucleospin® plasmid	740588.50	Nucleospin, Macherey-Nagel, Dueren, Germany
pCRII Topo cloning Kit	K462001	Invitrogen, Darmstadt, Germany
pGL3 basic vector	A gift from Dr. Chichung D. Lie	Promega GmbH, Mannheim, Germany
Polyfect transfection Kit	301105	Qiagen GmbH, Hilden, Germany
Qia-shredder column	79656	Qiagen GmbH, Hilden, Germany
Ready-To-Go T-Primed First-strand Kit	27-9263-01	Amersham Biosciences, Piscataway, USA
Renilla Juice	102531	P.J.K GmbH, Kleinblittersdorf , Germany
RNA extraction	74104	Qiagen GmbH, Hilden, Germany

d) Enzymes

Enzyme	**Manufacturer**
DNase1	Fermentas GmbH, St. Leon-Rot, Germany
HindIII	Fermentas GmbH, St. Leon-Rot, Germany
Pfu DNA polymerase	Fermentas GmbH, St. Leon-Rot, Germany
Proteinase K	Applichem GmbH, Darmstadt, Germany

RNase A	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
RNase free DNase1	Qiagen GmbH, Hilden, Germany
RNase Inhibitor	Fermentas GmbH, St. Leon-Rot, Germany
Sp6 polymerase	Roche Diagnostics, GmbH, Mannhein, Germany
T7 polymerase	Roche Diagnostics, GmbH, Mannhein, Germany
Taq DNA polymerase	Invitrogen, Darmstadt, Germany
XbaI	Fermentas GmbH, St. Leon-Rot, Germany
XhoI	Fermentas GmbH, St. Leon-Rot, Germany

e) Chemicals and biological material

Chemical	**Catalog no.**	**Manufacturer**
Acetic acid	1.00063	Merk KGaA, Darmstadt, Germany
Agarose	840004	Biozyme Scientific GmbH, Oldendorf, Germany
Ampicillin	K029.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Blocking reagent	11096176001	Roche Diagnostics GmbH, Mannheim, Germany
Boric Acid	A2940	Applichem GmbH, Darmstadt, Germany
Brdu (5-bromo-2′-deoxyuridine)	B5002	Sigma-Aldrich, Steinheim, Germany
Bromphenolblue	1.08122	Merk KGaA, Darmstadt, Germany
Citric acid	X863.2	Carl Roth GmbH & Co. KG, Karlsruhe, GermanyCarl Roth, Karlsruhe, Germany
Deoxycholate	D6750	Sigma-Aldrich, Deisenhofen, Germany
DEPC	18835	SERVA Electrophoresis GmbH, Heidelberg, Germany
Diethyl phthalate	W512206	Sigma-Aldrich, Schnelldorg, Germany
Difco LB-agar Miller	244520	Becton Dickinson & Company, Sparks, USA
Difco LB-Base Miller	241420	Becton Dickinson & Company, Sparks, USA
DMEM	E15-806	PAA Laboratories GmbH, Pasching, Austria
dNTPs	R0241	Fermentas GmbH, Leon-Rot, Germany
Donkey serum	D9663	Sigma-Aldrich, Deisenhofen, Germany
EDTA	8043.2	Carl Roth GmbH & Co. KG, Karlsruhe, Germany.

Ethanol	2246.2500	Th. Geyer GmbH & Co. KG Renningen, Germany
Ethidiumbromid	A1152	Applichem GmbH, Darmstadt, Germany
Fetal bovine serum	A15-104	PAA GmbH, Pasching, Austria
Ficoll 400	46324	Sigma-Aldrich, Steinheim, Germany
Formamide	P040.1	Carl Roth GmbH & Co. KG, Karlsruhe, GermanyCarl Roth, Karlsruhe, Germany
Gluteraldehyde(25 %)	G5882	Sigma-Aldrich, Steinheim, Germany
Glycerol	1.04093	Merk KGaA, Darmstadt, Germany
Glycine	G7126	Sigma-Aldrich Chemie, Steinheim, Germany
H_2O_2	8070.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany.
HCl	1.00319	Merk KGaA, Darmstadt, Germany
Heparin	7692.2	Carl Roth Carl Roth GmbH & Co. KGGmbH, Karlsruhe, Germany
Igepal CA 630	56741	Sigma-Aldrich, Steinheim, Germany
IPTG (Isopropylthio-β-D-Galactoside)	2316.3	Carl Roth GmbH & Co. KG, Karlsruhe, GermanyCarl Roth, Karlsruhe, Germany
KCl	4936	Merk KGaA, Darmstadt, Germany
Levamisole	31742	Sigma-Aldrich Chemie, Steinheim, Germany
Maleic Acid	800380	Merk, Schuchardt Hohenbrunn, Germany
Methanol	1.06009	Merk KGaA, Darmstadt, Germany
Methyl trans-cinnamate	173282	Sigma-Aldrich, Schnelldorf, Germany
MgCl2	2189.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany.
NaAc	1.06268	Merk KGaA, Darmstadt, Germany
NaCl	1.06404	Merk KGaA, Darmstadt, Germany
NaOH	1.06482	Merk KGaA, Darmstadt, Germany
OCT compound Tissue Tek	4583	Sakura Finetek, Torrance, CA, USA
Pencillin / streptomycin	P11.010	PAA GmbH, Pasching, Austria
PFA	0335.3	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Phenethyl acetate	290580	Sigma-Aldrich, Schnelldorf, Germany
Polymount	18606	Polysciences Inc., Warrington PA
Quantum 333	U15-813	PAA Laboratories GmbH, Pasching, Austria
RNase Zap	R2020	Sigma-Aldrich, Steinheim, Germany
Roti-Histol	6640	Carl Roth GmbH & Co. KG, Karlsruhe,

		Germany.
Roti-Mount	HP19	Carl Roth GmbH & Co. KG, Karlsruhe, Germany.
Sodium dodecyle sulfate	20760	Serva Feinbiochemica GmbH & c. KG, Heideberg, Germany
Sodium Citrate	3580.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany.
Sodium deoxycholate	D6750	Sigma-Aldrich, Steinheim, Germany
Sodium phosphate dibasic	1.06580	Merk KGaA, Darmstadt, Germany
Sodium phosphate monobasic	1.06346	Merk KGaA, Darmstadt, Germany
Sucrose	4621.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany.
Trizma Bases	1.08382	Merk KGaA, Darmstadt, Germany
Trypsin-EDTA (0.05 %)	25300	Invitrogen
Tween 20	9127.1	Carl Roth GmbH & Co. KG, Karlsruhe, Germany.
Water	1.15333	Merk KGaA, Darmstadt, Germany
X-gal	R0404	Fermentas GmbH, Leon-Rot, Germany
Xylene	1.08685	Merk KGaA, Darmstadt, Germany
Xylene cyanol	38505	SERVA Electrophoresis GmbH, Heidelberg, Germany
β-Mercaptoethanol	M7522	Sigma-Aldrich, Steinheim, Germany

f) Software and tools

Software / Tool	**website**
BioEdit software v 7.0.9.0	http://www.mbio.ncsu.edu/bioedit/bioedit.html
ECR	http://ecrbrowser.dcode.org/
End note v 9.0	http://www.endnote.com
Genomatix	http://www.genomatix.de/ Genomatix Software GmbH, Munich, Germany
iGrafx FlowCharter 2000 Professional v8.2.1.239	http://www.igrafx.com/
MGI	http://www.informatics.jax.org/
Microsoft office 2003	http://www.microsoft.com
NCBI	http://www.ncbi.nlm.nih.gov/
Primer design and evaluation:	http://www.sigma-genosys.com/calc/DNACalc.asp

Primer3	http://frodo.wi.mit.edu/primer3/
rVista 2.0	http://rvista.dcode.org/
Sequence and gene analysis:	http://www.ensembl.org/
Sequence scanner v1.0	http://www.appliedbiosystems.com
SigmaPlot	Systat software Inc., Chicago, USA http://www.sigmaplot.com/
StepOne™ Real time PCR sytemdata collection and analysis	https://www.appliedbiosystems.comApplied Biosystem Deutschland GmbH, Darmstadt, Germany
TESS	http://www.cbil.upenn.edu/cgi-bin/tess/tess

g) Primers

Table 1: Primers for genotyping of *aphakia* mice

Primer	Sequence	Annealing temperature (°C)	Product size (bp)	
			wt	*ak*
Pitx31/2NF	5'-ATTCGGTGCGGAGAGTAAGG-3'	63	1,165	399
Pitx32R	5'-ATTGGATTTGGCTCTGATGGTT-3'			

Table 2: Sequences of primers used in RT-qPCR

Gene	Primer	Sequence	Annealing (°C)	Primer efficiency (% age)	Product size (bp)
Tube1	Tube1-mqF	5'-CAGTGCTTCTTCATCATCCA-3'	60	90.	126
	Tube1-mqR	5'-GGAAGGATAAACCGCTGTC-3'			
Foxe3	Foxe3-lt	5'-GCCGCCCTACTCATACATC-3'	60	93	172
	Foxe3-rt	5'-ACAGTCGTTGAGGGTGAGG-3'			
Pax6	Pax6-12qF	5'-GTTCTTCGCAACCTGGCTA-3'	60	110	227
	Pax6-12qR	5'-TGAGCTTCATCCGAGTCTTCT-3'			
Tcfap2a	ap2a8qF	5'-TTCTCAACCGACAACATTCC-3'	60	90	229
	ap2a8qR	5'-GTAACCGCTGCACACACC-3'			
Sox2[#]	Sox2qF	5'-GCGGAGTGGAAACTTTTGTCC-3'	60	105	157
	Sox2qRF	5'-CGGGAAGCGTGTACTTATCCTT-3'			
Tcfap2a	ap2a6-7qF	5'-CCTCAGCTCCACCTCGAA-3'	60	104	191
	ap2a6-7qR	5'-CAGCTTTCAGTCTCCCTGCT-3'			
Cdh1	Cdh1qF	5'-ACTGTGAAGGGACGGTCAAC-3'	60	102	123
	Cdh1qF	5'-GGAGCAGCAGGATCAGAATC-3'			
Cdh2	Cdh2qF	5'-TTCTGTGTATCATCATCCTGCT-3'	60	102	161

	Cdh2qR	5'-GTCTTCTTCTCCTCCACCTTCT-3'			
Bmp4	Bmp4qF	5'-GGATTACATGAGGGATCTTTACC-3'	60	102	196
	Bmp4qR	5'-GAGGTTGAAGAGGAAACGAAA-3'			
E4f1	E4FqF	5'-AGTACATTATTGAGGCCACTGC-3'	60	95	219
	E4FqR	5'-CAATGGTGATCGTGTCTGC-3'			
Otx2	Otx2qF	5'-GAATCCAGGGTGCAGGTATG-3'	60	102	250
	Otx2qR	5'-CAGACAGTGGGGAGATGGA-3'			
Tuba**#	TubeaF	5'-CCAGATGCCAAGTGACAAGA-3'	60	101	117
	TubeaR	5'-GTGGGTTCCAGGTCTACGAA-3'			
Prox1	Prox1qF	5'-ATGCTGTGTCTCCTGTTTCTCT-3'	60	106	101
	Prox1qR	5'-GCTTATCAGGCTCAAATCAAAC-3'			
Hes1**#	Hes1qF	5'-ATAGCTCCCGGCATTCCAAG-3'	60	96	133
	Hes1qR	5'-GCGCGGTATTTCCCCAACA-3'			
Hes5**#	Hes5qF	5'-AGTCCCAAGGAGAAAAACCGA-3'	60	100	183
	Hes5qR	5'-GCTGTGTTTCAGGTAGCTGAC-3'			
Wnt1*	Wnt1qF	5'-GAGGTGATTGCGAAGATGAA-3'	60	90	116
	Wnt1qR	5'-AAATGGCAATTCCGAAACC-3'			

- * = Primer from primer depot (http://mouseprimerdepot.nci.nih.gov)
- # = Gift from Dr. Chichung D. Lie

Table 3: Primers used for the cloning of riboprobes

Gene	Primer	Sequence	Annealing Temperature (^{o}C)	Product size (bp)
Tcfap2a	Forward	5'-GCCGATCCATGAAAATGC-3'	62	639
	Reverse	5'-TCGTTGGGGTTTACCACG-3'		
Tube1	Forward	5'-AGGAACAGGCTCTGGGCT-3'	62	957
	Reverse	5'-CACATCTGGCACAGGCAG-3'		
Pax6	Forward	5'-GGGAGTGCCCTTCCATCT-3'	62	885
	Reverse	5'-CCCATGGGCTGACTGTTC-3'		

Table 4: Primers for cloning of *Tube1* cDNA

Gene	Primer	Sequence	Annealing Temperature (^{o}C)	Product size (bp)
Tube1	Tube-egfF	5'-TAGGTACCATGACCCAGTCGGTG-3'	66	1441
	Tube-egfR	5'-TAGGATCCCAAGGCCACACTCAAC-3'		

Table 5: Primers used for cloning of promoters

Gene	Primer	sequence	Annealing temperature (^{o}C)	Product size (bp)	
Tcfap2a	Ap2F1	5'-CGGGCCTACAGGTCATAGGGC-3'	65	1,009	
	Ap2R1	5'-CTCCATGCGGTGTCGTACATGC-3'			
	Ap2F2	5'-CAAACACTTGGATTTGCCGATGTC-3'	65	594	
	Ap2R2	5'-CTCCATGCGGTGTCGTACATGC-3'			
	Ap2F3	5'-CCAGAGAAATCCCTCTATATCAGAGTGTCAC-3'	65	352	
	Ap2R3	5'-CTCCATGCGGTGTCGTACATGC-3'			
	Ap2NF1	5'-TTCCTACACCTATCAGCCAAAGT-3'	65	251	
	Ap2NR1	5'-GAAGACATCGGCAAATCCAAGTG-3'			
	Ap2-6R	5'-CGGGCCTACAGGTCATAGGGC-3'	60	214	
	Ap2-6R	5'-ACTTTGGCTGATAGGTGTAGGAA-3'			
	Ap27F	5'-ACCTTGCGGAGCTTTACTTAGA-3'	61	118	
	Ap27R	5'- CCTAGAGATGCCTTTCCACATT-3'			
	Ap2F8	5'-ATGTGGAAAGGCATCTCTAGGGGTTA-3'	67	220	
	Ap2R8	5'-GAAGACATCGGCAAATCCAAGTGTTTGC-3'			
Cdh1	Cdh1F	5'-GGCTCAGGTTCACCATTAACAC-3'	61	2,059	
	Cdh1R	5'-GGGCAGGAGTCTAGCAGAAGT-3'			
	Cdh2F	5'-CTGGAACAGGAGAGCTTGAGTT-3'	61	1,498	
	Cdh2R	5'-GGGCAGGAGTCTAGCAGAAGT-3'			
	Cdh3F	5'-ACATACACGGAGGGAGAACAAT-3'	61	1,053	
	Cdh3R	5'-GGGCAGGAGTCTAGCAGAAGT-3'			
	Cdh4F	5'-CCCTAAGCAAACAAACTCATCC-3'	61	383	
	Cdh4R	5'-GGGCAGGAGTCTAGCAGAAGT-3'			
Pitx3	Pitx34F	5'-CAACGCTACCCTTACCCACAG-3'	62	**wt**	***ak***
	Pitx31/4R	5'-AACAGGGCTCCAATTCCAAC-3'		1,610	187
Pax6	Pax6P1F	5'-AGATGTTGGAATGGAGAGAGGA-3'	62	1,739	
	Pax6P1R	5'-GAACACACAGGTTGCACGTC-3'			
Tube1	Tube1F1	5'-TAAGCTGTTTCTGCCATCTTG-3'	58	806	

	Tube1R1	5'-CATCTTGTTTCCATAAGTGTGC-3'		
	Tube1F2	5'-CTGGCGGCTGAATAAGGTA-3'	60	874
	Tube1R2	5'-AGCTAGGTACACTCCGACCAA-3'		
	Tube1F3	5'-CGGCTGTTGGAAGTTGGAT-3'	62	694
	Tube1R3	5'-GAGAGAGAGGTGCATTAGGAAGG-3'		
Foxe3	Foxe3ch-2F	5'-TAAGACGGCCAGTGAAGGTG-3	58	283
	Foxe3ch-2R	5'-CTTTGGACAAGGGTGGGAAT-3		
Prox1	Prox1-NF1	5'-CCAGGGAGAGGACCATTC-3	61	1,370
	Prox1R1	5'-GAGTGATCTGGGCGAGTGCT-3		
	Prox1ch-1F	5'-TGCTGTAAAGATCGCCCAAG-3	60	272
	Prox1ch-1R	5'-CCCTCCAGATACCAGCGAAG-3		

h) Antibodies

Table 6: List of primary antibodies

Antibody	Company	Host species	Catalog no.	Dilution used
Anti-GFP	Aves Labs Inc.	Chicken	GFP-1020	1:200
BrdU[#]	AbD Serotec	Rat	OBT0030CX	1:500
E-cadherin	Sigma-Aldrich	Rat	U3254	1:200
Foxe3 (M-57)	Santa Cruz	Rabbit	sc-134536	1:200
N-Cadherin (H-63)	Santa Cruz	Rabbit	sc-7939	1:50
Otx2*	----------	Rabbit	----------	1:200
Pax6	Chemicon	Rabbit	PRB-278P	1:500
Pitx3(N-20)X	Santa Cruz	Goat	sc-19307X	1:1000
Prox1	Millipore	Rabbit	AB5475	1:1000
Sox2 (Y-17)	Santa Cruz	Goat	sc-17320	1:500
Tcfap2a (3B5)	Santa Cruz	Mouse	sc-12726	1:500
γ-crystallin (FL-175)	Santa Cruz	Rabbit	sc-22746	1:100
ε-tubulin	Sigma-Aldrich	Mouse	T1323	1:200

= A gift from Dr. Chichung D. Lie.
* = A gift from Dr. Antonio Simeone

Table 7: List of secondary antibodies

Antibody Name	Company	Reactivity	Catalog #	Dilution used
Alexa Fluor® 488	Invitrogen	Rabbit	A-21206	1:250
Alexa Fluor® 488	Invitrogen	Rat	A-21208	1:250

CY3	Jackson immuno	Goat	705-165-147	1:250
Cy3	Jackson immuno	Rat	712-165-153	1:250
Cy3	Jackson immuno	Mouse	715-165-150	1:250
CY5	Jackson immuno	Mouse	715-175-150	1:250
DAPI	Sigma-Aldrich	---------	D9564	1:10,000
FITC[#]	Jackson immuno	Chick	703-095-155	1:250

= A gift from Dr. Chichung D. Lie

2.2 Methods

2.2.1 Animals and tissue preparation

All the animals used in this study (C57BL/6J, *aphakia*, *Foxe3*$^{-/-}$ [185]- kindly provided by Prof. Peter Carlsson (University of Gothenburg, Sweden), *Pax6* mutants (*Aey11* and *Aey18*) [186]) were kept in the mouse facility of Helmholtz Zentrum Munich. They were treated and bred according to the German Law for Animal Protection.

To get the embryos, respective animals were bred and vaginal plug was used to detect the pregnancy. The day of positive plug was used as post coitum day zero and the females were sacrificed in a CO_2 chamber, around noon of the respective post coitum day for the required embryos. After dissecting the animals, uterei were removed and embryos were recovered in PBS using fine forceps. They were then fixed in 4% PFA overnight and either transferred in 30% sucrose solution (in PBS) until they sink to the bottom (usually overnight) and embedded in OCT compound (2.1) (for cryosections) or were dehydrated in serial dilution (25%, 50%, 75%) of methanol (in PBS) for 10 minutes each. Following the bleaching in 6% H_2O_2 (in Methanol) for 1 hour, embryos were washed twice in absolute methanol for 10 minute and then either stored at -20^{o}C (for whole mount) or embedded in paraffin for sections (to be used for *in situ* hybridization or immunofluorescence).

For the realtime qPCR, littermate embryos were used for the respective genotype after genotyping, using the embryonic tail tissues and the heads were stored immediately at -80^{o}C (to be used for RNA extraction).

2.2.2 Basic Molecular Biology techniques

a) DNA Isolation

DNA isolation from tail tissues

Either adult or embryonic tail samples were used to extract the genomic DNA. Tissue samples with 500 µl of lysis buffer (appendix) containing 20 µg of proteinase K and 0.5% SDS were incubated over night at 55^{o}C with shaking. Following lyses, proteins were precipitated using 2.5M of NaCl with vigorous shaking and incubating on ice for 10 minutes. Samples were centrifuged at 6000 rpm for 10 minutes and supernatants were transferred into new tubes. DNA was precipitated with double volume of ethanol and pelleted by centrifugation at 13000 rpm for 15 minutes. Pellets were washed with one volume of 70% ethanol, dried till semi transparent and

resuspended in 50 µl to 200 µl of MilliQ water, depending on the size of the pellet. DNA quantity was measured using Biophotometer (section 2.1) and stored at 4^{o}C till use.

DNA isolation from E. coli

DNA was extracted from *E. coli* using different kits, depending on the volume of the media following manufacturer's instructions. For minipreps Nuclespin Plasmid kit (section 2.1) while for maxiprep Qiagen plasmid purification kit (section 2.1) was used.

Purification of DNA from Gel and PCR

For the purification of DNA from the agarose gel, band of interest from the gel was excised with the help of clean scalpel and washed the cut band with MilliQ water. DNA was purified either from the agarose or PCR product using the Nucleospin extract II kit (section 2.1) following the manufacturer's instructions.

b) Primer design

DNA and RNA reference sequences were retrieved from ENSEMBL database (section 2.1) and primers were designed using primer3 software [187]. For RT-qPCR, cautions were taken to include the intron-exon boundaries to enhance the specificity. Best primers based on their characteristics like, specificity, complementarities and secondary structures were selected and synthesized from Sigma (section 2.1).

c) Genotyping

For the genotyping of *aphakia* mice, primers spanning the distal promoter deletion were used (Table 1) to amplify the DNA using the standard PCR reactions [188]. Reaction mix was prepared as mentioned in table8. PCR product was then resolved on 2% agarose gel (see appendix) along with 100 bp ladder as size marker (section 2.1). Genotypes were assigned on the basis of appropriate band size.

Table 8: PCR reaction mix

Reagent	Volume per reaction (µl)
Water	13.75
Taq Buffer;10x (15 mM $MgCl_2$)	2.0
dNTPs	1.0
Primer Forward (10 µM)	1.0
Primer Reverse (10 µM)	1.0
Taq DNA polymerase (5 U / µl)	0.25
DNA (100 ng)	1.0
Total volume	**20**

d) Reverse transcription- qPCR

Total RNA extraction

RNA was extracted using RNeasy mini Kit (section 2.1) following the manufacturer's protocol with some modifications. Samples were homogenized in 350 µl of RLT buffer containing 1% β-**m**ercaptoethanol and passed through Qia-shredder column (section 2.1) by centrifugation at 13000 rpm for 2 minutes at room temperature. Then, I added 100 µl of RNase free water in the lysate and precipitated the RNA by adding 250 µl of absolute ethanol. RNA is then recovered by RNeasy mini spin columns. Samples (along with column) were incubated for 5 minutes and then centrifuged at 1300 rpm for 15 seconds. Flow through was passed through the column again to increase the binding of RNA to the RNeasy column. Columns were washed with 350 µl of buffer RW1 for 1 minute, and centrifuged for 15 seconds at maximum speed.

DNA was eliminated by using 'on column DNase1' kit (section 2.1). Columns were incubated at room temperature for 15 minutes with 80 µl of the buffer RDD containing 30 units of DNase1 followed by two times washing with buffer RW1 as above. During the 2nd wash columns were incubated for 3-5 minutes. To further remove the contaminants, columns were washed twice with buffer RPE; with incubation for 5 minutes during the first wash. Transferred the column into new tube and centrifuge for 2 minutes at full speed and further for 5 minutes with open lid. Columns were left with open lids for 5-10 minutes to completely dry and the RNA was recovered by adding 45 µl of RNase free water.

RNA yield and purity were measured using NanoDrop ND-1000 (section 2.1). Only the RNA with A_{260} / A_{280} and A_{260} / A_{230} ratios above 1.8 were used in further downstream experiments. To measure the RNA concentration, the convention that 1 OD at 260 nm equals to 40 µg / ml was used.

cDNA synthesis

cDNA was synthesized using Ready-To-Go T-primed first-stranded kit (section 2.1), following essentially manufacturer's instructions. 1 µg of the total RNA was used in 33 µl of reaction volume, of which 1 µl of one tenth dilution was used in PCR reactions.

Realtime-qPCR

Realtime qPCR was performed using the EvaGreen qPCR mix (section 2.1), following essentially the manufacturer's protocol. 1 µl of the one tenth dilution of the cDNA was used as template and prepared the reaction mix as mentioned in table 9.

Table 9: Reaction mix for RT-qPCR

Reagent	Volume per reaction (µl)
Water	14
EvaGreen mater mix (5x)	4
Primer Forward (10 µM)	0.5
Primer Reverse (10 µM)	0.5
cDNA (1:10)	1
Total volume	**20**

Following the initial denaturation and enzyme activation at 95^{o}C for 15 minutes, reaction was cycled for 30 times with denaturation at 95^{o}C for 30 seconds and annealing-extension temperature depending on the primers used (as mentioned in table 2) for 30 seconds. Data was collected at the extension phase and processed using the StepOne software (section 2.1). Relative gene expression was calculated following $2^{-\Delta\Delta CT}$ method [189]. *Tuba* was used as a reference gene.

Standardization of realtime qPCR primers

All the RT-qPCR primers (Table 2) either from the primer data base (section 2.1) or self designed were standardized using the standard curve method. Samples were run in duplicate with five different dilutions in a series of 1 to 2. Reaction mix was prepared as mentioned in table 10. Standard curve was generated using stepone software v 2.0 (section 2.1) and the reaction mix was prepared as mentioned in table 10. Only those primers with an efficiency of 90 to 110 were used in the expression analysis.

Table 10: Reaction mix for standardization of realtime-qPCR Primers

Reagent	Volume per reaction (µl)
Water	11
EvaGreen mater mix (5x)	4
Primer Forward (10 µM)	0.5
Primer Reverse (10 µM)	0.5
cDNA (dilution)	4
Total volume	**20**

2.2.3 Microbial techniques

a) TA- cloning

For TA cloning amplified PCR products using *Taq* DNA polymerase were run on 2% agarose gel along with 100 bp ladder as size marker (section 2.1) and the products with right sizes were purified using the pocedure as mentioned in section 2.2.2. For those DNA fragments which were amplified using *Pfu* DNA polymerase, the reaction mix was incubated with *Taq* DNA polymerase (1 unit per reaction) at 72^{o}C for 10 minutes to add the polyA tail that is important for TA cloning. Purified DNA fragments were cloned in the pCRII Topo vector (section 2.1), according to the manufacturer's protocol, using 4 µl of the PCR product.

b) Transformation of *E. coli*

Competent DH5α cells (section 2.1) were thawed on ice. The DNA was added to the bacteria by gently mixing and incubated on ice for 30 minutes. The heat-shock was performed at 42°C for 45-60 seconds followed by cooling on ice for 2 minutes. After adding 950 µl of LB media (without any antibiotics), cells were incubates at 37°C for 1 hour at a speed of 180 rpm with shaking. Bacteria were plated out on agar plates with the appropriate antibiotics (ampicillin) for selection and incubated over night at 37°C.

c) Analysis of positive colonies

Selected colonies were grown in 5 ml (for miniprep) of LB media at 37^{o}C for overnight. 2 ml of the cultures media were used to isolate the DNA using Nucleospin plasmid kit (section 2.1) following the manufacturer's protocol and saved the rest of 3 ml culture at 4^{o}C. DNA was then digested overnight using *Hind*III and *Xho*I or *Kpn*I and *Xho*I restriction enzymes (section 2.1) at 37^{o}C and run on 2% agarose gel along with 1 kb DNA ladder (section 2.1) as size marker. Positive samples were marked and send to the GATC Biotech (GATC Biotech AG, Konstanz, Germany) for sequencing.

Sequences were processed using ABI software and aligned with reference sequences using BioEdit software [190]. Samples with perfectly matched sequences were selected and used in further experiments.

d) Subcloning

For sub-cloning of the TA-cloned fragments, destination vector (pGL3; for luciferase assay) and pCRII Topo vector with inserts were cut with *Hind*III and *Xho*I or *Kpn*I and *Xho*I restriction enzymes (Fermentas GmbH, Leon-Rot, Germany) at 37^{o}C overnight, depending on the orientation of the insert. Restriction digests were run on 2% agarose gel along with 1 kb ladder (section 2.1) as size marker. DNA bands were cut out from the gel and purified as mentioned in section 2.2.2. Vector and insert DNA were mixed in a ration of 1:3 and ligated using rapid DNA dephosphorylation and ligation kit (section 2.1). Ligation reaction was transformed and colonies were analyzed as in section 2.2.3. Positive cultures were amplified in a volume of 150 to 200 ml and DNA was isolated as in section 2.2.2 and stored at -20^{o}C till further use.

2.2.4 Histological techniques

a) In situ hybridization

RiboProbe preparation

For *in situ* hybridization, riboprobes were synthesized in the lab by using the cloned cDNA (for primers see table 3) for the respective gene in the pCRII Topo vector (section 2.1). Primers used for the cloning of riboprobes are listed in table 3. Riboprobes were prepared using either T7 or Sp6 polymerase (section 2.1)

***In situ* hybridization on sections**

Paraffin sections were washed twice in Roti-Histol for 15 minutes each to deparaffinized and then rehydrated in series of ethanol dilutions (2x100%, 95%, 90%, 80%, 70%, 50% and 30%) for 2 minutes each. After fixation of the slides in 4% PFA for 30 minutes, slides were incubated with proteinase K (2 mg / ml) in proteinase K buffer for 3 minutes and fixed again in 4% PFA for 30 minutes. Following the washing twice in PBS for 5 minutes each and in 2x SSC (see appendix) for 2 minutes each, slides were incubated with hybridization solution (containing Tris / Glycine) for 30 minutes. Slides were then incubated overnight at 65^{o}C in hybridization solution with respective DIG labelled probes.

Unbound probes were removed by washing three times for 20 minutes each, with 5x SSC at room temperature and 0.5x SSC with 20% formamide at 65^{o}C; it cools down to 37^{o}C in the same

solution (approx. 30 minutes). Slides were further treated with RNase A (10 µg / ml) for 30 minutes at 37°C and washed before and after treatment with NTE buffer (see appendix) for 15 minutes at 37°C.

After washing with 0.5x SSC three times at 65°C and 2x SSC once for 30 minutes each, slides were incubated in 1% blocking solution for 45 minutes and then overnight with antibody (1:5000) at 4°C.

Unbound antibodies were removed by washing with TBST (see appendix) for 2 hours and 3 times with NTMT (pH 9.5) (see appendix) for 10 minutes each and developed in staining solution at 4°C. Slides were then washed with NTMT 2 times for 15 minutes each, fixed in 4% PFA for 5 minutes, dried and mounted using Roti-mount and viewed under the microscope.

Whole mount *in situ* hybridization

Embryos were rehydrated by passing through serial dilution (75%, 50% and 25%) of methanol for 10 minutes each on ice and then washed with PBS (see appendix) twice for 10 minutes and once for 5 minute. Following proteinase K (10µg / µl) treatment in proteinase K buffer at 37°C (4 minutes for E 11.5 and 5 minutes for E 12.5), embryos were washed with PBT / Glycine (see appendix) and PBT (see appendix) twice for 5 minutes each on ice to block the proteinase K. After treating with RIPA buffer (see appendix) for 10 minutes embryos were fixed with 4% PFA containing 0.2% gluteraldehyde (see appendix). Prehybridized the embryos at 68°C for 3 hours in prewarm hybridization solution (see appendix), containing tRNA (100 µg / µl). Embryos were then incubated overnight at 68°C with respective DIG labelled probes (1:100) in hybridization solution.

Unbound probe was removed by washing twice with hybridization solution at 65°C and further by treating with RNase (100µg / ml) in RNase solution (see appendix) for 1 hour at 37°C. Washed the embryos for 10 times in SSC / FA / Tween20 solution (2 x 5 minutes, 3 x 10 minutes, 5 x 30 minutes) at 65°C and twice in TBST (see appendix) and MABT (see appendix) for 10 minutes each respectively. Embryos were then incubated in DIG antibody for overnight at 4°C, preadsorbed in blocking solution (see appendix) for 1 hour. Unbound antibody was reomoved by washing the embryos for 11 times in TBST solution (3 x 5 minutes and 8 x 1 hour) at room temperature.

Following the washing with alkaline phosphatase (2 x 5 minutes) (see appendix), embryos were developed in staining solution (see appendix) at 4°C for one to five days depending on the intensity of the stain. Fixed the embryos in 4% PFA and stored at 4°C.

b) Immunofluoresence staining

Immunocytochemistry

After 48 hours of transfection, cells were fixed in 4% PFA (in PBS) for 10 minutes at room temperature and rinse in PBS for three times. Cells were then blocked with blocking solution for one hour and incubated with antibody of interest (diluted in blocking solution) at 4^{o}C for over night. Unbound antibody was removed by washing with PBS for 3 times. To minimize the background and non-specific binding, cells were again blocked for 30 minutes in blocking solution and incubated with appropriate secondary antibody conjugated with fluorescence tags for 2 hours. Following the staining with secondary antibody nuclei were stained with DAPI (in PBS) for 5 minutes. Cells were then washed with PBS for 2 minutes each, dried and mounted on slides using aqua-mount by putting the inverted cover slips on the glass slides and viewed under the fluorescence microscope after drying.

Immunohistochemistry

For immunohistochemistry tissues were processed differently;

1) Paraffin sections

Tissue sections were deparaffinized in Roti-Histol by incubating for 15 minutes twice, followed by serial rehydration, passing through ethanol series (2x100%, 96%, 80%, 60%, and 30%) for 4 minutes each. Finally, I washed sections three times in water for 5 minutes each. For antigen retrieval, slides were boiled in 0.01 M sodium citrate buffer (pH 6.4) for 15 minutes in microwave oven at 630 watts and then cooled slowly by adding MilliQ water intermittently for approximately the same time. Slides were then washed with water and PBS for 5 minutes each and incubated in blocking solution. Following 1 hour incubation with blocking solution at room temperature, slides were incubated with respective primary antibody(ies) (Table 4) at 4^{o}C for overnight. Next day, after washing three times in PBS for 5 minutes each, slides were incubated with secondary antibody (depending on the primary antibody) (Table 5) for 90 minutes and then washed 3 times with PBS for 5 minutes each. After first washing, tissues were stained with DAPI for 10 minutes. Slides were then air dried, mounted using polymount (section 2.1) and photographed using epifluoresence (section 2.1) or confocal (section 2.1) microscope.

2) Cryo-sections

For cryo-sections, slides were washed four times in PBS for 10 minutes each. After the 2nd wash, slides were treated with 3% H_2O_2 for 5 minutes to block the endogenous peroxides. After washing, slides were incubated in blocking solution for 1 hour and processed as in the previous section

2.2.5 BrdU labelling

To label the dividing cells with the thymidine analog, 5-bromo-2'-deoxy-uridine (BrdU), pregnant mice were injected peritonially with BrdU solution (0.05 mg / g) on the required embryonic day. Two hours after injection, mice were sacrificed and embryo were isolated, fixed and embedded in paraffin as mentioned in section 2.2.1. Tail tips were used to genotype the embryos following the same procedure as mentioned in section 2.2.2. BrdU was detected by immunofluoresence staining as mentioned in section 2.2.4.

2.2.6 Promoter assay

a) Cell culture

HEK293 cells and MEF cells (kindly provided by Dr. Chichung D. Lie) were cultured in sterile cell culture flasks at 37°C with 5% CO_2 in Dulbecco's modified Eagle media (DMEM) (section 2.1) supplemented with 10% Fetal bovine serum (section 2.1) and 1x penicillin / streptomycin (section 2.1). When the cells were 80-90% confluent, they were detached by digesting with trypsin (section 2.1) and split in a ration of 1:30 and track the passage numbers. For luciferase assay, HEK293 cells of less than 30 passage numbers were used.

b) Luciferase assay

For luciferase reporter assay, sequences from the 5'-end of the gene of interest were cloned into the pGL3 basic vector (section 2.1) using primers listed in table 5 (section 2.1) and following the procedure mentioned in section 2.2.2.

HEK293 cells (30,000 to 50,000 cells) were cultured (without any antibiotic) in 24-well plates for 24 hours at 37°C with 5% CO_2 and transiently transfected using polyfect transfection reagent (section 2.1). The DNA mix (1.05 µg plasmid-DNA) in the transfection reagent contained 0.2 µg reporter plasmid, 0.2-0.8 µg effector (e.g., Pitx3-pcDNA3.1 or one of the other transcription factor, or parental plasmid pcDNA3.1 as negative control) and 0.05 µg pRL-SV40 as an internal transfection control. Total DNA amount was adjusted with empty pcDNA3.1 plasmid wherever

required. Cells were harvested 48 hours after transfection and lyzed using lysis buffer (section 2.1) and stored at -80^{o}C till analyzed. 15-20 µl of cellular extracts was assayed with Dual-Luciferase Reporter Assay System (section 2.1) or P.J.K luciferase reagents (section 2.1). Standard deviation was calculated from three or five independent experiments performed in triplicate.

2.2.7 Testing olfacotory abilities

For the olfactory testing 6 animals from each group (wild-type: C57BL/6J and *aphakia*: 3 males and 3 females) were tested in a conditioned paradigm using the procedure form Mihalick et al. [191] with some modifications [192] (in collaboration with Dr. Hoelter, Helmholtz Center, Munich). Mice were housed in groups and on restricted food (approx. 3 g per animal) to maintain their body weight around 90% of their free feeding weight. Body weight of all the animals was monitored daily before starting the paradigm.

Test was performed in standard mouse cages, two third of which was covered with plastic lid and separated from the rest of the cage with the help of transparent plastic barrier to keep away the subjects from the stimulation presentation apparatus (SPA) during the intertrail interval, which is presented in the open lid part of the cage. SPA is a plastic platform on which two plastic dishes (covers of 50 ml falcon tubes: 3 cm diameter) are attached. These plastic dishes are separated with the help of a divider to prevent the mice from making contact with both the dishes simultaneously. Odorants were mixed with fresh mouse beddings and presented in plastic dishes on the SPA. For each trial fresh bedding was used.

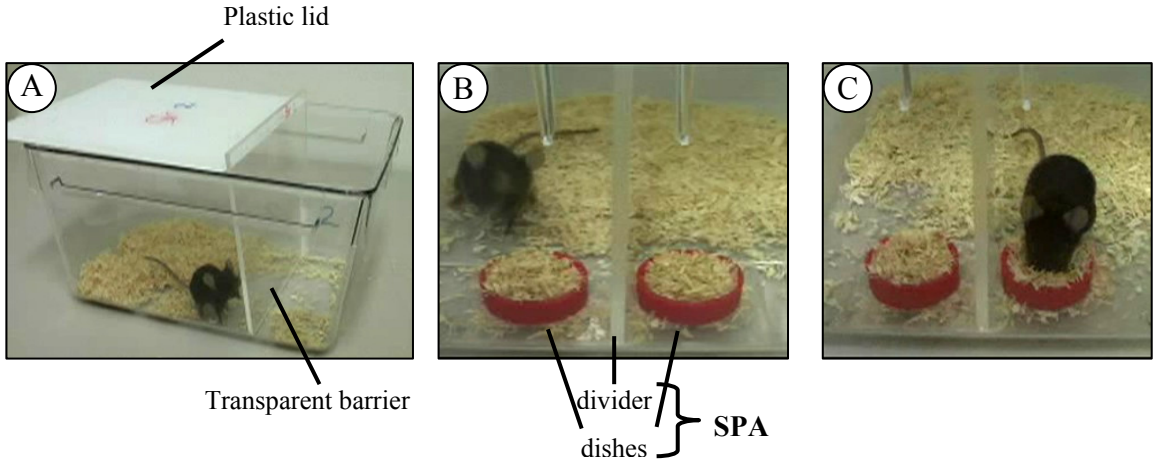


Figure 2.1: Olfactory discrimination paradigm. (A) Experimental setup with barrier and apparatus in place. (B) stimulus presentation apparatus (SPA): dishes attached with adhesive tape. (C) Mouse digging the shavings to retrieve a small piece of chocolate.

Before starting the paradigm animals were pretrained for one week to dig a small piece of chocolate hidden in the unscented bedding presented on one side of the SPA equally on either

side. Then the animals were presented with one of the odorant; either Phenethyacetate (smells like apple) or Methyl trans-cinnamate (smells like cherry) diluted to 10%. For each mouse one smell was randomly assigned as conditioned (S^+) and the other as non-conditioned (S^-). During this phase two dishes were presented simultaneously; one containing conditioned odorant and the 2nd containing solvent (Diethyl phthalate) and the animals were trained for three days (with 3 sessions per days and each session contains 6 trials). Mice were allowed to dig only the conditioned smell and were getting a piece of chocolate buried under the (S^+) bedding as a reward; in case of wrong digging SPA was removed. To improve this training and confirm that animals are responding for the odorant and not the chocolate, mice were given the chocolate with the help of forceps after the correct choice. Percentage of correct responses was recorded.
In the 2nd phase mice had to discriminate between the conditioned (S^+) and non-conditioned (S^-) smell. For this phase animals were tested for three days (with 3 sessions per days and each session contains 6trials) as above and the percentage of correct responses was recorded. In the 3rd phase which is more challenging, mice had to discriminate between different binary mixtures of (S^+) and (S^-) odorants (85% : 15%; 70% : 30%; 55% : 45% respectively). Response for each mixture was tested for one day (3 sessions; 6 trials per session). During the final phase mice were tested for the sensitivity of the smell by using different concentration of odorant, starting with 10% dilution.

2.2.8 Bioinformatics and statistics

All the nucleotide sequences were retrieved using ENSEMBL data base (section 2.1). Promoter sequences were retrieved and analyzed for the transcription factor binding using MatInspector (section 2.1) and TESS (section 2.1). Evolutionary conserved elements were detected using ECR browser (section 2.1) and analyzed to find the conserved transcription factor binding sites using rVista 2.0 (section 2.1). Data analysis was done using SigmaPlot software (section 2.1) and iGrafx flowchart 2000 Pro. was used to draw the models.

3 Results

3.1 Olfaction in aphakia

Aphakia mice being a model of Parkinson's disease are tested for the non-motor symptoms of Parkinson's disease. Six animals, including 3 males and 3 females of eight weeks old were tested for any impairment in olfaction. Wild-type C57BL/6J animals of comparable age and gender were used as controls.

Before starting the test paradigm, animals were weighted daily to access their general health status as they were on restricted diet. Although during early days of experiment, weight of all animals including controls were decreased but stabilized on latter days and no significant change in body weight from the beginning and latter days (for the 2nd and 3rd phase of experiment that are explained latter) of experiments is observed in all the animals (Fig. 3.1).

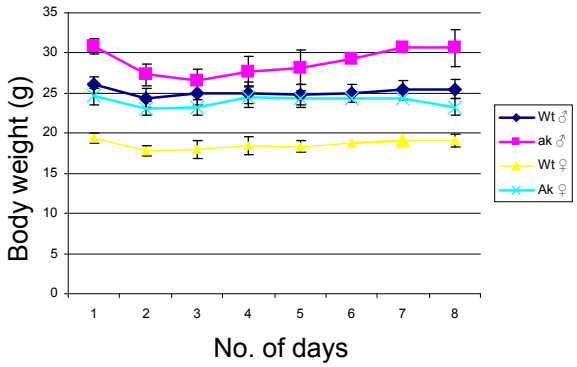


Figure 3.1: No change in body weight of animals during olfactory paradigm. Weights of all animals were recorded daily before starting the experimental paradigm. Data is shown as mean weight in grams ± standard deviation.

In the 1st phase of olfactory discrimination, animals were tested for three consecutive days to differentiate between conditioned smell and no smell. Wild-type animals showed 40% correct responses on the day one, while *aphakia* animals performed better with 50% of correct responses, however, the difference in not significantly different.

During the 2nd phase of olfactory functioning, animals were tested to differentiate between conditioned and non-conditioned smell for three consecutive days. Although the percentage of correct responses is increased as compared to the 1st phase of this test (Fig. 3.2), no difference in this phase of testing was observed between the control and *aphakia* mice.

In the 3rd phase, animals were tested to detect the difference in binary mixtures of conditioned and unconditioned smell for three consecutive days. Performance of *aphakia* in this paradigm was as good as wild-type mice. These results show that olfactory discrimination is not impaired in *aphakia* mice. Animals could not be tested for any deficit in the olfactory sensitivity and

memory as the control animals were not responding to the paradigm, which could be because of their hausing in a single cage.

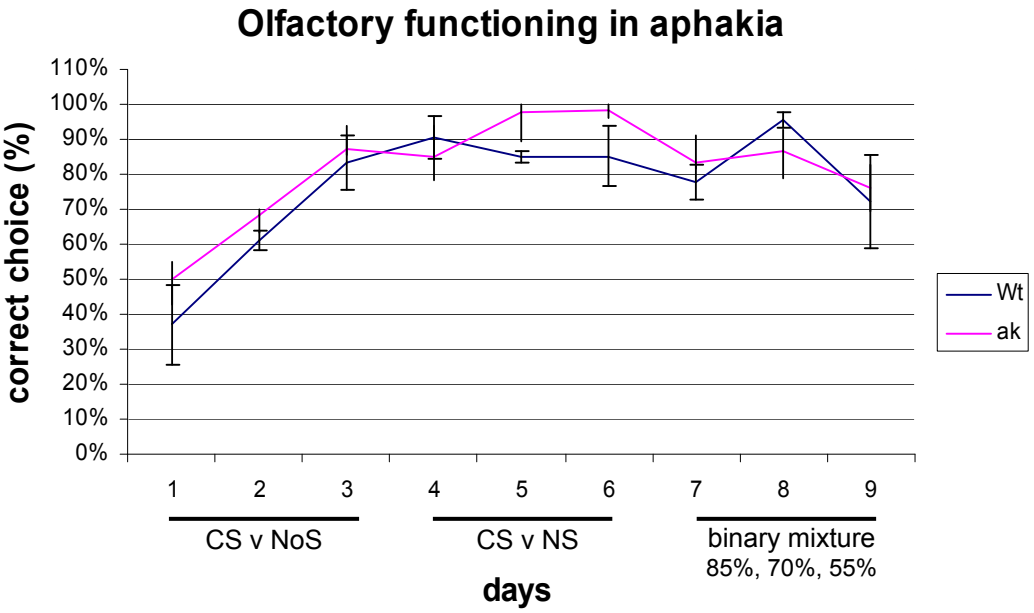


Figure 3.2: Olfactory discrimination is not impaired in *aphakia*. 3 male and 3 female animals of 8 weeks old, from each wild-type and *aphakia* were tested for olfactory discrimination using operant conditioning paradigm. No difference in the performance of *aphakia* animals was observed. Data are shown as percentage of correct choices from 18 trials per day. Values are means ± standard deviation. CS: conditioned smell; NoS: no smell; NS: non-conditioned smell; v: verses.

3.2 Expression profiling of lens genes in aphakia

To further focus on the molecular targets and interaction of *Pitx3* with other factors, I compared the quantitative and spatiotemporal expression of different genes in *aphakia* and wild-type mice that determine lens formation during early stages of development

To investigate the expression of the genes critical for lens development, RNA from the head of the *aphakia* and wild-type littermate controls was quantified using realtime quantitative RT-PCR. For the expression analysis, genes (transcription factors) were selected on the basis of their role in lens vesicle separation (which is impaired in *aphakia*) like, *Pax6*, *Ap2a* and *Foxe3*. The latter two genes are also important of the differentiation and proliferation of lens cells. In addition to these two other genes that are also important for lens proliferation and differentiation; *Sox2* and *Prox1* are also analyzed. Expression of all those genes mentioned above was detected at different developmental stages from E9.5 to E12.5.

Pax6, which is the master controlling gene [33,193] in eye development, has higher expression in *aphakia* at an early developmental stage (E9.5) (Fig. 3.3). Although the difference in the expression of *Pax6* between wild-type and *aphakia* was not statistically significant, there was a trend towards higher expression in *aphakia*.

Sox2 is a an important transcription factor that initiates lens development in co-operation with Pax6 [52]. Therefore, its expression was analyzed in the *aphakia* mice during different developmental stages (form E9.5 to E12.5). Amount of *Sox2* mRNA in *aphakia* was not changed significantly at any of the developmental stage tested but there was a trend towards decrease in its amount in *aphakia* at E12.5, when fiber cells are differentiating.

Tcfap2a, which is critical for the separation of lens epithelium from the surface ectoderm [194] was reduced to 20% ($P = 0.04$) at E10.5 (Figure 3I), exactly the point that follows this separation. However, no change in expression was observed a day earlier (Fig. 3.3) or at latter stages (Figure 3J).

Expression of lens epithelium maintaining factor, *Foxe3* [84,97] is severely affected in *aphakia* mice. At E9.5 (Fig. 3.3) no difference in expression of *Foxe3* was found compared to the littermate wild-type controls but was detectable from E10.5 (Fig. 3.3), when it was decreased to 50% ($P = 0.018$) and further to 65% ($P = 0.015$) a day later (Fig. 3.3). At E12.5, its expression could not be detected within reliable range in *aphakia* (Fig. 3.3).

Examination of *Prox1* expression, which is involved in lens fiber cell differentiation [92], has shown a reduction in its expression (Fig. 3.3) compared to the littermate controls. Expression of this gene was reduced to 20% ($P = 0.016$) at E11.5 while at the earlier and later stages a trend towards decrease in RNA was observed but was not statistically significant.

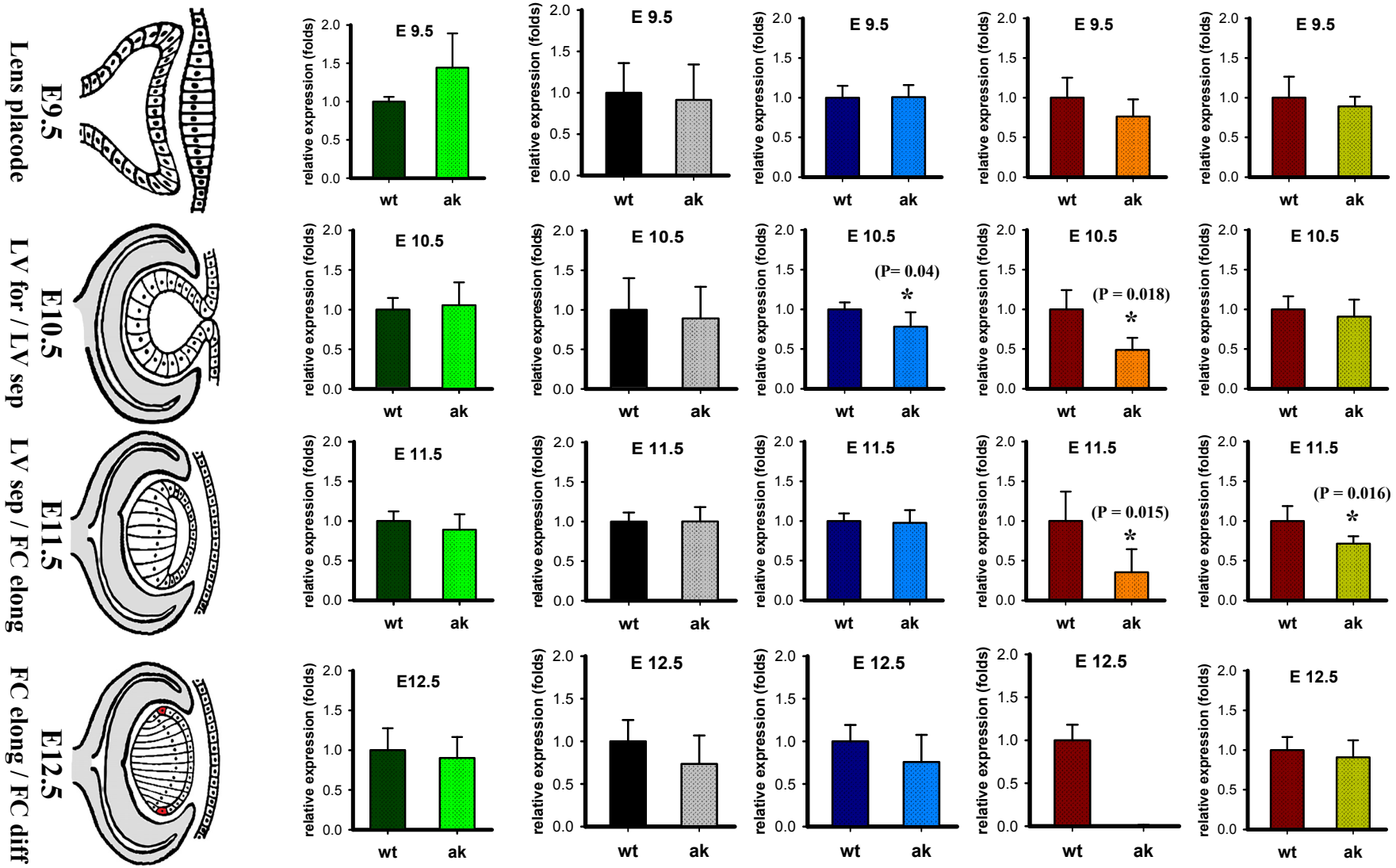

Dev. Stage
Pax6
Sox2
Ap-2α
Foxe3
Prox1
E9.5
Lens placode
E10.5
LV for / LV sep
E11.5
LV sep / FC elong
E12.5
FC elong / FC diff
relative expression (folds)
E 9.5
E 10.5
E 11.5
E12.5
E 12.5
wt
ak
(P= 0.04)
(P = 0.018)
(P = 0.015)
(P = 0.016)
*

Figure 3.3: **Quantification of important ocular lens determining factors in *aphakia* by RT-qPCR.** PCR was done for *Pax6*, *Sox2*, *Ap-2α*, *Foxe3* and *Prox1* at different developmental stages (dev. stage) (adopted from Lang [24] and http://www.mc.vanderbilt.edu/) using RNA form the head of littermate embryos. Expression is shown as folds of values normalized to *Tuba* and calculated using $2^{-\Delta\Delta CT}$ method [189]. Values from wild-type samples are represented as one. Data represents means ± standard deviations from five samples run in duplicate. Statistical analysis was done using student's t test and p-values are given wherever applied. Abbreviations: Dev, developmental; LV for, lens vesicle formation; LV sep, lens vesicle separation; FC, elong, fiber cell elongation, FC diff, fiber cell differentiation.

3.3 Molecular interaction between Pax6 and Pitx3

a) Expression analysis of *Pax6*

In situ hybridization for *Pax6* was done at E11.5 to look for the localization of its expression in *aphakia* lens. *Pax6* expression is found to be higher in *aphakia* lens compared to the wild-type lens (Fig. 3.4). These results are in line with the RT-qPCR data. Hybridization for the sense probe was done in parallel as quality control.

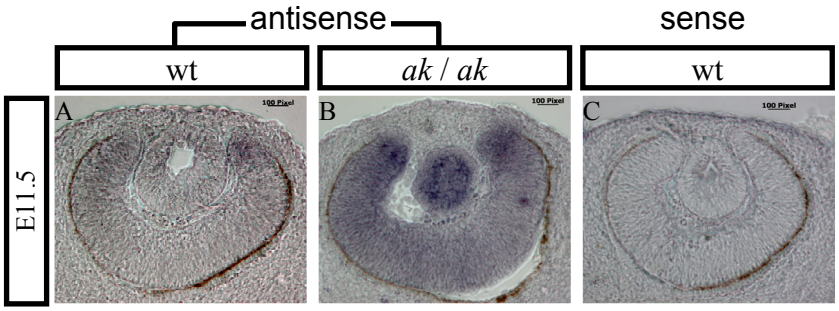


Figure 3.4: Expression of *Pax6* is increased in *aphakia* at E11.5. *In Situ* hybridization was done on 8µm thick PFA fixed, paraffin sections from the wild-type and *aphakia* embryos at E11.5 using DIG labelled probes. Wild-type samples using sense probe were run in parallel as control. Scale bars=100pixels.

To look for the expression of Pax6 at protein level, immunofluoresence staining was done at different developmental stages using anti-Pax6 antibody (Fig. 3.5). At E10.5 no visible difference in the expression pattern was observed; however, from E11.5, alteration in its expression was observed in *aphakia* lens compared to the wild-type lens. All the cells filling the lens vesicle, a characteristic of *aphakia* lens, were found to express Pax6.

Co-expression of Pitx3 and Pax6 has shown a complete overlap in the wild-type lens at all the stages studied (Fig. 2.5 D, L). However, expression of Pax6 in *aphakia* indicates that Pitx3 is not necessary for its expression.

To further investigate the interaction between these two factors, I looked for the expression of Pitx3 in *Pax6* heterozygous mutants (*Aey18*$^{+/-}$) at E12.5, as homozygous *Pax6* mutants do not develop eyes. In *Pax6* heterozygous mutants, expression of Pax6 was restricted to the surface ectoderm, anterior lens epithelium, and the very anterior margins of the neuro-retina but the expression of Pitx3 is maintained in these mutants irrespective of the reduction in the Pax6 expression (Fig. 3.6).

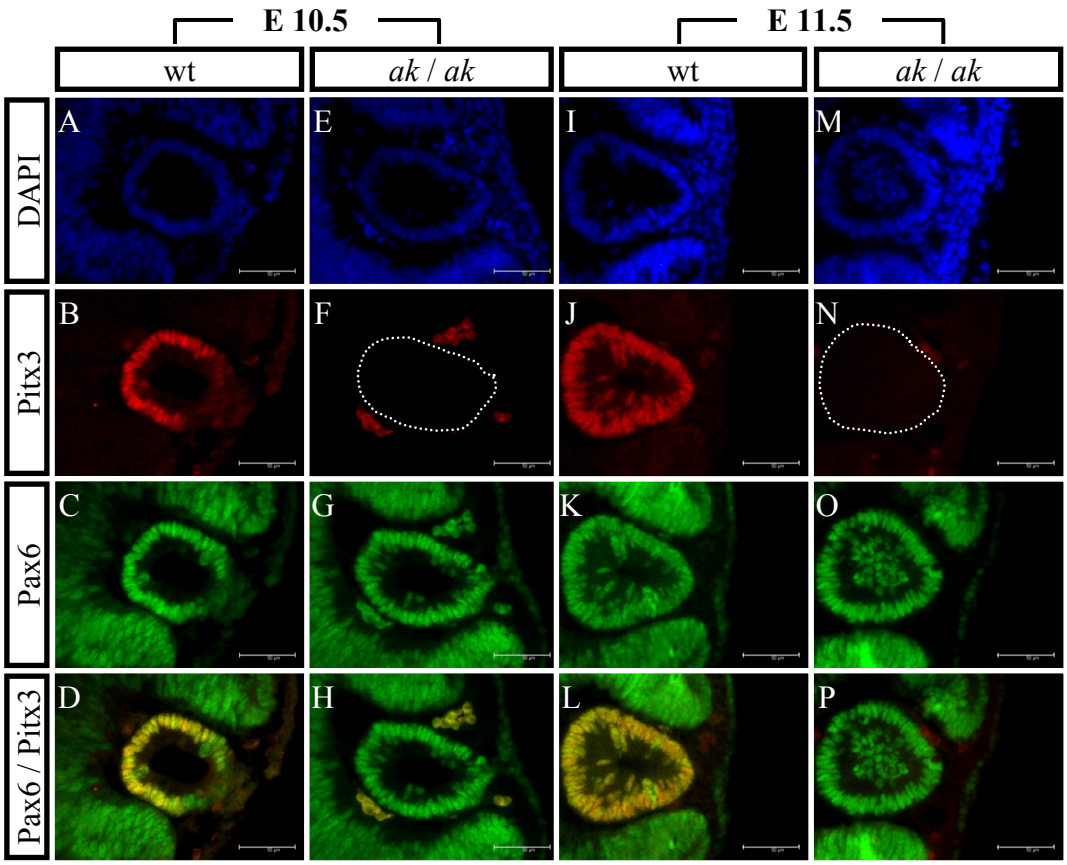


Figure 3.5: Spatiotemporal expression of *Pax6* in *aphakia* during early development. Expression of Pax6 was spread to more anterior in the lens pit in *aphakia* at E10.5 (G, H) compared to the wild-type samples (C, D). At E11.5 (I-P), its expression was persistent in all the cells forming lens vesicle including those filling the vesicle lumen in *aphakia* (O, P). Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

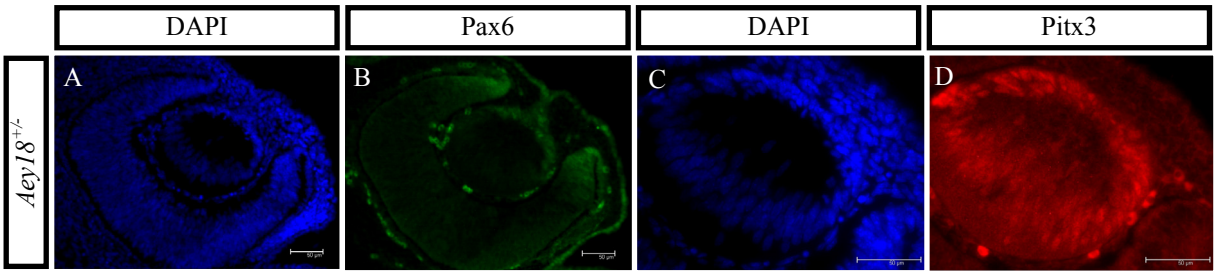


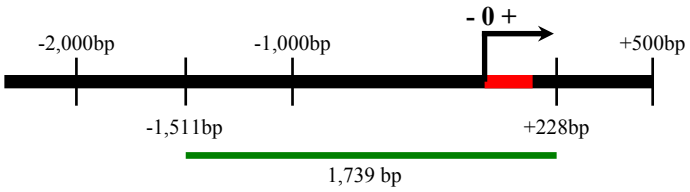
Figure 3.6: Expression of Pax6 and Pitx3 in Pax6 heretozygous (*Aey18*$^{+/-}$) mice. Pax6 was reduced in this mutant at E12.5. However, Pitx3 expression was maintained in these mutants. Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

b) Cross regulatory interaction between Pax6 and Pitx3

To look for the potential interaction between *Pax6* and *Pitx3*, I analyzed the 5'-upstream sequences for the binding of these factors using MatInspector (section 2.1). Analysis of the 2 kb upstream sequence of the *Pax6* gene did not show any Pitx3 putative binding site (Fig 3.7A). Contrary to this, analysis of *Pitx3* promoter spanning the proximal deletion in *aphakia* (-884 / +414) revealed five putative Pax6 binding sites. Those binding sites along with their matrix similarity values are shown in figure 3.8A.

To analyze the regulatory interaction between *Pitx3* and *Pax6 in vitro*, I cloned the proximal promoter region of these genes in the pGL3 basic luciferase vector and analyzed their activity using dual luciferase reporter assay system. 1,739 bp of the 5'-upstream sequence of the *Pax6* containing 1,511 bp upstream and 228 bp downstream of the transcription start site (Fig. 3.7 A) were cloned upstream of the luciferase gene and expressed in the HEK293 cells along with the *Pitx3* expression plasmid (cloned in the pCDNA3.1 vector). Dual luciferase reporter assay revealed that this promoter sequence regulate the expression of luciferase in the presence of Pitx3 in dose dependent manner (Fig. 3.7B). By adding 200 ng of *Pitx3* expression plasmid luciferase activity increased to 4 folds compared to the control, where empty pCDNA3.1 plasmid instead of *Pitx3* expression plasmid was added. By increasing the amount of *Pitx3* expression plasmid luciferase activity was increased and reached to 12 folds when its amount was increased to 800 ng.

A)



B)

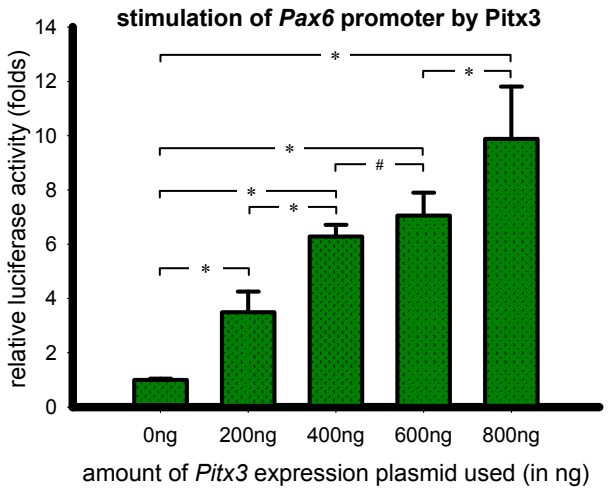
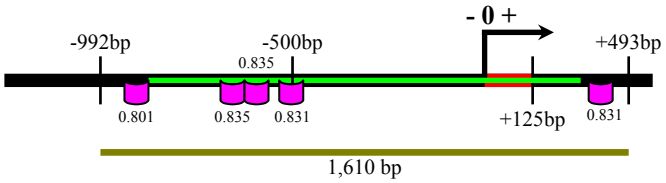


Figure 3.7: *In vitro* regulation of *Pax6* by Pitx3. 1,739 bp (-1,511 / +228) 5'-upstream sequence of the *Pax6* (shown as green line and the red line indicates first exon) was cloned in the pGL3 basic luciferase vector (A) and expressed in the HEK293 cells along with *Pitx3* expression plasmid. Pitx3 regulated the expression of luciferase under this promoter sequence in dose dependent fashion (B). Values are shown as folds of relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity considering the value of control as one. Data are means ± standard deviation from three different experiments performed in triplicate. Statistical analysis was done using student's t test; *, $P \leq 0.001$; #, $P \leq 0.05$.

Contrary to this, Pax6 was expressed in the HEK293 cells along with the luciferase vector carrying the 5'-upstream sequence of the *Pitx3* gene corresponding to the proximal deletion in *aphakia* (-992 bp / +493 bp) (Fig 3.8A). Luciferase reporter assay revealed that Pax6 has

inhibitory action on the *Pitx3* promoter. Significant reduction in the luciferase activity (0.7 folds) was observed with 400 ng of the *Pax6* expression plasmid and this trend continued till the maximum amount of *Pax6* plasmid (800 ng), where it is decreased to 0.4 folds (Fig 3.8B). These luciferase reporter assays indicate that Pax6 has an inhibitory function on the *Pitx3* promoter contrary to the *vice versa*, at least for the tested regulatory regions.

A)



B)

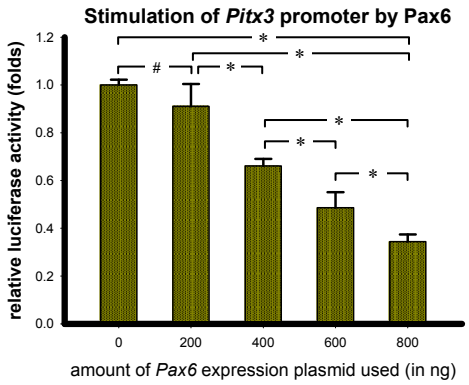


Figure 3.8: *In vitro* regulation of *Pitx3* by Pax6. Putative binding sites for Pax6 on the *Pitx3* proximal promoter were analyzed by MatInspector and shown as pink boxes with their matrix similarity values. (A). Red line indicates the position of the 1st exon and the green line represents the proximal deletion in *aphakia* mice. 1,610 bp (-992 / +493) 5'-upstream sequence of the *Pitx3* (shown as dark yellow line) spanning the *aphakia* proximal deletion was cloned in the pGL3 basic luciferase vector (A) and expressed in the HEK293 cells along with *Pax6* expression plasmid. Pax6 negatively regulated the expression of luciferase under this promoter sequence in dose dependent fashion (B). Values are shown as folds of relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity considering the value of control as one. Data are means ± standard deviation from three different experiments performed in triplicate. Statistical analysis was done using student's t test; *, $P \leq 0.001$; #, $P \leq 0.05$.

3.4 Molecular interaction between Sox2 and Pitx3

a) Expression analysis of Sox2

Immunostaining for Sox2 (Fig. 3.9) has shown that its expression is present in the whole *aphakia* lens at E10.5 but in the wild-type lens expression is only in the posterior half of the lens and moving towards the anterior part as the development proceeds. At E11.5 its expression is more obvious in the cells lining the anterior half of the lens and seems to diminish in cells towards the posterior half. In *aphakia* lens where cells are not organized in circular fashion and some cells keep moving toward the central cavity at E10.5 and fill the lens vesicle at E11.5, they all keep on expressing the Sox2 at almost the similar level. No difference in its expression level was observed in the retina of the *aphakia* and wild-type eye.

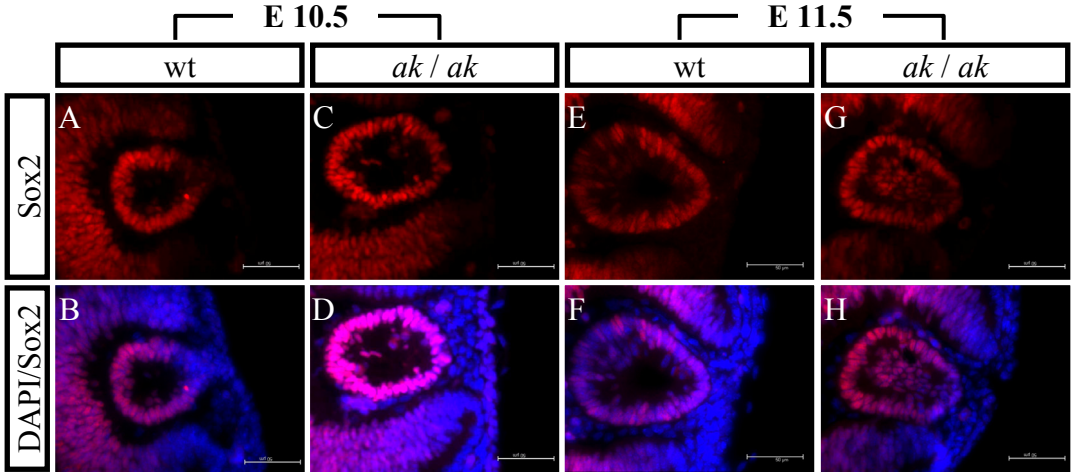


Figure 3.9: Altered expression of Sox2 in *aphakia*. Expression of Sox2 is altered at E10.5 (C, D) and E11.5 (G, H) in *aphakia* lens. At E10.5 its expression is not reached till the most anterior part of the lens in wild-type (A, B) contrary to the *aphakia* lens, while at E11.5 all the cells in the wild-type lens vesicle express Sox2. Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50µm.

b) Regulation of Pitx3 by Sox2

Analysis of 1,610 bp (-992 / +493) 5'-upstream sequence of the *Pitx3* gene (Fig. 3.8A) spanning the *aphakia* proximal deletion was analyzed for the putative Sox2 binding sites but no binding site was detected using MatInspector (Genomatix). However, response of Sox2 to this promoter region was tested in luciferase reporter assay using increasing amount of Sox2 expression plasmid (Fig. 3.10). Sox2 slightly increases the expression of luciferase regulated by *Pitx3* proximal upstream region. However, these slight altertions (although statistically significant) are difficult to interpret in the biological impact.

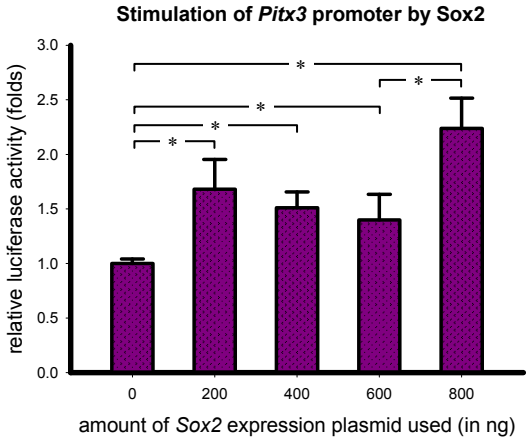


Figure 3.10: Figure 2.12: *In vitro* regulation of *Pitx3* by Sox2. 1,610 bp (-992 / +493) 5'-upstream sequence of the *Pitx3* (as in Fig. 3.9A) spanning the *aphakia* proximal deletion was cloned in the pGL3 basic vector (A) and expressed in the HEK293 cells along with *Sox2* expression plasmid. Sox2 slightly increased the expression of luciferase put under the control of *Pitx3* promoter (B). Values are shown as folds of relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity considering the value of control as one. Data are means ± standard deviation from three different experiments performed in triplicate. Statistical analysis was done using student's t test; *, $P \leq 0.001$.

3.5 Molecular interaction between Ap-2α and Pitx3

a) Expression analysis of *Ap-2α* in aphakia

Whole mount *in situ* hybridization has shown a slight reduction in the expression of *Ap-2α* in the neural tube of the *aphakia* at E11.5 but is not visible at E12.5. In the eye region no difference in expression was detected either at E11.5 or E12.5 between the *aphakia* and wild-type embryos (Fig. 3.11).

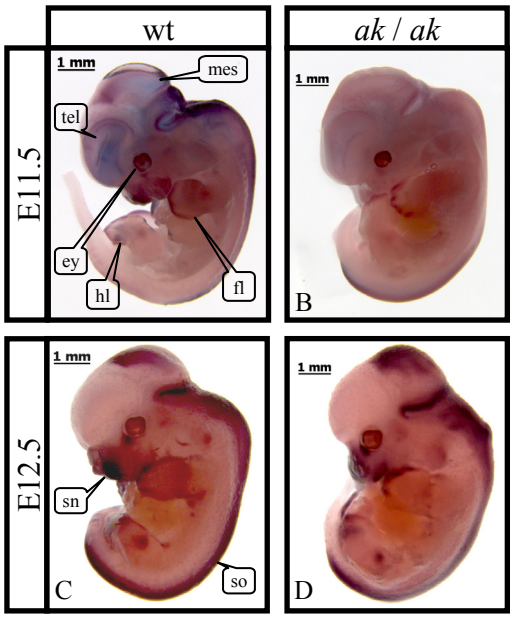


Figure 3.11: Whole mount *in situ* hybridization for *Ap-2α* in *aphakia*. Embryos from day E11.5 and E12.5 were hybridized to DIG-labelled probe for *Ap-2α*. At E11.5 no expression is visible in the neural tube of the *aphakia* embryo (B) but in the rest of the areas no difference was observed compared to the wild-type embryos (A). At E12.5 (C, D) no difference in expression between the *aphakia* and wild-type embryos were observed. Scale bar = 1mm. Abbreviations; ms, mesencephlon; tel, telencephlon; fl, fore limb; hl, hind limb; ey, eye; sn, snout; so, somite

To look for the expression of the Ap-2α in more detail in the *aphakia* eye, I did immunofluoresence staining using anti-Ap-2α antibody (Fig. 3.12). Staining revealed no change in its expression in the epithelial cells at all the stages studied and in the lens vesicle cells at E10.5. However, from E11.5 all the cells in the *aphakia* lens epithelium and those filling the lumen of lens vesicle, consistently express Ap-2α, similarly as the expression of Pax6 and Sox2. Contrary to this, its expression in wild-type lens is restricted to the anterior epithelium from E11.5 and is diminished in the posterior part of the lens.

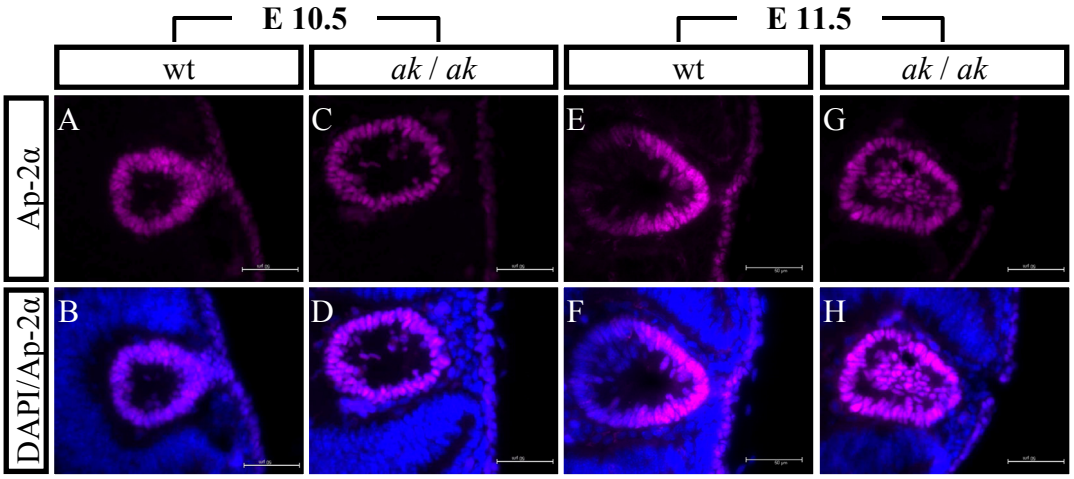


Figure 3.12: Aberrant expression of Ap-2α in *aphakia* lens. At E10.5 (A-D) no apparent change is expression of Ap-2α was observed in *aphakia* lens (C, D) compared to the wild-type lens (A, B). But at E11.5 (E-H) its expression in *aphakia* lens was observed in all the cells present in the lens vesicle including those in the lens lumen (G, H), while in the wild-type lens (E, F), its expression was persistent in the anterior half of lens vesicle but diminished from the posterior half. Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50µm.

b) Cross regulatory interaction between Pitx3 and Ap-2α

Analysis of the *Ap-2a* promoter sequence using MatInspector (section 2.1) did not show any putative Pitx3 binding site. However, two concensus sites (TAAGCC) for bicoid homeodomain protein binding are observed (Fig. 3.13A). To find the potential binding sites of Ap-2α on the *Pitx3* promoter, analysis of the *Pitx3* 5'-upstream sequence spanning the proximal deletion in *aphakia* (-884 / +414) revealed a putative Ap-2α binding site at 125 bp downstream of transcription start site using MatInspector (section 2.1). The binding site along with its matrix similarity value is shown in figure 3.14A.

For *in vitro* analysis of the regulatory interaction between *Pitx3* and *Ap-2α*, I cloned the proximal promoter region of these genes in the pGL3 basic luciferase vector and analyzed their activity using dual luciferase reporter assay system. 1,009 bp of the 5'-upstream sequence of the *Ap-2a* containing 942 bp upstream and 67 bp downstream of the transcription start site (Fig. 3.13A) were cloned upstream of the luciferase gene and expressed in the HEK293 cells along with the *Pitx3* expression plasmid (cloned in the pCDNA3.1 vector) (Fig. 3.13).

Dual luciferase reporter assay revealed that this promoter sequence regulated the expression of luciferase in the presence of Pitx3 in a dose dependent manner. By adding 200 ng of *Pitx3* expression plasmid luciferase activity increased to ~18 folds compared to the control, where empty pCDNA3.1 plasmid instead of *Pitx3* expression plasmid was added. By increasing the amount of *Pitx3* vector luciferase activity was increased to ~60 folds when its amount was increased to 600 ng. This *Ap-2α* promoter region is then analyzed in the luciferase reporter assay

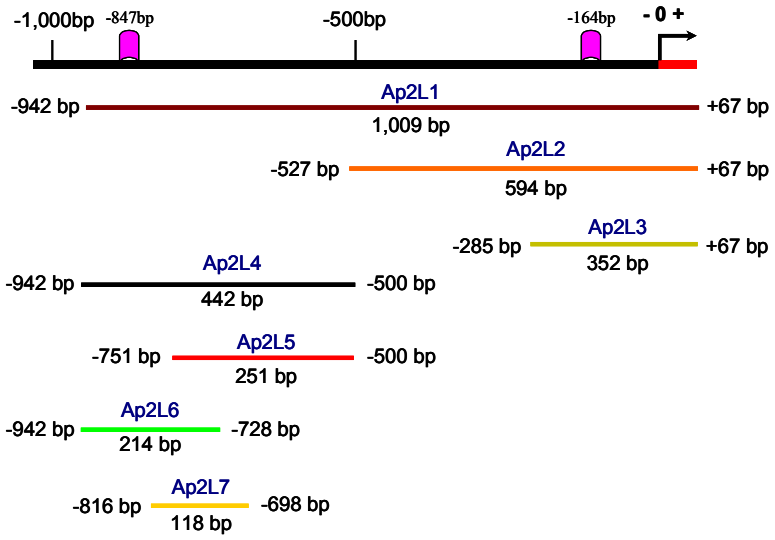
by using two promoter constructs, Ap2L2 (-527 bp / +67 bp) and Ap2L3 (-285 bp / +67 bp) in which part of the 5'-sequence of the 1st promoter construct (Ap2L1) was deleted (Fig. 3.13B). However, these former promoter sequences (Ap2L2 and Ap2L3) did not show comparable luciferase activity as in case of 1st promoter sequence (Ap2L1). These results indicate that a strong binding site for Pitx3 is present between -942 bp and -527 bp of the *Ap-2a* promoter.

To further analyze this upstream sequence, I cloned 4 further *Ap-2a* promoter deletion constructs (Ap2L4, Ap2L5, Ap2L6 and Ap2L7) of varying sizes to find out the minimal sequence responsible for the binding of Pitx3 (Fig. 3.16 A). Luciferase reporter assay revealed that a novel binding site for Pitx3 is present within 198 bp between -698 bp and -500 bp (Fig. 3.13A & B) and Pitx3 can diretly regulate the expression of *Ap-2α* at least *in vitro*.

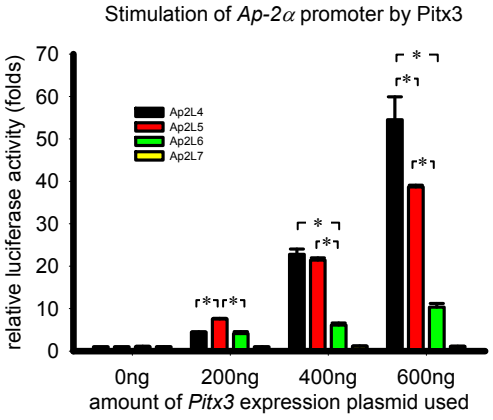
In addition to this, I also investigated if Ap-2α can directly regulate the expression of Pitx3, as a putative binding site for Ap-2α on the proximal promoter sequence of *Pitx3* has been detected by using MatInspector (section 2.1) (Fig. 3.14A). To analyze this, I used the luciferase-*Pitx3* promoter construct spanning the proximal *aphakia* deletion (as in Fig. 3.8A) and the comparable sequence from the *aphakia* mice (Fig. 3.14A). Although, 2 folds increase in luciferase activity (compared to the controls) is observed with the *aphakia-Pitx3* promoter construct but the wild-type-*Pitx3* promoter construct did not show any increase in luciferase activity (Fig. 3.14B).

These results demonstrate that Ap-2α is not a trans-regulator of *Pitx3* at least for its proximal cis-regulatory element. Increase in the luciferase activity with the *aphakia-Pitx3* promoter construct could be a result of change in the sequence (as a result of deletion in the wild-type sequence) that may result in the generation of additional binding site for some factors that are present in the cells and may have some interaction with Ap-2α. But the *aphakia-Pitx3* promoter did not show any additional Ap-2α binding site (compared to the wild-type sequence) as analyzed using MatInspector (section 2.1).

A)



B)



C)

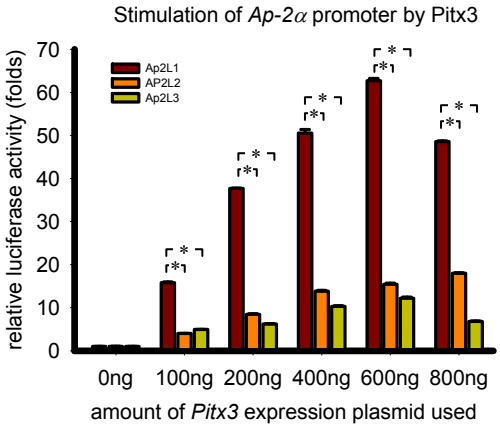
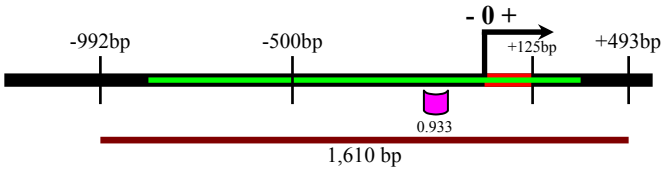


Figure 3.13: *In vitro* regulation of *Ap-2a* by Pitx3. 1,009 bp (-527 bp / +67 bp) 5'-upstream sequence of *Ap-2a* was cloned in the pGL3 basic vector (red color indicate the 1st exon) (A) and expressed in HEK293 cells along with *Pitx3* expression plasmid. Pitx3 regulated the expression of luciferase under this promoter sequence in dose dependent fashion (B, C). Pink boxes in A indicate 'TAAGCC' consensus bicoid homeodomain binding sites with their positions mentioned on the top. Values are shown as folds of relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity considering the value of control as one. Data are means ± standard deviation from three different experiments performed in triplicate. Statistical analysis was done using student's t test. *, $P \leq 0.001$.

A)



B)

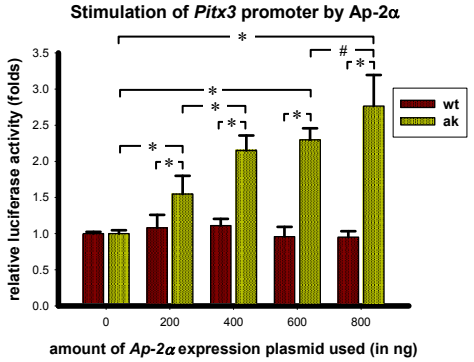


Figure 3.14: *In vitro* regulation of *Pitx3* by Ap-2a. Putative binding sites for Ap-2α on the *Pitx3* proximal promoter were analyzed by MatInspector and shown as pink box with its matrix similarity value (A). 1,610 bp (-992 / +493) 5'-upstream sequence of the *Pitx3* (shown as dark red line) spanning the *aphakia* proximal deletion (shown as green line) was cloned in the pGL3 basic vector (A) and expressed in HEK293 cells along with *Pitx3* expression plasmid. Pitx3 regulated the expression of luciferase under this promoter sequence in dose dependent fashion (B). Values are shown as folds of relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity considering the value of control as one. Data are means ± standard deviation from three different experiments performed in triplicate. Statistical analysis was done using student's t test. *, $P \leq 0.001$; #, $P \leq 0.05$.

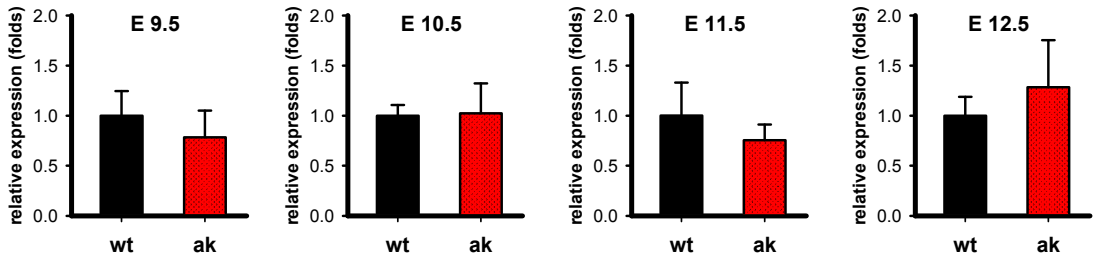
3.6 Expression analysis of E- and N-cadherin in aphakia

Cadherins are members of transmemebrane proteins and involved in cell-cell adhesion [195,196]. In the lens, classical cadherins (E and N) have very distinct expression pattern. E-cadherin is expressed in the surface and anterior lens epithelium. Contrary to this, N-cadherin is expressed in all the lens cells during lens development but not in the surface ectoderm [73,197,198].

To investigate, if changes in expression of *Ap-2α* affect the expression of cadherins, especially E-cadherin, which is considered as a direct downstream target of Ap-2α [78,199,200], mRNA of E-cadherin and N-cadherin were analyzed. Quantification of E-cadherin using RT-qPCR has shown that its expression is not significantly changed from E9.5 to E12.5 (Fig. 3.15); however, a trend towards change in expression of *Cdh1* was observed at all stages. I found that, its expression is reduced at E9.5 (20%) and E11.5 (25%) but increased at E12.5.

RT-qPCR for N-cadherin mRNA did not significant change at all the stages analyzed compared to the littermate controls (Fig. 3.15). However, there was a trend towards decrease in its transcript at E11.5 but opposite was the case at E9.5 and E12.5.

***Cdh1* (E-cadherin)**



***Cdh2* (N-cadherin)**

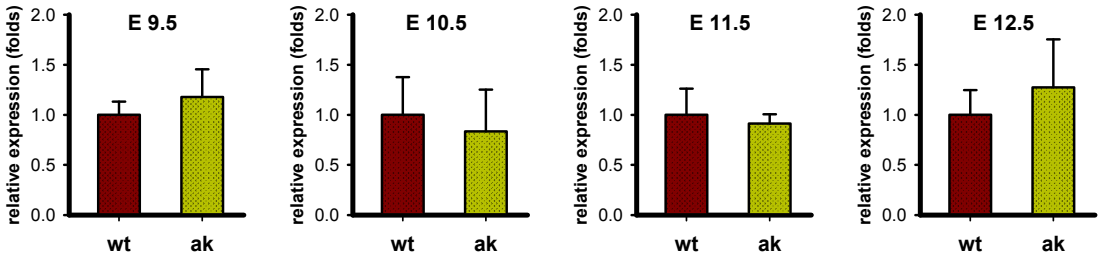


Figure 3.15: Expression of E- and N-cadherin is not significantly changed in *aphakia*. RT-qPCR was done at different developmental stages (shown as bold letter at the top of each graph) using RNA from the head of littermate embryos. Expression is shown as folds of values normalized to *Tuba* and calculated using $2^{-\Delta\Delta CT}$ method [189]. Values from wild-type samples are represented as one. Data represents means ± standard deviations from five samples run in duplicate. Statistical analysis was done using student's t test.

Further, spatiotemporal expression of these cadherins in *aphakia* mice were studied in detail using immunofluoresence staining at different developmental stages using anti-E-cadherin and anti-N-cadherin antibodies (Fig. 3.16).

At E10.5 expression of E-cadherin has shown irregular pattern in *aphakia* lens compared to the wild-type lens, where its expression is nicely organized in the cells forming a round lens vesicle (Fig. 3.16). Surface epithelium did not show any abnormality in its expression in *aphakia*. All the cells forming the lens vesicle including those involved in the attachment of lens vesicle to the surface epithelium and filling the lens vesicle, continue expressing E-cadherin at E11.5 and at latter developmental stages (E12.5) but still in irregular pattern (and reduced especially at E12.5) (Fig. 3.16R), indicating that the cells in the rudimentary lens vesicle maintain the identity of the epithelial cells. In wild-type lens expression of E-cadherin is reduced after the lens vesicle separation (at E12.5); diminished in the posterior lens vesicle cells and resticted to the anterior lens vesicle in line with the published data [198]. However, expression of N-cadherin is increase at this lens developmental stage. Therefore, I explored its expression at the stage of lens vesicle separation and one day later.

Expression of N-cadherin in *aphakia* at E11.5 was strongly reduced and present in the form of few patches; however, in the wild-type lens, it was detected in the whole lens (Fig. 3.17). Similarly at E12.5, expression of N-cadherin was observed in the all the lens cells in wild-type but in *aphakia*, its expression is almost dimished at this stage in the lens (Fig. 3.17K).

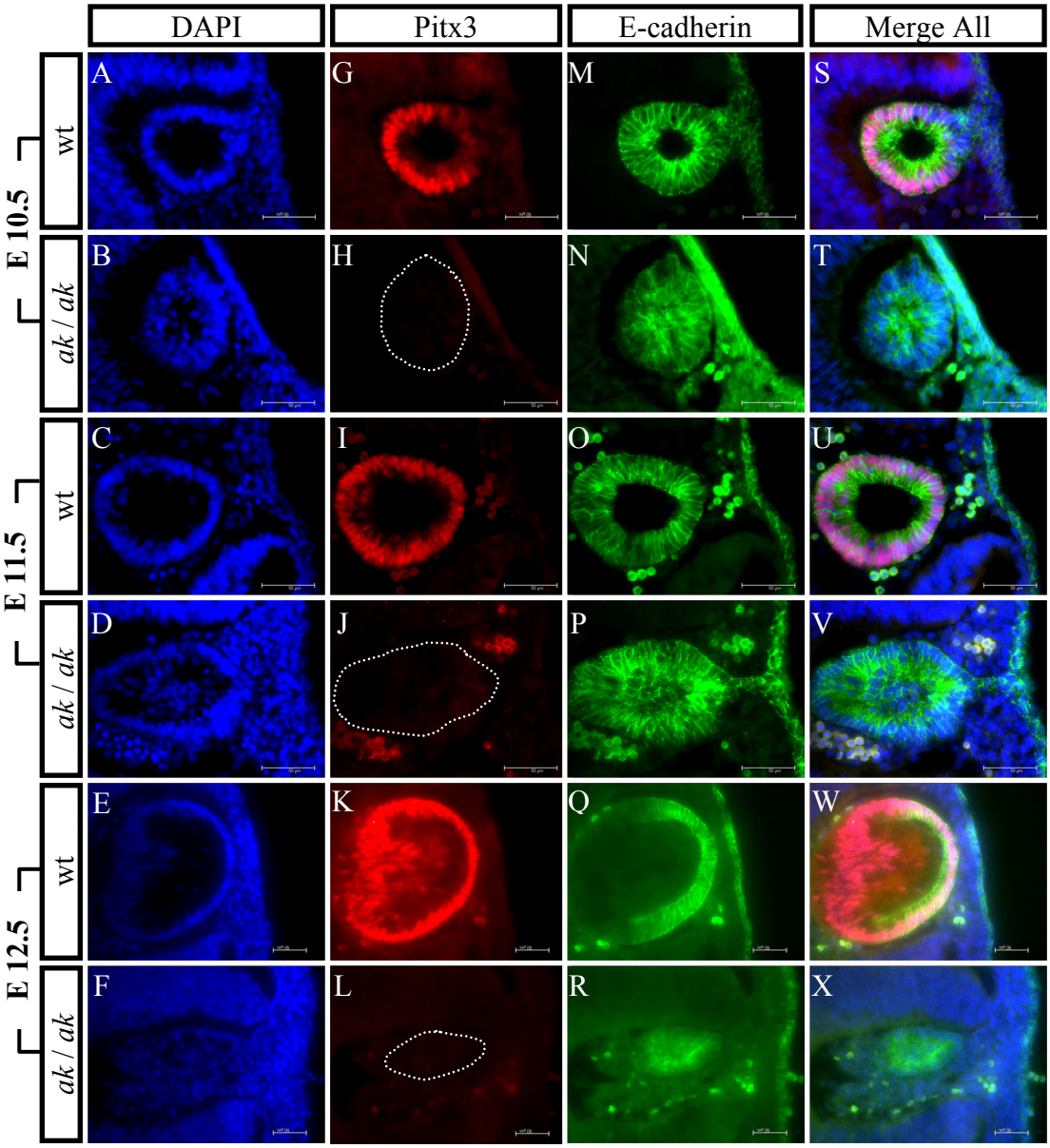


Figure 3.16: Disorganized expression of E-Cadherin in *aphakia*. Expression of E-cadherin was detected in *aphakia* at all the stages studied (from E10.5 to E12.5) (N, T, P, V, R, X) but highly disorganized. All the cells in the *aphakia* lens including those filling the lens lumen were found positive for E-cadherin at E11.5. At E12.5, its expression was found restricted to the anterior epithelium and dimished from the posterior part in the wild-type lens, while in the *aphakia* was detected in the whole rudimentary lens although at low level. Immunofluoresence staining was performed on 12 µm thick, PFA fixed frozen sections. Scale bars = 50 µm**.**

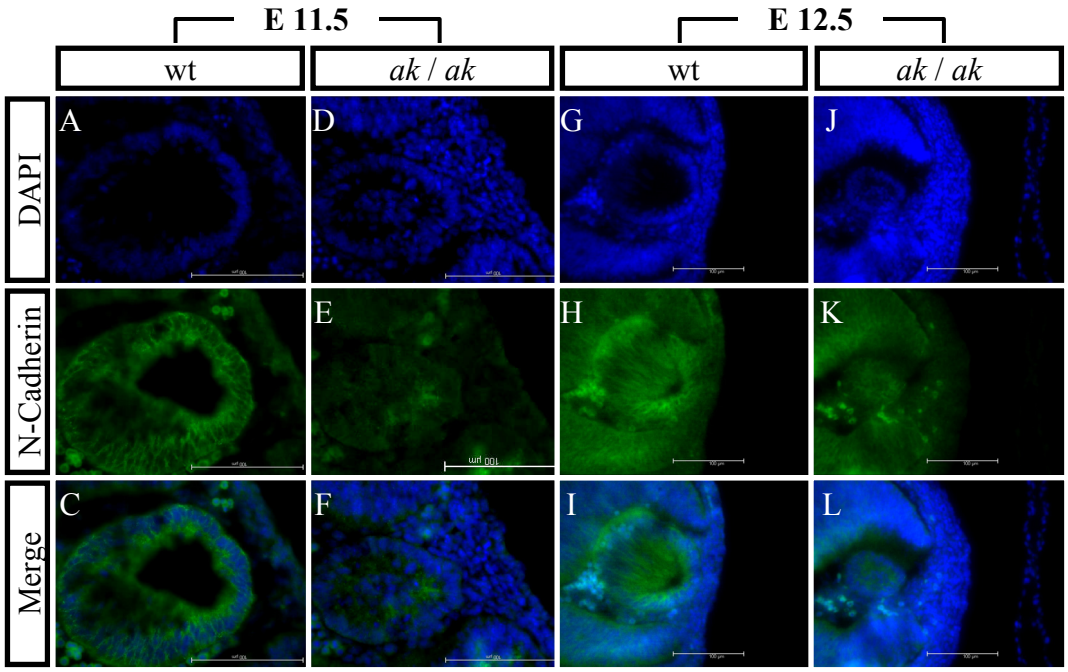


Figure 3.17: Reduced expression of N-Cadherin in *aphakia*. In wild-type lens expression of N-cadherin was observed in almost whole lens (I, M), while in *aphakia* its expression was detected in the patches form (E, K) and reduced at both E11.5 and E12.5. Immunofluoresence staining was performed on 12 µm thick, PFA fixed frozen sections. Scale bars = 100 µm.

a) E-cadherin as a target of Pitx3

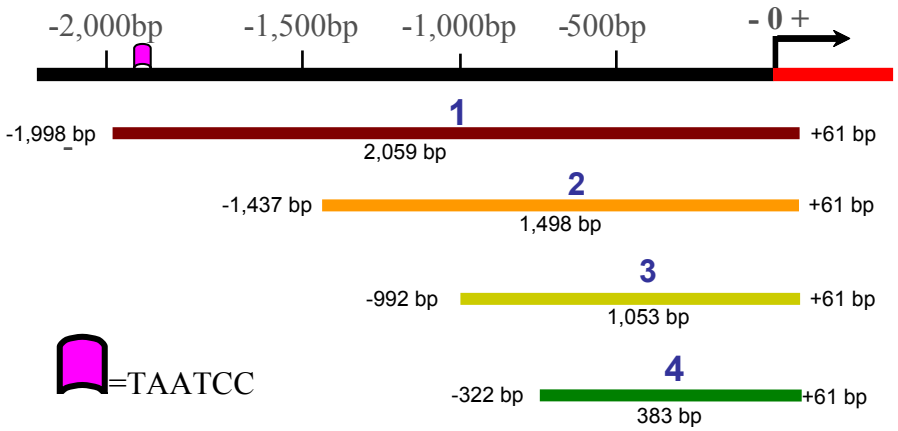
To investigate the molecular interaction between E-cadherin (*Cdh1*) and the *Pitx3,* I analyzed 2,000 bp 5'-upstream region of *Cdh1* for the putative Pitx3 binding sites using MatInspector (section 2.1) but no binding site was found. However, detailed analysis of this sequence revealed a Pitx3 putative binding site (Fig. 3.18A), similar to the one observed in the upstream region of the tyrosine hydroxylase gene (*TH*) and has been shown to regulate the expression of TH in dopaminergic neurons [178,201]. These binding sites contain the 'TAATCC' sequence element that has also been observed in the promoter region of other target genes of Pitx family members [6,7].

To look experimentally for an interaction between *Cdh1* and Pitx3, I cloned the 2,059 bp 5'-upstream sequence of the *Cdh1* including 1,998 bp upstream and 61 bp downstream of the transcription start site (Fig 3.18A). This construct regulated the expression of luciferase in the presence of Pitx3 in a dose dependent manner. Co-transfection of 200 ng of *Pitx3* expression plasmid increased the luciferase activity to ~18 folds compared to the control. This increase in luciferase activity was almost doubled when the amount of *Pitx3* expression plasmid was increased to 800 ng (Fig. 3.18).

Luciferase reporter assays using *Cdh1* promoter deletion constructs of different sizes indicate that Pitx3 can bind to other regulatory sites as well in addition to the 'TAATCC' mentioned

above. Additionally, it seems that there are two regulatory elements in the investigated *Cdh1* promoter region, 1st between -1,437 bp and -992 bp and the 2nd is between -322 bp and +61 bp, as the difference in the luciferase activity between these promoter-luciferase constructs is almost double.

A)



B)

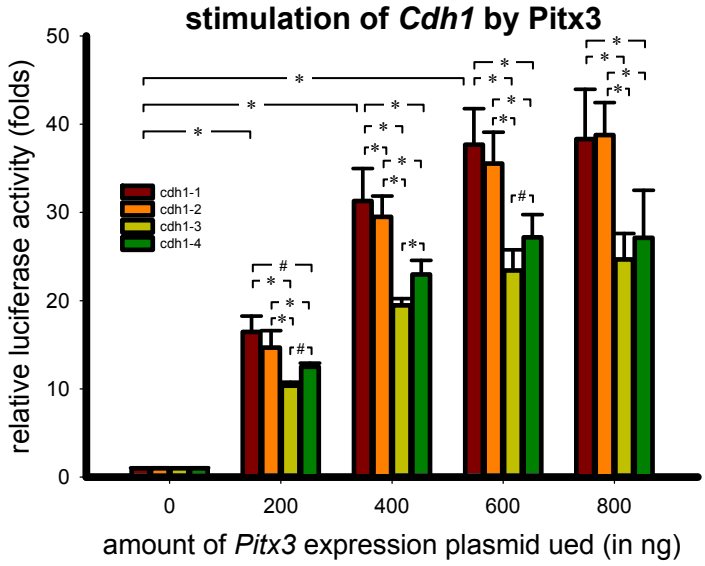


Figure 3.18: *In vitro* regulation of *Cdh1* by Pitx3. 2,059 bp (-1,998 / +61) 5'-upstream sequence of the *Cdh1* gene was cloned in the pGL3 basic vector (red line indicates 1st exon) (A) and expressed in the HEK293 cells along with *Pitx3* expression plasmid. Pitx3 regulated the expression of luciferase under this promoter sequence in dose dependent fashion (B). Values are shown as folds of relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity considering the value of control as one. Error bars ± standard deviations from three different experiments performed in triplicate. Pink colored box in 'A' indicates the putative Pitx3 binding site with its sequence mentioned below. Statistical analysis was done using student's t test. *, $P \leq 0.001$; #, $P \leq 0.05$.

3.7 Foxe3 is a molecular target of Pitx3

a) Expression analysis of *Foxe3*

Immunofluoresence staining using the antibody against Foxe3 has shown that its expression is reduced in *aphakia* at E10.5 and E11.5 but severe reduction in staining was observed at E12.5 (Figure 3.19), where only very few cells expressing Foxe3 are seen. Compared to the wild-type lens at this stage, where its expression is restricted to the anterior lens epithelium, Foxe3 positive cells were present arbitrarily in the *aphakia* lens. These results suggest that Pitx3 is not necessary for the initiation of *Foxe3* expression but it is crucial for the maintenance of spatiotemporal expression of Foxe3.

b) Sequence analysis of the putative Pitx3 binding sites

RT-qPCR for the *Foxe3* in *aphakia* indicates that *Foxe3* is a potential downstream target of Pitx3 (Fig. 3.3). To explain this, sequence analysis of the 5'-upstream sequence of the *Foxe3* was done to find the putative Pitx3 binding sites. Analysis of the 4 kb upstream stream sequence of the gene from the transcription start site using MatInspector (section 2.1) did not show any potential binding site for Pitx3; however, binding sites for the other bicoid-like homeodomain transcription factors, like Otx2 and Crx were found. Then I looked for the conserved regulatory elements in the upstream sequence of this gene using the ECR browser (section 2.1). An element of 654 bp, which is located 2,954 bp upstream of the *Foxe3* transcription start site, is conserved throughout mammals (Fig. 3.20 A). Analysis of this conserved sequence using rVista (section 2.1) revealed two Pitx3 putative binding sites (TAATCC) similar to the one explained in section 3.6.

c) Regulation of *Foxe3* by Pitx3

To analyze this conserved 5'-upstream sequence *in vitro*, I cloned 283 bp (-3,517 / -3234) containing 'TAATCC' site (known binding site of Pitx3, see previous section) in the pGL3 basic luciferase vector (Figure 3.20B) and transfected into the HEK293 cells along with *Pitx3* expression plasmid. This sequence regulated the luciferase expression with increasing amount of *Pitx3* expression plasmid. An increase in ~65 folds luciferase activity was observed when 600 ng of *Pitx3* expression plasmid was used (Figure 3.20 C). These results demonstrate the functional impact of these Pitx3 putative binding sites on the *Foxe3* promoter and provide an evidence of direct regulation of *Foxe3* by Pitx3.

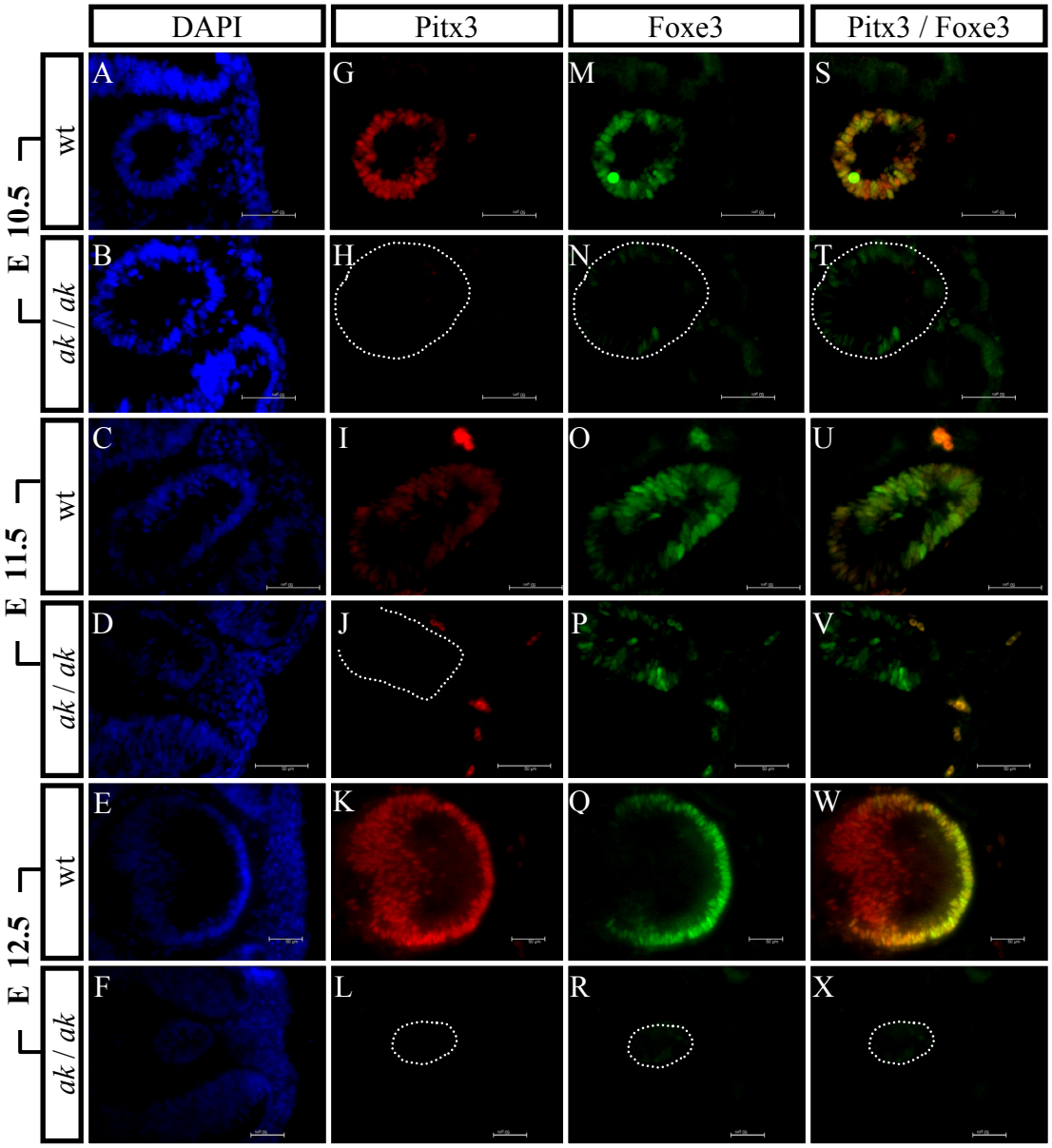


Figure 3.19: Diminished Foxe3 expression in *aphakia*. At E10.5, low expression of Foxe3 was observed in *aphakia* (N, T) as compared to the wild-type lens (M, S) but all the cells in the lens vesicle express Foxe3 at E11.5 in *aphakia* (P, V). However, at E12.5 only few Foxe3 positive cells were observed in *aphakia* (R, X) but in wild-type lens whole anterior lens epithelial cells were found positive for Foxe3. Co-staining of Pitx3 and Foxe3 has shown that their expression is completely overlapped at all the stages in wild-type lens (S, U, W). Immunofluoresence staining was performed on 12 µm thick, PFA fixed frozen sections. Scale bars = 50 µm.

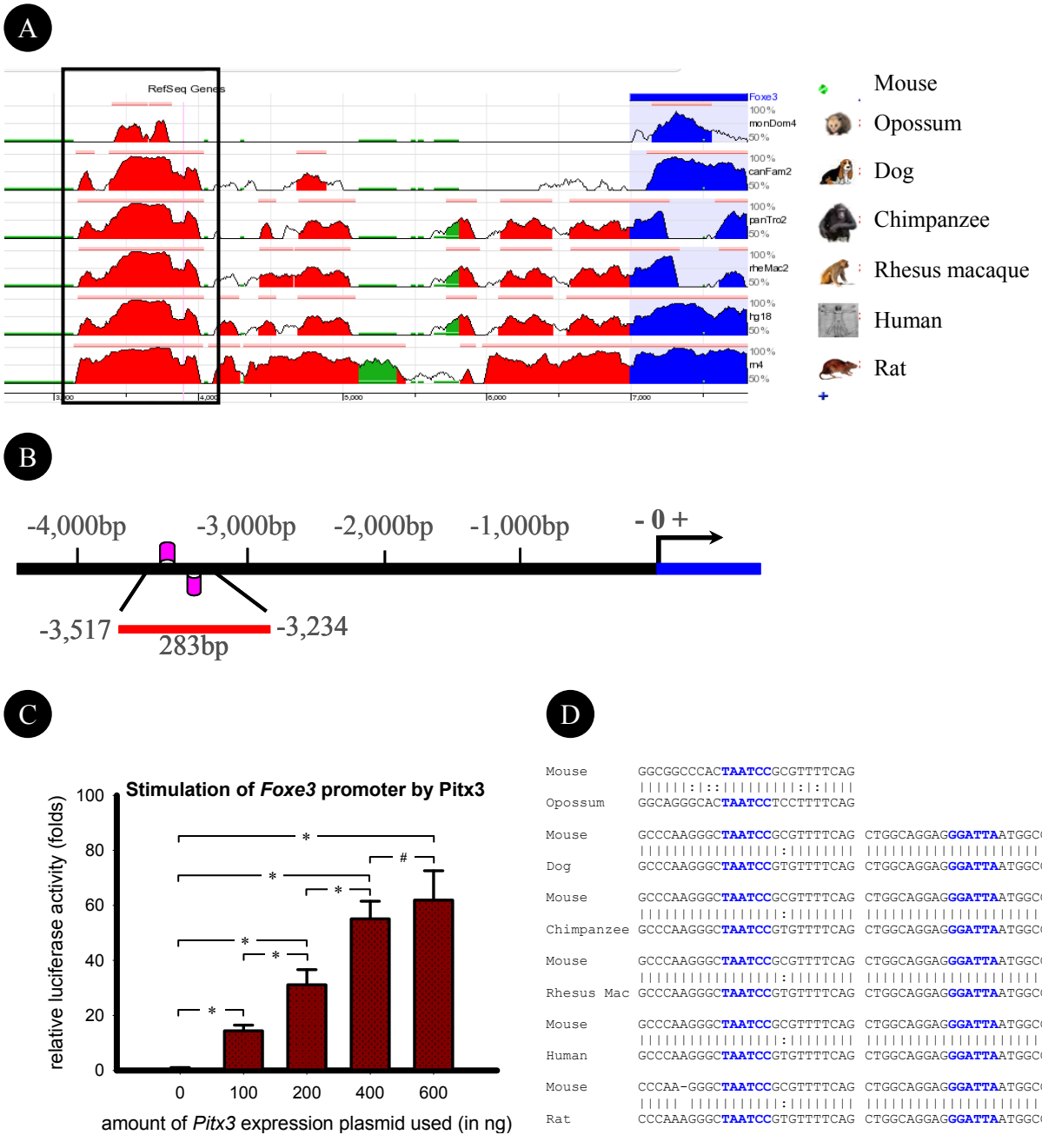


Figure 3.20: *In vitro* regulation of *Foxe3* by Pitx3.
An evolutionary conserved genomic region 5'-upstream of *Foxe3* is detected (shown as black box and blue color indicates coding region) (A) that contains conserved putative Pitx3 binding sites, shown as pink boxes (B). This conserved 283 bp (-3,517 / -3,234) was cloned into pGL3 basic luciferase vector (B) and expressed in the HEK293 cells along with *Pitx3* expression plasmid. Pitx3 regulated the expression of luciferase under this promoter sequence in dose dependent fashion (C). Sequences of evolutionary conserved Pitx3 putative binding sites are shown (D). Values are shown as folds of relative luciferase activity, calculated as the ratio of firefly luciferase activity to renilla luciferase activity considering the value of control as one. Data represents means ± standard deviation from six different experiments performed in triplicate. Statistical analysis was done using student's t test. *, $P \leq 0.001$; #, $P \leq 0.05$.

3.8 Prox1 is directly regulated by Pitx3

a) Expression analysis of Prox1

To observe the expression of Prox1 at the protein level, I did immunofluoresence staining (Fig. 3.21) at different developmental stages. Only few cells in the posterior part of the lens were found positive for Prox1 at E10.5 in the wild-type lens, indicating that Prox1 is detectable around this developmental stage, as *Prox1* transcript has already been detected at E9.5 in the lens [92]. Further, analysis at latter stages (E11.5 and E12.5) clearly show that the expression of Prox1, after its initiation from the posterior lens cells, spreads towards the anterior cells, but not in the very anterior part of the lens (Fig 3.21M, O, Q) that comprises of actively dividing cells. Co-staining of Prox1 and Pitx3 in the wild-type lens has shown that the expression of these two transcription factors is higly overlapping (Fig. 3.21S, U, W) but the presence of more number of posivite cells for Pitx3 at E10.5 compared to Prox1 indicate that the expression of *Pitx3* probably starts little earlier than *Prox1* (Fig. 3.21G, M, S). Furterhmore, co-expression of these genes at latter developmental stages (E11.5 and E12.5) demonstrate that the expression of Prox1 follows the expression of Pitx3 indicating that these two transcription factors have some regulatory interaction. This notion is further supported by the complete lack of Prox1 expression in the *aphakia* lens at all developmental stages (E10.5, E11.5 and E12.5) investigated (Fig. 3.21N, P, R) and provides evidence that Pitx3 is necessary to elicit the expression of *Prox1*.

b) Sequence analysis of the putative Pitx3 binding sites

Lack of *Prox1* expression in Pitx3-deficient *aphakia* mice indicates that it is a potential direct downstream target of Pitx3. To explain this, sequence analysis of the 5'-upstream sequence of the *Prox1* was done to find the Pitx3 putative binding sites. Analysis of the 4 kb upstream sequence of this gene from the transcription start site using MatInspector (section 2.1) did not revealed any potential binding site for the Pitx3; however, binding sites for the other bicoid-like homeodomain transcription factors, like Otx2, Crx, Pitx1 and Pitx2 were found. Then, I looked for the conserved regulatory element in the upstream sequence of this gene using ECR browser (section 2.1). A sequence element of 494 bp, which is ~10,000 bp upstream of the *Prox1* transcription start site, is conserved throughout the mammals (Fig. 3.22A). Analysis of this sequence revealed a conserved Pitx3 putative binding site (Fig. 3.22B, D) similar to the one found in the *Foxe3* 5'-upstream sequence (see sections 3.6 and 3.7).

c) Regulation of *Prox1* by Pitx3

To analyze this conserved 5'-upstream sequence of *Prox1 in vitro*, I cloned 272 bp (-10,173 / -9,901) sequence containing 'TAATCC' site (known binding site for Pitx3, see section 3.7) in the pGL3 basic luciferase vector (Fig. 3.22B) and transfected into HEK293 cells along with *Pitx3* expression plasmid. This sequence regulated the luciferase expression with increasing amount of *Pitx3* expresion plasmid. An increase in ~50 folds luciferase activity was observed when 600 ng of this *Pitx3* expresion plasmid was added (Fig. 3.25 C). Proximal 1,370 bp (-1,136 / +234) 5'-upstream sequence of *Prox1*, which was also analyzed along with the distal conserved sequence, did not regulated the expression of luciferase in the presence of Pitx3 comparable to the distal sequence (Fig. 3.22C). These results show that Pitx3 binds to this conserved putative binding site and regulates the expression of *Prox1* in the ocular lens.

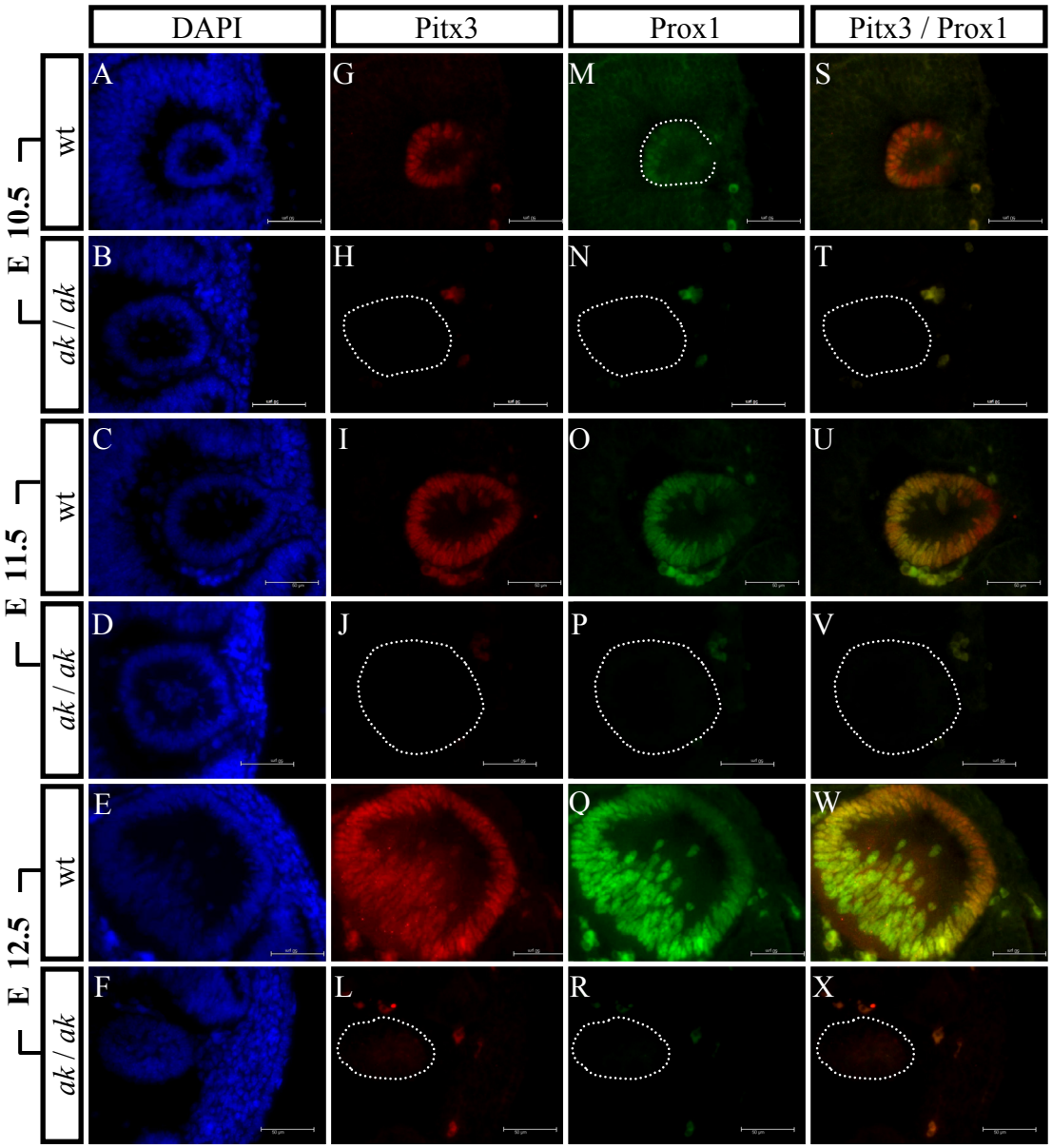


Figure 3.21: Abolished expression of Prox1 in *aphakia*. At E10.5 only very few cells in the posterior part of the lens were found positive for Prox1 in the wild-type lens (M) but its expression increased one day later (O) and found in all the lens cells in the posterior and equatorial region (Q). No expression of Prox1 in the *aphakia* lens was observed at all the stages observed (N, T, P, V, R, X). Complete overlap of Pitx3 and Prox1 is evident in wild-type lens at all the stages investigated (S, U, W). Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

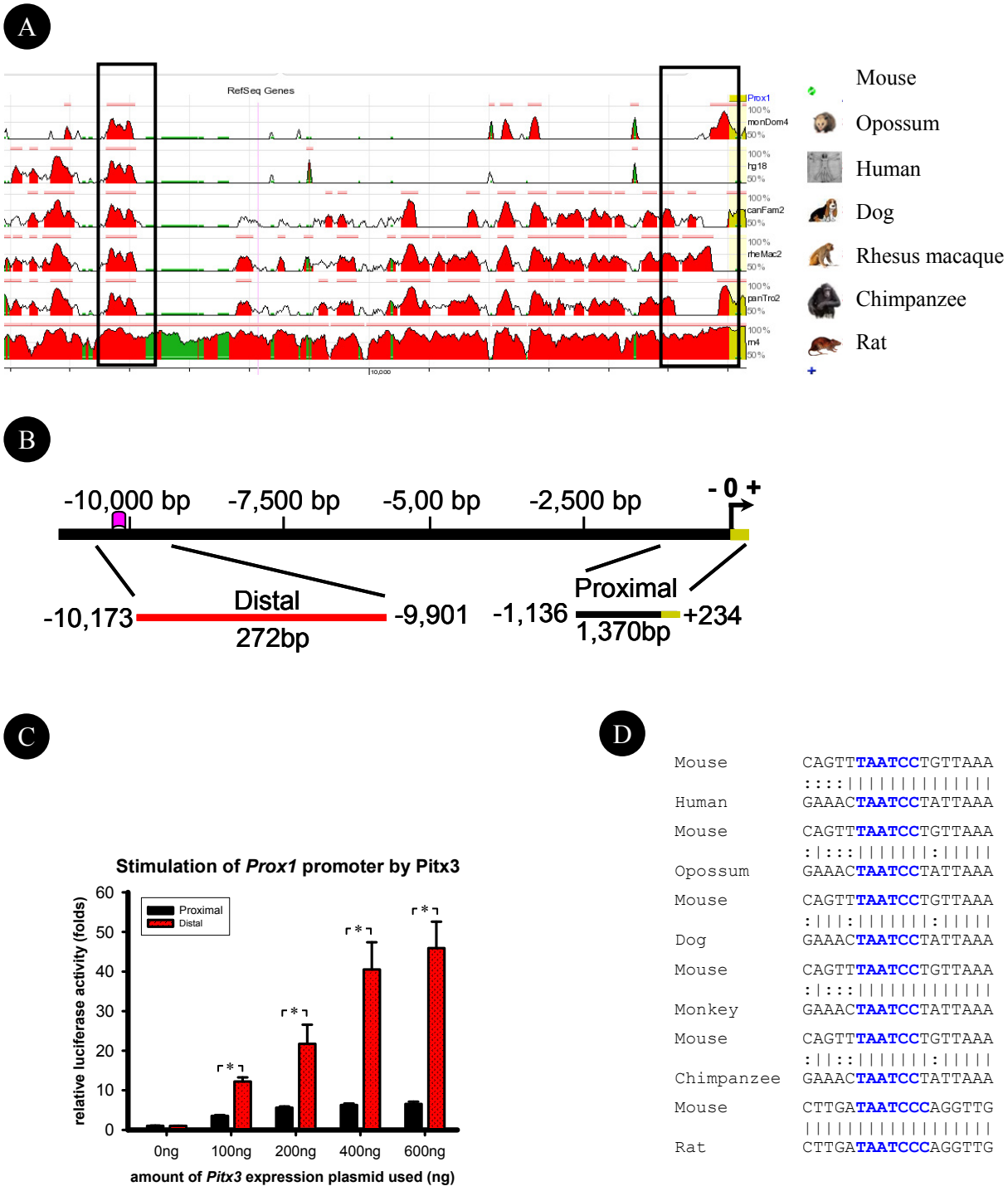


Figure 3.22: Pitx3 binds to the putative binding site in the 5'-upstream sequence of *Prox1* and regulates its expression. A conserved regulatory element is observed ~10,000 bp upstream of the *Prox1* (shown as black box and the yellow color indicate the transcribing region) (A) that contains a Pitx3 putative binding site, shown as pink colour box (B). Alignment of the sequences with conserved base pairs among mammals is shown (D). Analysis of the 272 bp sequence (-10,173 / -9,901) containing the conserved putative Pitx3 binding site, in the luciferase reporter assay showed dose dependent regulation by Pitx3 but the 1,370 bp proximal sequence (-1,136 / +234) did not show comparable activity (C). Data is shown as firefly luciferase activity relative to the renilla luciferase activity. Error bars are means ± SD from at least 3 different experiments run in triplicate. Statistical analysis was done using student's t test. *, $P \leq 0.001$; #, $P \leq 0.05$.

3.9 Lens proliferation and differentiation in aphakia

Diminished expression of Foxe3 in *aphakia* lens provoked me to investigate the proliferation in *aphakia* lens, as Foxe3 is known to be responsible for maintaining the lens proliferative activity [85]. To observe the proliferation in developing *aphakia* lens, dividing cells were labelled with BrdU at E11.5. Staining using antibody against BrdU has revealed severe defects in proliferation in the *aphakia* lens (Fig. 3.23). Only very few BrdU positive cells were observed in the *aphakia* lens compared to the littermate wild-type controls (Fig. 3.23D, F, H, J). In the wild-type lens, anterior cells were found actively dividing contrary to the *aphakia* lens.

Co-staining for BrdU and Prox1 revealed that cells with low or missing expression of Prox1 in the anterior lens epithelium are most actively dividing; however, the posterior part of the lens comprises of differentiating cells were found expressing high level of Prox1 (Fig. 3.23E) but Pitx3 expression was detected in all the lens wild-type lens at this stage (Fig. 3.23G).

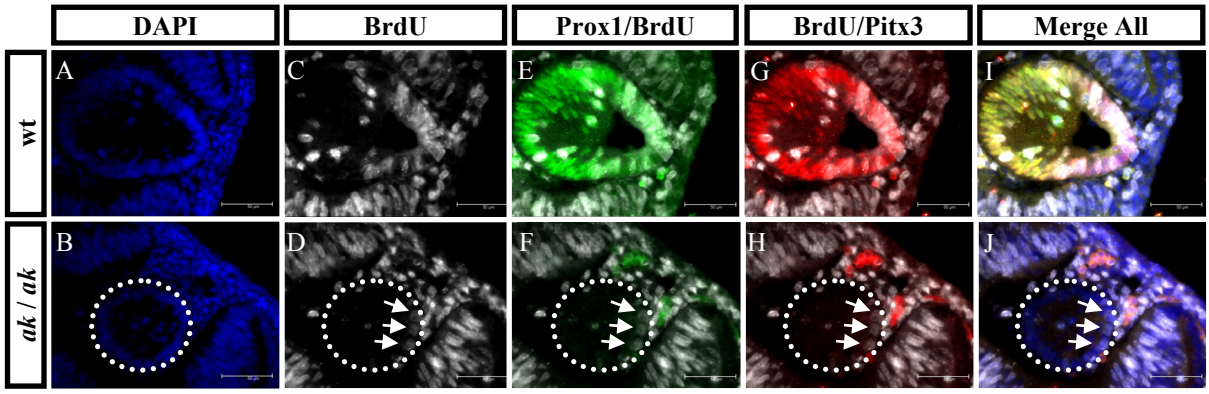


Figure 3.23: Reduced proliferation in *aphakia* lens at E11.5. BrdU was injected in the pregnant female at E11.5, two hours before sacrifice. Immunofluoresence staining using anti-BrdU showed very few positive cells in the *aphakia* lens (D) compared to the littermate controls (C). Co-staining with Prox1 is evident that its expression is higher in differentiating cells (E). Staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

To further explore the mitotic activity in *aphakia* at different developmental stages, I investigated the expression of *E4f1* transcription factor that is expressed ubiquitously and is crucial for mitotic activity [202] by RT-qPCR (Fig 3.24). Expression of *E4f1* was found to be reduced at E11.5 compared to the littermate wild-type controls, confirming the results from BrdU labelling. However, a trend towards increase in *E4f1* expression was observed in *aphakia* at E10.5, indicating that the *aphakia* lens may have higher proliferation rate one day earlier than the wild-type littermates. Undetectable difference in *E4f1* expression at E12.5 is due to decreased mitotic activity in the wild-type lens that is restricted to the anterior epithelium at this stage.

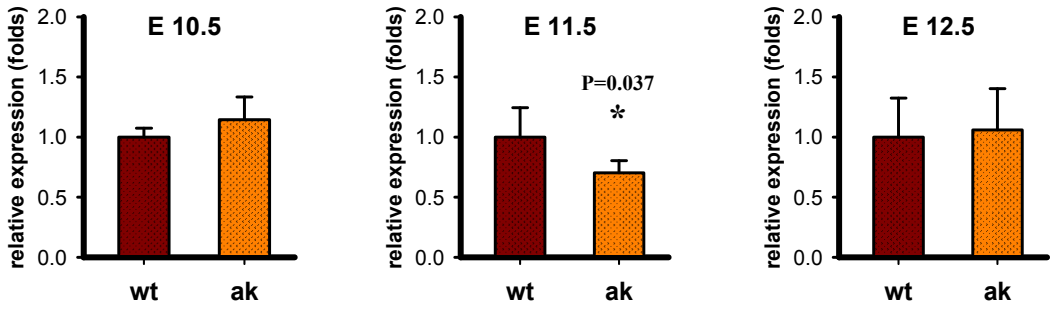


Figure 3.24: Expression of *E4f1* in *aphakia* during development. *E4f1* expression was observed at embryonic day E10.5 (A), E11.5 (B) and E12.5 (C) by RT-qPCR using RNA from the head of embryos at these stages. At E10.5 (A), a trend towards increase in *E4f1* expression was observed in *aphakia* compared to the wild-type littermate controls, but its expressed was significantly reduced at E11.5 in *aphakia* (B); however, no difference was observed in *E4f1* expression at E12.5 (C). Expression is shown as folds of expression normalized to *Tuba*. Data are means ± standard deviation from five littermate samples run in duplicate. Statistical analysis was done using student's t test and p-values are given wherever applied.

Deficits in *aphakia* lens proliferation and absence of Prox1, which is considered an important factor in differentiation of lens fiber cells [92], prompted me to investigate the differentiation process in the developing *aphakia* lens. To pursue this, I explored the expression of γ-crystallin, highly expressed in differentiating fiber cells. Immunofluoresence staining using antibody against γ-crystallin (Fig. 3.25) has shown that its expression starts at E11.5. At this stage, all the wild-type lens cells were found positive for γ-crystallin but its expression was found restricted to the differentiating posterior lens fiber cells at latter stages. From developmental stage E12.5, its expression was not observed in the anterior lens epithelium, representing the proliferation zone, while cells in the posterior part of the lens continue expressing γ-crystallin, representing the differentiation zone. Most striking and noticeable finding was the earlier detection of γ-crystallin expression (at E10.5) in *aphakia* lens compared to the wild-type lens. This earlier expression of γ-crystallin indicates that *aphakia* lens cells start differentiation earlier than the wild-type and the In addition to this, all the cells in the rudimentary *aphakia* lens were found expressing γ-crystallin, although its expression decreased at latter stages (E12.5 and E14.5). Anterior lens epithelial cells that lack the expression of γ-crystallin in the wild-type lens were not observed in the *aphakia* lens. These results indicate that *aphakia* lenses lack the proliferative anterior lens epithelium.

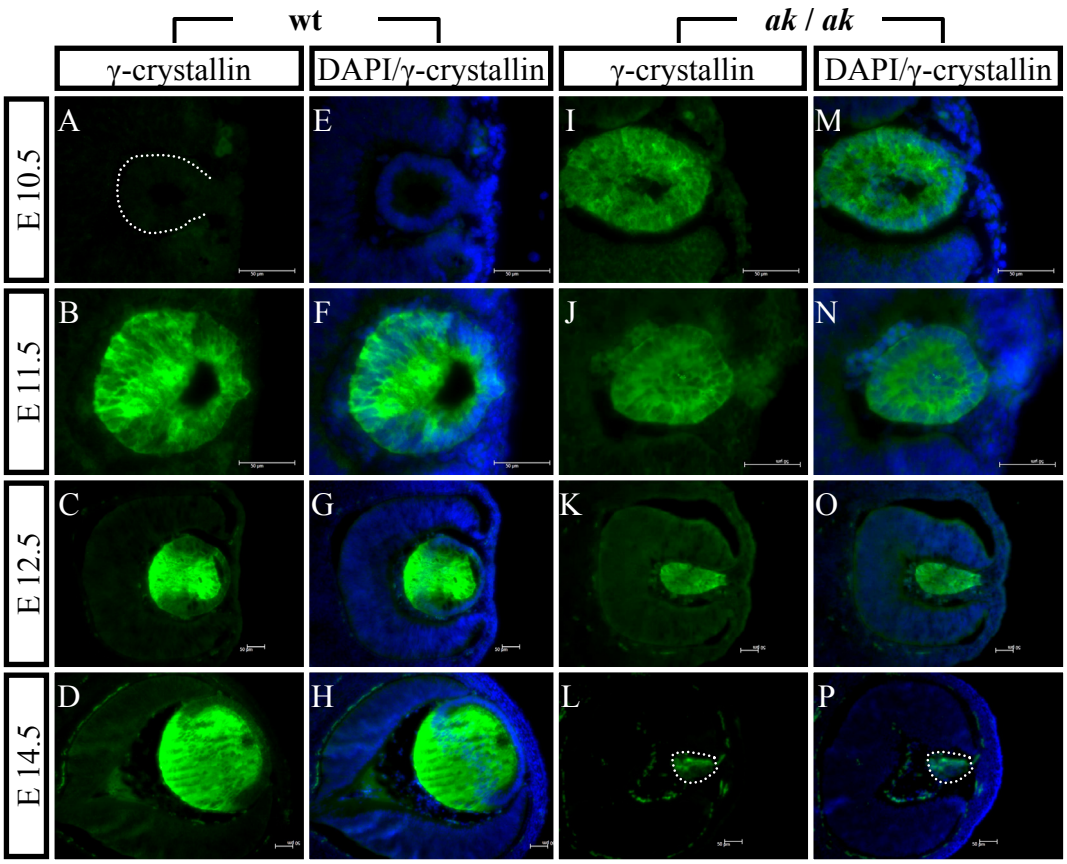


Figure 3.25: Earlier and persistent expression of γ-crystallin in *aphakia*. Immunofluoresence staining of wild-type and *aphakia* lens for γ-crystallin during different developmental stages (E10.5, E11.5, E12.5, E14.5) revealed that it is expressed earlier in *aphakia* (C, D) compared to the wild-type embryos (A, B). From E12.5, a clear demarcation of lens epithelium and differentiating lens fibre cells can be seen that express γ-crystallin in wild-type embryos (I, J) and is more apparent at E14.5 (M, N), while no such demarcation is observed in *aphakia* and γ-crystallin expressed throughout the rudimentary lens (K, L). Immunofluoresence staining was performed either on, PFA fixed 8 µm thick paraffin sections (E14.5) or 12 µm thick frozen sections (E10.5, E11.5, E12.5). Scale bars = 50 µm.

3.10 *Tube1 as a target of* Pitx3

ε-tubulin which is one of the most recently identified tubulins and is required for centriole duplication and microtubule organization. Expression of this gene was found down-regulated in *aphakia* embryos in the expression array analysis done in our lab (Muenster, 2005).

To confirm these findings, I looked for the expression of *Tube1* at different developmental stages using realtime-qPCR (Fig. 3.26). Expression of this gene was found to be down-regulated at early developmental stages (E9.5 and E10.5) but no difference was observed at E11.5 compared to the littermate controls.

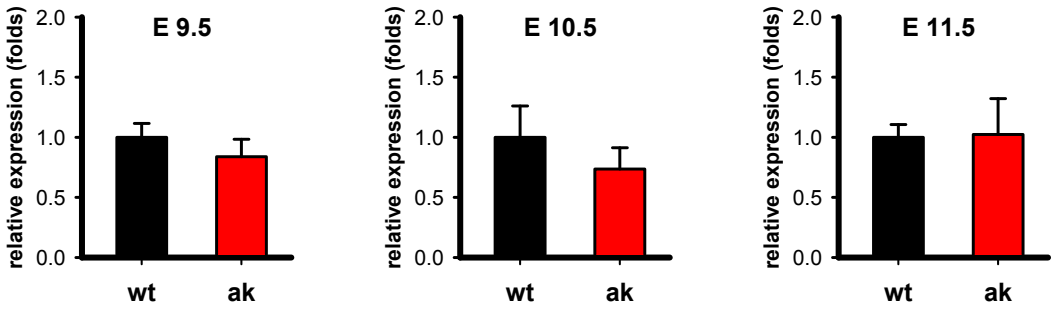


Figure 3.26: Quantification of *Tube1* expression in *aphakia* at different developmental stages. RT-qPCR was done at different developmental stages (shown in bold letters on the top of each graph) using RNA form the head of littermate embryos. Expression is shown as folds of values normalized to *Tuba* and calculated using $2^{-\Delta\Delta CT}$ method [189]. Values from wild-type samples are represented as one. Data represents means ± standard deviations from five samples run in duplicate. Statistical analysis was done using student's t test.

To get an overview of the ε-tubulin (*Tube1*) expression in *aphakia*, I did the whole mount *in situ* hybridization (Figure 3.27). Expression was observed in the forebrain, midbrain (neural tube) and eye areas in the wild-type at E11.5. *Aphakia* embryos have shown reduced expression in the forebrain at E11.5 and E12.5. At latter stage *Tube1* expression is reduced in other areas like, midbrain and eye region.

For the expression analysis of ε-tubulin at protein level, I did immunostaining. However, the only available commercial antibody recommended for immunostaining (Table 6) is not documented for immunohistochemistry. So, first I tested this antibody on cells. To test this, I cloned the *Tube1* cDNA in the pcDNA3.1 expression plasmid and expressed in the HEK293 and MEF cells. Additionally, I tagged this construct with GFP and used as a control for the antibody. This commercial antibody (from Sigma: Table 6) stained the cells very specifically (Fig. 3.28).

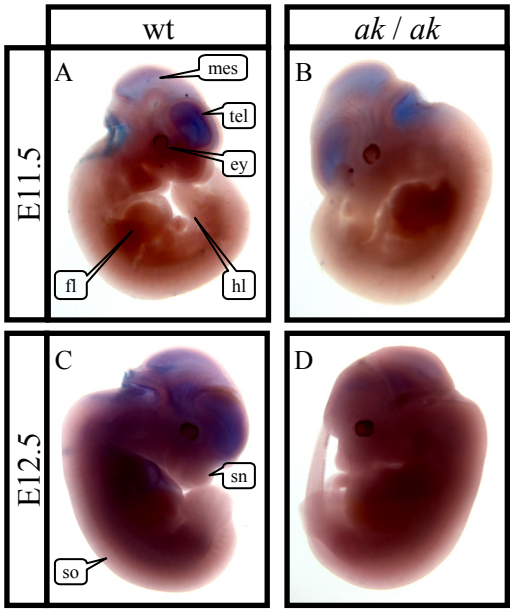


Figure 3.27: Whole mount *in situ* hybridization for *Tube1* in *aphakia*. Mouse embryos of ages E11.5 and E12.5 were fixed in PFA and hybridized with DIG-labelled *Tube1* probe. At E11.5, no difference in its expression was observed in *aphakia* (A) compared to the wild-type (B) in the midbrain but was reduced staining was observed in the forebrain. At E12.5 (C, D), low expression was observed in the forebrain, midbrain and eye regions in *aphakia* embryos compared to the wild-type embryos. Abbreviations; ms, mesencephlon; tel, telencephlon; fl, fore limb; hl, hind limb; ey, eye; sn, snout; so, somite

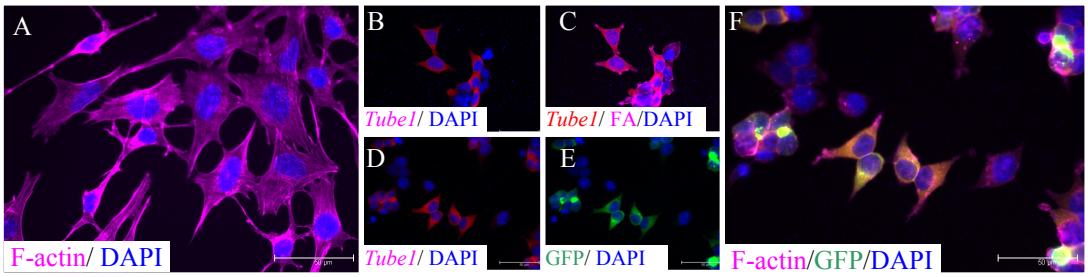


Figure 3.28: ε-tubulin antibody stained the cells very specifically. HEK293 cells were stained with anti-F- actin to visualize cytoskeleton as a control (A). Mouse *Tube1* was cloned in pcDNA3.1 expression vector and expressed in MEF cells (B) and HEK293 cells (D), which is detected using antibody against that. Additionally Tube1 fused with GFP was also expressed in HEK293 cells (E). Co-staining for GFP and Tube1 confirmed that this antibody detected the ε-tubulin very specifically (F) both in human and mouse cell lines. Control cells were stained with F-actin. Scale bar = 50 µm. FA: F-actin.

After successfully testing this antibody in immunocytochemistry, I did immunohistochemistry from E12.5 embryos (Fig. 3.29). Immunofluoresence staining has shown higher expression of ε-tubulin in the anterior portion of the wild-type lens, which has actively dividing cells. Contrary to this, no expression of ε-tubulin was observed in the *aphakia* lens. These results confirmed my finding of very limited mitotic activity in the *aphakia* lens and lack of anterior lens epithelium, and also indicate the role of Pitx3 in the regulation of *Tube1* expression.

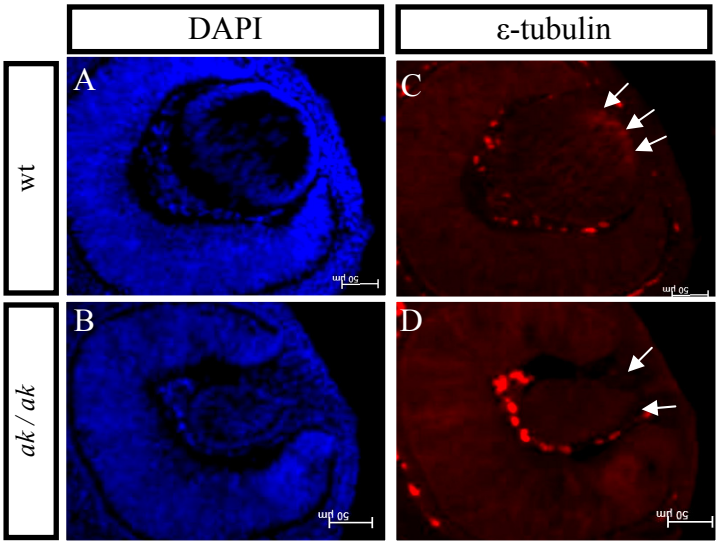


Figure 3.29: Expression of ε-tubulin is reduced in *aphakia*. Immunofluoresence staining at E12.5 showed a concentrated crescentric expression of ε-tubulin in the anterior lens epithelium in the wild-type lens (white arrows) (C), which was not observed in the *aphakia* lens (white arrows) (D). The red fluorescent dots in C and D are disrupted mesenchymal cells that give autofluoresence. Staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

To consider *Tube1* as a potential direct downstream target of Pitx3, I analyzed 2 kb 5'-upstream sequence of this gene for the putative Pitx3 binding sites using MatInspector (Genomatix) but no binding site for this transcription factor was detected on this sequence. However, three Pitx biding sites similar to the POMC [6] that are consensus binding sites for homeoproteins [4] were observed in the 5'-upstream sequence of *Tube1*. DNA fragments containing these binding sites were cloned into pGL3 basic luciferase vector and analyzed in the luciferase reporter assay (Fig. 3.30A). 1st fragment of 694 bp (-534 bp / +160 bp) contained a 'TAAGCC' site at -393 bp, 2nd fragment of 874 bp (-1,288 / -414) having no putative site while the 3rd one was 806 bp (-1,862 bp / -1,078 bp) contains two 'TAAGCT' sites (-1,293 bp and -1,856 bp).

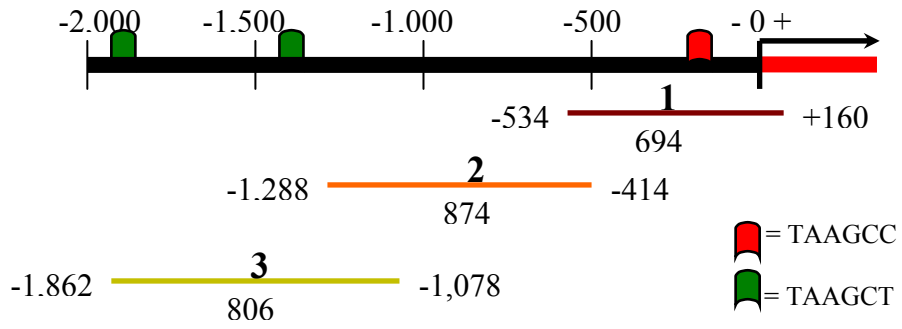
Luciferase assay has shown the maximum luciferase reporter activity with the fragment 3 with increasing amount of *Pitx3* expression plasmid (Fig. 3.30B). This strong stimulation of *Tube1* promoter by Pitx3, suggest that it is binding to the putative POMC binding sites.

Additionally, I also looked for the possibility of molecular interaction of *Tube1* with Ap-2α, as their expression domains are similar in the lens (anterior lens epithelium) and *Ap-2α* also has reduced expression in *aphakia* (Fig. 3.3). Luciferase reporter assay revealed that Ap-2α regulates the same *Tube1* promoter construct that is responsive for Pitx3 (Fig. 3.30C). These results indicate some interaction between Ap-2α and Pitx3 on the *Tube1* promoter.

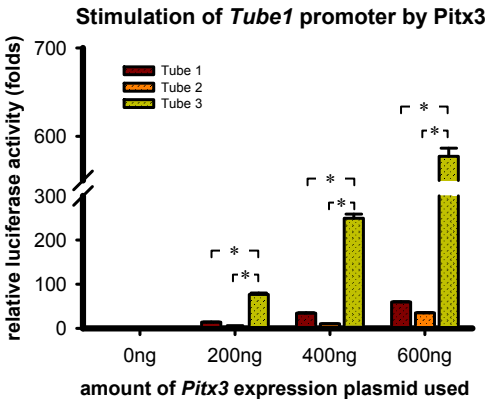
Considering the co-operative role of Pitx3 and Ap-2α in the regulation of *Tube1*, I co-transfected these two expression plasmids along with 1st and 3rd luciferase promoter plasmids for *Tube1* (Fig. 3.30A), which also have shown some activity in the previous experiments. These experiments confirmed the previous findings (Fig. 3.30B & C), as addition of *Pitx3* expression

plasmid enhanced the expression of luciferase that was under the control of 3rd promoter fragment of *Tube1* (Fig. 3.30D). These luciferase reporter experiments provide evidence that Pitx3 regulates the expression of *Tube1* independently as well as in co-operation with Ap-2α at least *in vitro*.

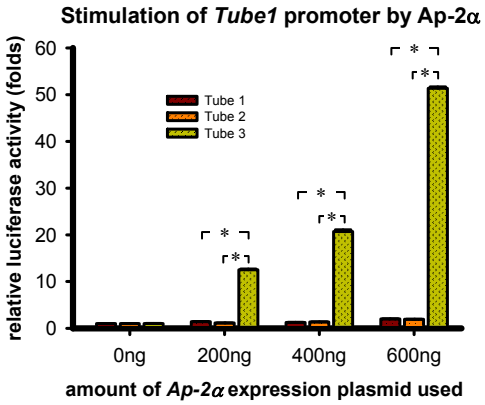
A)



B)



C)



D)

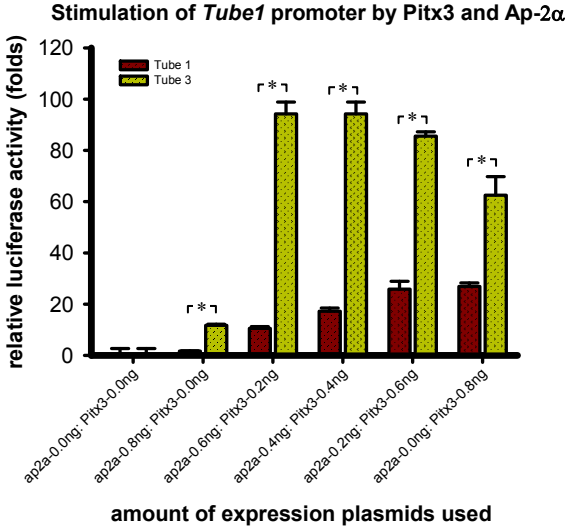


Figure 3.30: Pitx3 regulates the expression of *Tube1*. Different sized 5'-upstream genomic region of *Tube1* (red line indicates the 1st exon) that has POMC putative binding sites (shown in green and red boxes) were cloned in the pGL3 basic luciferase vector (A) and analyzed in the reporter assay for their stimulation by Pitx3 (B), Ap-2α (C) and their combination (D). Fragment 3 has shown the maximum activity, indicating the binding of Pitx3 on the POMC putative binding sites on this fragment shown as green boxes (A). Values are means ± standard deviations from 3 different experiments run in triplicate. Statistical analysis was done using student's t test. *, $P \leq 0.001$.

3.11 Downregulation of Otx2 in aphakia

Retinal pigment epithelium (RPE) is a higly specialized tissue and consists of a single layer of hexagonal cells outside the neuro-retina. It serves as a multifunctional component, critical for the eye development and supporting the photoreceptor survival and function. Its dysfunction results in the death of photoreceptor cells [203], as found in age-related macular dystrophy (AMD) and retinitis pigmentosa (RP). RPE has gained a lot of attention from developmental biologist due to its capability of transdifferentiation into neuro-retina in some species [204]. However, the mechanism is still not clear, although only very limited number of transcription factors are required for the onset of RPE including *Pax6*, *Mitf* and *Otx2*. Expression of Pax6 in *aphakia* mice has already been discussed in the previous sections. Here, I investigated the expression of Otx2 in the RPE of *aphakia* eye.

Otx2 expression was observed in the retinal pigment epithelium (RPE) at E11.5 in the wild-type eye that spread to the cells forming the Iris, while in *aphakia* its expression was detected in the RPE only (Figure 3.31). This change in Otx2 expression is then quantified by realtime-qPCR. RT-qPCR analysis confirmed the reduction of its expression at this developmental stage and at E12.5 as well (Fig. 3.32), although this reduction is not statistically significant (reason could be the difference in the expression domain of Otx2 is small but it is critical).

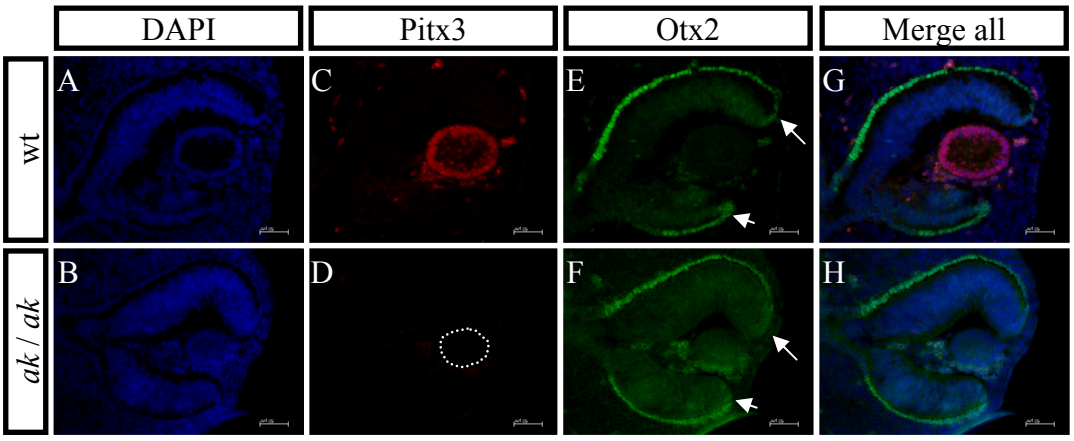


Figure 3.31: Expression of Otx2 is reduced in the anterior RPE at E11.5. Immunofluoresence staining has shown that the expression of Otx2 is almost absent in the anterior RPE in *aphakia* (F) compared to the wild-type eye (E) shown as white arrows. Red dots outside the lens in Pitx3 staining (C, D) are disrupted mesenchymal cells that give autofluoresence. Nuclei were stain with DAPI (A, B) Staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

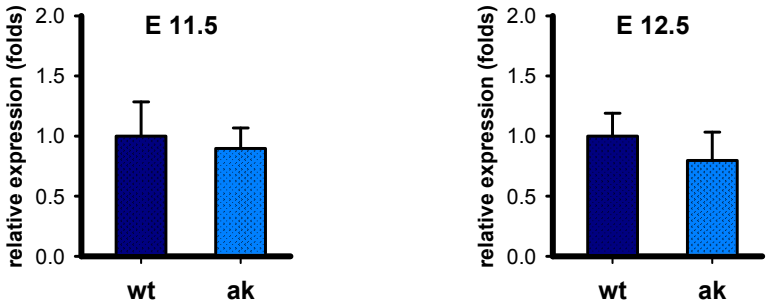


Figure 3.32: Quantification of *Otx2* expression has shown its reduction in *aphakia*. RT-qPCR was done at E11.5 and E12.5 using RNA form the head of littermate embryos. Expression is shown as folds of values normalized to *Tuba* and calculated using $2^{-\Delta\Delta CT}$ method [189]. Values from wild-type samples are represented as one. Data represents means ± standard deviations from five samples run in duplicate.

3.12 *Notch, Wnt and BMP4 signalling in aphakia*

As lens formation is a complex phenomenon, involving the input of may regulatory genes and signalling also coming from the retina, I looked for changes in some important signalling molecules (Fig. 3.36). Expression analysis of *Hes5* and *Hes1*, which are direct targets and indicator of Notch signalling have not shown altered expression at E10.5 and E11.5 (Fig. 3.33A B & C). Wnt signalling, which is critical for proliferating lens epithelium was also observed by investigating the expression of *Wnt1* at E10.5 and E11.5. However, no reduction in the amount of *Wnt1* transcript was observed in *aphakia* as compared to wild-type littermate controls (Fig. 3.33D, E). For the BMP signalling, expression of *Bmp4* was observed at E11.5 (Fig. 3.33F). No change in expression level of this gene was observed compared to wild-type controls.

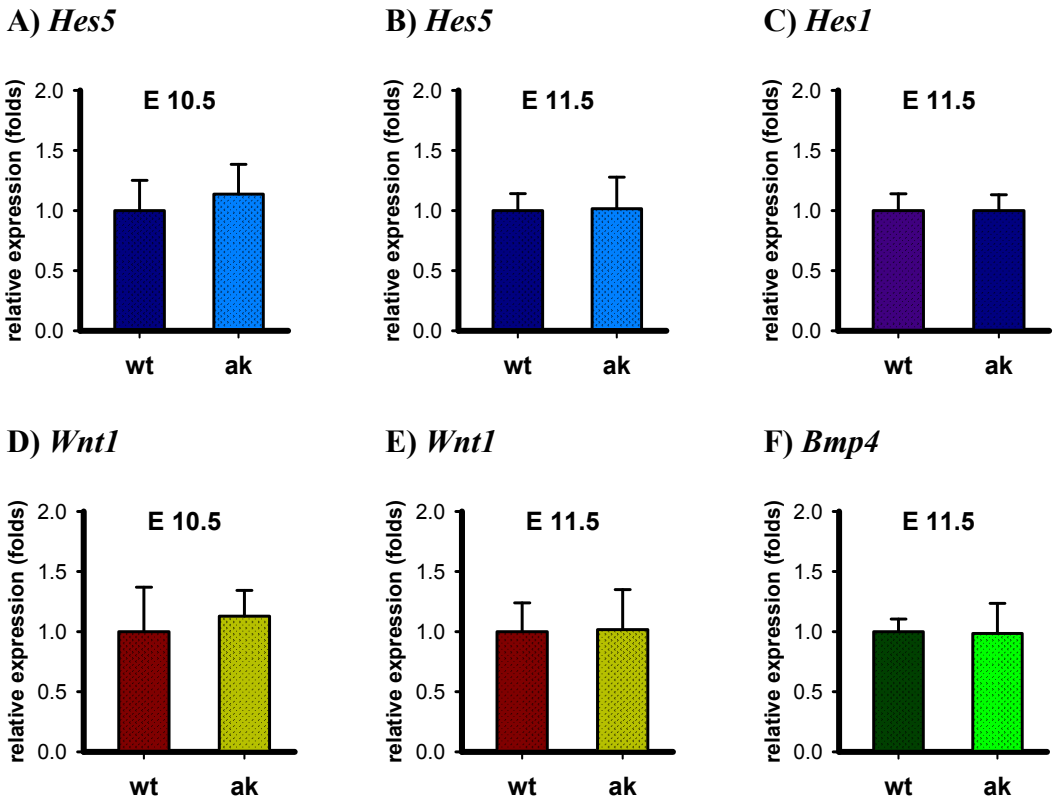


Figure 3.33: Expression of signalling molecules and their targets in *aphakia.* RT-qPCR was done for *Hes5* (A, B), *Hes1*(C), *Wnt1* (D, E) and *Bmp4* (F) at different developmental stages using RNA form the head of littermates. Expression is shown as folds of values normalized to *Tuba* and calculated using $2^{-\Delta\Delta CT}$ method [189]. Values from wild-type samples are represented as one. Data represents means ± standard deviations from five samples run in duplicate.

3.13 Interaction between Pitx3, Pax6, Sox2, AP-2α, Foxe3 and Prox1

To investigate the interaction of Pax6 with Sox2 and Ap-2α I looked for the expression of these factors in the *Pax6* mutant (*Aey18*) (Fig. 3.34A-J). Staining for the Sox2 in the *Aey18*$^{+/-}$ mice showed less intensity at E12.5 especially in the neuro-retina. However in *ak*$^{-/-}$ */ Pax6* (*Aey11*$^{+/-}$) double mutants no such difference was observed except in the ventro-anterior part of neuro-retina where Sox2 expression is diminished and the expression of Pax6 is increased (Fig 3.34Q) indicating that Sox2 may have an inhibitory action on *Pax6*.

Ap-2α has shown no difference in expression in both of the above mutants despite of the fact that its expression completely overlapped with Pax6 in the lens and overlying epidermis, indicating some interaction between these two transcription factors. To look for the interaction between *Pax6* and *Ap-2α in vitro*, expression of varying amount of *Pax6* expression plasmid in HEK293 cells along with *Ap-2α* promoter plasmids in the pGL3 basic luciferase vector revealed the suppressive role of Pax6 on *Ap-2α* promoter (Fig. 3.35B). However, no luciferase activiy was observed when it was put under the control of *Pax6* promoter (Fig. 3.35C) in the presence of Ap-2α (Fig. 3.35D).

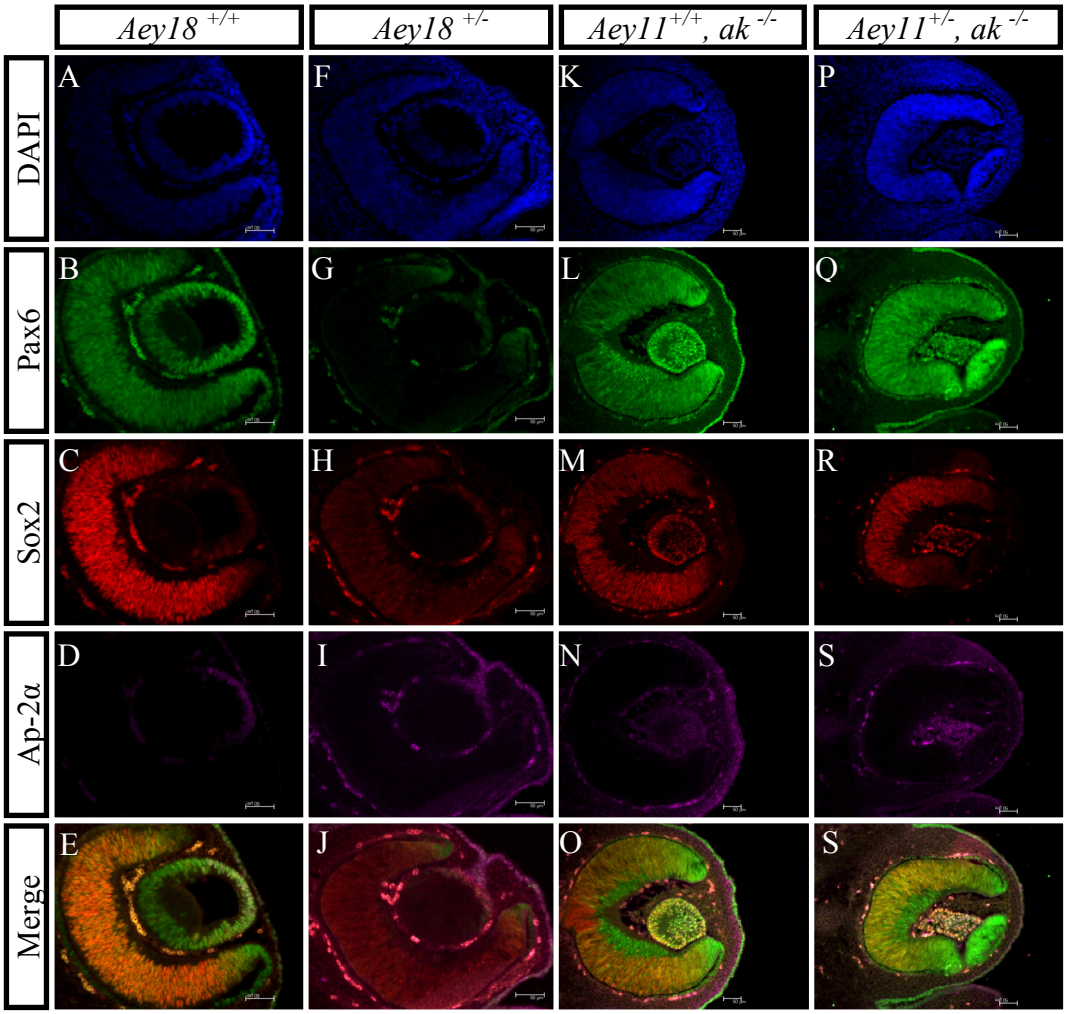


Figure 3.34: Expression of Pax6, Sox2 and Ap-2α in *Pax6*$^{+/-}$ and *Pax6*$^{+/-}$ / *ak*$^{-/-}$ at E12.5. Expression of Sox2 is decreased in the neuro-retina in *Pax6*$^{+/-}$ (H) compared to the *Pax6*$^{+/+}$ (C) but difference was observed in *Pax6 / ak* double mutant (M, R). Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

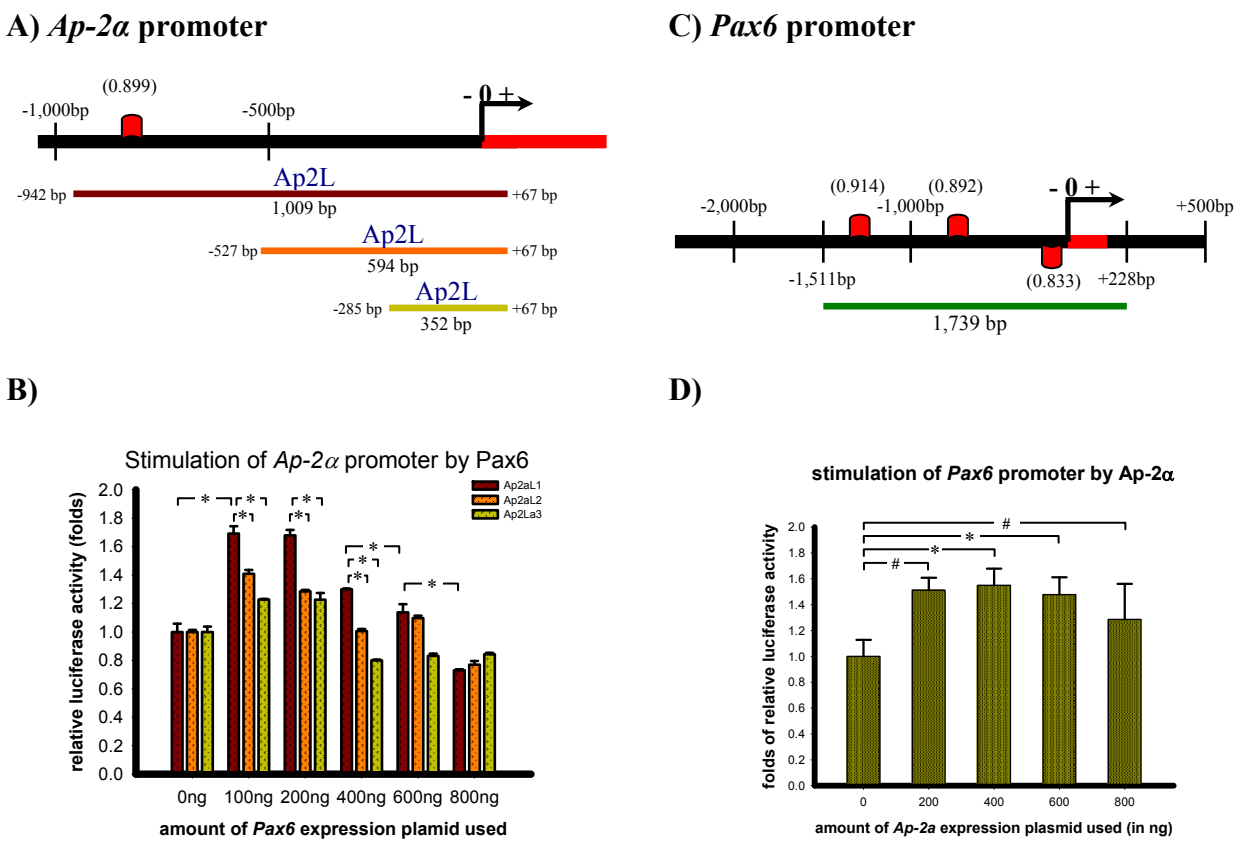


Figure 3.35: Figure 2.40: Pax6 down-regulate the expression of *Ap-2α* in HEK293 cells. *Ap-2α* promoter constructs (A) has shown negative regulation in luciferase reporter assay when co-expressed with *Pax6* expression plasmid (B). However, *Pax6* promoter, which has Ap-2α putative binding sites as detected by MatInspector (section 2.1) and shown as red boxes with their matrix similarity values in parentheses (C) did not responded to the Ap-2α when co-transfected with its expression plasmid (D). Data is shown as ratio of firefly luciferase activity to the renilla luciferase activity. Values are means ± standard deviations from 3 different experiments run in triplicate. Statistical analysis was done using student's t test. *, $P \leq 0.001$; #, $P \leq 0.05$.

In relation to the phenotypic features (persistent lens stalk), I also looked of the expression of Foxe3 in *Pax6* heterozygous (*Aey18*$^{+/-}$) and *Pax6* / *ak* double mutants (*Aey11*$^{+/-}$ / *ak*$^{-/-}$). Expression of Foxe3 was detected in *Pax6* heterozygous (*Aey18*$^{+/-}$) mutants (Fig. 3.36C), regardless of the size of the lens (small lenses in *Pax6* mutants) but no expression of this gene was observed in *Pax6* / *ak* double mutants (Fig. 3.36E) showing the dominance of *aphakia* phenotype that lack the expression of Foxe3 at this stage of development. These results provide additional evidence that *Pitx3* has a role in the regulation of *Foxe3*, and Pax6 either has no direct role or *Pax6* heterozygosity is not sufficient enough to pose an effect on the expression of *Foxe3* (compared to the *ak* / *ak* mice).

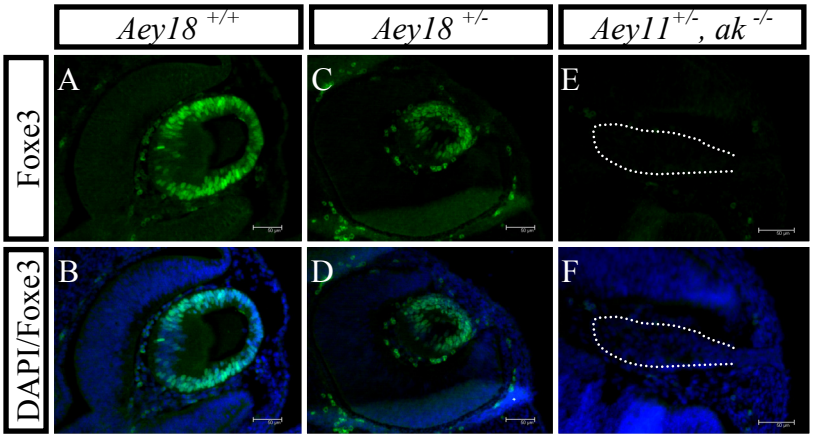


Figure 3.36: Expression of Foxe3 in *Pax6* and *Pax6 / ak* double mutant at E12.5. In *Aey18*$^{+/-}$ mutant expression of Foxe3 is unaffected (C, D) compared to the *Aey18*$^{+/+}$ (A, B), while no expression was detected in *Aey11*$^{+/-}$, *ak*$^{-/-}$ double mutant (E, F). Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

As the expression of Foxe3 is not significantly altered in *Pax6* heterozygous mutant (*Aey18*$^{+/-}$), I explored the expression of Pax6 in *Foxe3* null mice to look for the interaction between these two factors that show a similar anterior lens phenotype. In *Foxe3* homozygous mutants, expression of Pax6 at E11.5 and E12.5 (Fig. 3.37C & D) is not affected compared to the wild-type (Fig. 3.34B), meaning that either Pax6 is upstream of *Foxe3* or they are part of independent regulatory cascades. Similar conditions may apply for *Sox2* and *Ap-2α*, as the expression of these two genes is not changed considerably in *Foxe3* null mutants (Fig. 3.37E-H).

In order to examine, if the loss of Foxe3 has an impact on the expression of Pitx3 and to develop their regulatory cascade, I investigated the expression of Pitx3 in *Foxe3* null mutant. These mutant mice have shown almost normal expression of Pitx3 (Fig. 3.38A) compared to the wild-type (Fig: 3.5J); further confirming the finding that Foxe3 is not upstream of Pitx3. Furthermore, to confirm the finding that Pitx3 directly regulates the expression of Foxe3 and not through Prox1, which is absent in the Pitx3-deficient (*aphakia*) mice, I also looked for the expression of *Prox1* in *Foxe3* mutant mice. I have found that the *Prox1* expression is maintained in this mutant at least at E11.5 (Fig 3.38B). However, its expression has also been detected in the anterior lens cells that do not express Prox1 (Fig. 3.21O) in the wild-type lens, in line with the previously published *in situ* hybridization data [85]. These results indicate that *Foxe3* is not only directly regulated by Pitx3 but also has an inhibitory action on *Prox1* expression.

As the expression of Prox1 is also considered to be controlled by Pax6 [34], its expression in Pax6 mutant (*Aey18*$^{+/-}$) has also been investigated. Immunofluoresence staining for Prox1 has shown no change in its expression in *Pax6* mutant (Fig. 3.39A) compared to the wild-type (Fig. 3.21Q). Pax6 / ak double mutants lack the expression of Prox1 (3.39C & E) similar to ak / ak mice (Fig 3.21). Thus *Pax6* (at least one defective allele) has no affect on its expression (Fig.

3.39C & E) either negative (as in *Aey18*$^{+/-}$ mutant) or positive (as *aphakia* phenotype prevails in *Aey11*$^{+/+}$ / *ak*$^{-/-}$ and *Aey11*$^{+/-}$ / *ak*$^{-/-}$ double mutants,).

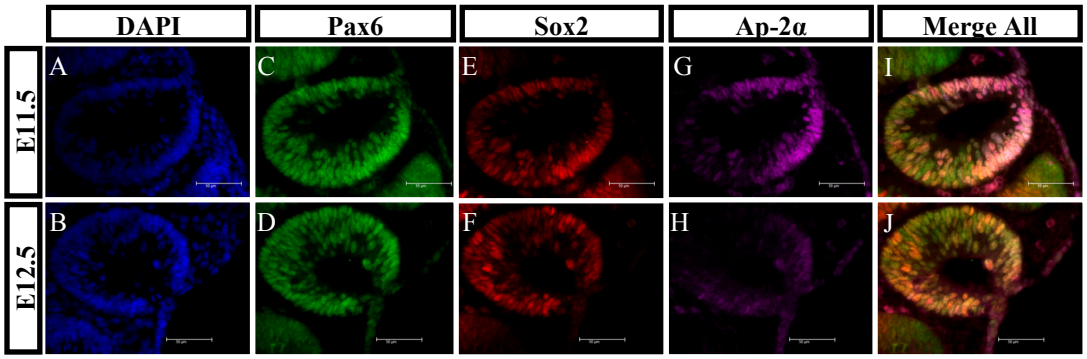


Figure 3.37: Expression of Pax6, Sox2 and Ap-2α is maintained in *Foxe3* null mutant. No change in expression of these transcription factors were observed in *Foxe3* mutant lenses compared to the wild-type lenses (see Fig 3.5, 3.9 and 3.12 respectively, for wild-type expression) Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

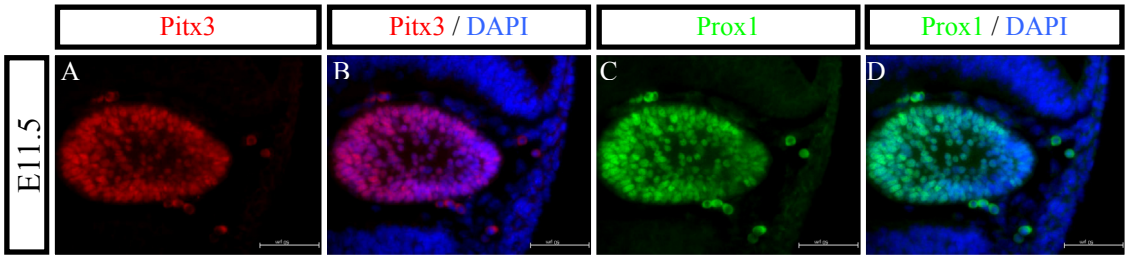


Figure 3.38: Analysis of Pitx3 and Prox1 expression in *Foxe3* mutant. Immunofluoresence staining for Pitx3 (B) and Prox1(C) was performed on *Foxe3* mutant embryos at E11.5. Staining for both of these genes revealed that their expression is maintained in this mutant at least at E11.5. Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars=50µm.

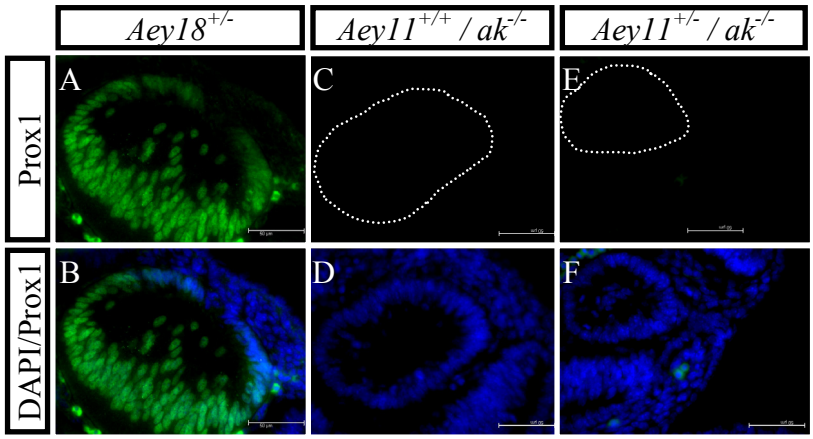


Figure 3.39: Expression of Prox1 in *Pax6*$^{+/-}$ (at E12.5) and *Pax6* / *ak* double mutant (at E11.5). Expression of Prox1 in *Pax6*$^{+/-}$ (A, B) was detected as un-altered compared to the wild-type lens (Fig. 3.21) at this stage, while no expression was observed in *Pax6* / *ak* double mutant at 11.5 (C-F). Immunofluoresence staining was performed on 8 µm thick, PFA fixed paraffin sections. Scale bars = 50 µm.

4 Discussion

Pitx3 is a transcription factor known to play an important role in the development of eye and mesencehalic dopaminergic (meDA) neurons [205,206]. However, most of the studies focused on its role in context of dopaminergic neurons and found to directly regulate the expression of genes involved in the development and maintenance of these neurons including, *Th* [178,205], Vmat2, Dat [179], *Bdnf* and *Gdnf* [207]. But not so many targets and regulatory networks of Pitx3 in the development of the ocular lens are known and most of the targets that are mentioned above for this gene are not even expressed in the lens. More interestingly, *Nurr1*, which is important for the function of Pitx3 and considered its co-factor in brain, is also not expressed in the lens.

In the lens, Pitx3 seems to have very diverse roles; causing varied abnormalities in different organisms although genomic and protein sequence homology is conserved among species, especially in human and mice. In humans, different mutations in *PITX3* causes ASMD, cataract and microphthalmia but only micropthalmia has been observed in *Pitx3* mouse mutants (*aphakia* and *eyeless*). But interestingly, almost all of the mutations found so far in humans are present in the 4th exon of this gene (around a 17 bp hot spot region) [1,16,17,208], similar to an insertion found in the *eyeless* mutant. These mutations affect the OAR domain, present on the C-terminus of Pitx3 (Fig. 1.1). It is considered to play a role in transactivation and thus different mutations in this region result in diverse affects by influcing its interation with other proteins. However, all these affects are generated through the alteration in expression of different genes regulated by Pitx3 and need to be explored.

In the present study, I have focused on the role of Pitx3 in the development of lens and identified its targets using *in vivo* (*aphakia* mice) and *in vitro* approaches. I have shown that Pitx3 can directly regulate the expression of some important genes influencing various processes in the ocular lens including *Foxe3*, *Prox1* and *Ap-2α*. Additionally, one novel target; *Tube1* that was indentified in the microarray studies previously in our lab (Muenster, 2005) has also been verified in this study. Furthermore, I have also assessed Pitx3-deficient *aphakia* mice for the presence of olfactory impairment; an important non-motor symptom of Parkinson's disease [160,164].

Aphakia mice at the age of 8 weeks do not show any change in the sense of smell; despite of the fact that they exhibit loss of DA neurons in the VTA region [175] that project to the olfactory tubercle although the depletion is not as severe as in the SNc [172,175]. The loss of meDA in *aphakia* is developmental contrary to the Parkinson's disease in human that show progressive loss of these neurons with growing age [154,209]; there may exist some compensatory

mechanisms to overcome these deficits at least at the investigated age as in the nigrostriatal pathway [175,210-212]. These mice also do not show significantly altered motor functions and the increase in striatal 5-HT is responsible for the hyperactivity of these mutants [213]. But if and how the mesolimbic compensation takes place in *aphaki*a is not known. However, these mice may develop some symptoms with growing age as a result of age dependent neuronal deficit (a phenomenon of aging [209]) therefore I handed these mice over to Dr. Hoelter (behaviour team) to test these mice at an older age (≥ 1 year) and more extensively including smell sensitivity. Interestingly, they have observed reduced smell sensitivity and motor deficits (in catwalk test) in these mice (Dr. Hoelter, unpublished data) motivating further studies in this orientation to explore the Parkinson's disease features in this mutant; hence, the pathogenesis of this disease. I then, further focused on the morphological and molecular changes in lens.

4.1 Pitx3 changes the morphological and molecular signature of lens

Lens placode formation is the first step in the development of the lens. In *aphakia* mice although lens placode is formed, expression of some of the critical genes including the master controlling gene, *Pax6* is disturbed. During lens development, expression of Pax6 takes place in two phases [214]. In the preplacodal phase, Pax6 is expressed in the head surface ectoderm and 2nd phase is the lens placodal phase, where it is expressed in the lens. In *aphakia*, Pax6 expression is increased at the placodal stage of lens development but the expression of Prox1 and interestingly Foxe3 is decreased, which is considered as downstream of Pax6 [84,214]. This notion is supported by the loss of Foxe3 expression in the *Pax6* homozygous mutant that lacks the ectodermal enhancer element (EE element): present on the proximal 5'-upstream region of *Pax6* and important for lens induction [214]. This discrepancy indicate that Foxe3 does not lie directly downstream of Pax6 but rather involves some other factors, possibly Pitx3, as the expression of Pitx3 is also reduced in *Pax6* heterozygous mutants [215].

Expression of Prox1 starts around this time in the wild-type lens but the analysis of *aphakia* mice revealed that it is not even initiated in this mutant; leading to the fact that the presence of Pitx3 is important for its initiation. Surprisingly, Ho et al [97] detected high expression of Prox1 in Pitx3 null lens at E10.5 by immunostaining. This could be due to the use of a different antibody but they have not mentioned the source of the antibody used. Other possibilities could be the use of mice with different background or the presence of another isoform of Prox1 in the lens. But so far no splice variant has been reported for this gene. However, *Prox1* transcript has also not been detected in the *aphakia* lens by *in situ* hybridization in another study [95] in line with the present study. I have provided comprehensive evidence that Prox1 in not expressed in the lens of *Pitx3*-deficient mice.

In the lens, *Pitx3* is detectable around E9.5 in wild-type lens [94,95] but the molecular changes already present in *aphakia* raise two important points. Firstly, either its expression starts even earlier (exists below detectable level) and secondly, even very low concentration of Pitx3 is enough to generate its effect on some genes. However, despite of these molecular changes lens placode proceeds towards the formation of lens vesicle in this mutant.

Although lens vesicle is formed in *aphakia* but it fails to separate from overlying surface ectoderm and show persistent lens stalk. A similar lens phenotype has also been observed in *Foxe3* [84,85], *Pax6* heterozygous [186,216] and conditional *Ap-2α* mouse mutants [72,194] but the molecular mechanism behind this phenotypic feature is not fully revealed yet. As *Pax6* mutants show reduced expression of Foxe3 and Pitx3 [185,215], it is expected that either Foxe3 or Pitx3 is responsible for peristent lens stalk phenotype. Further, *Pitx3* expression is maintained in *Foxe3* mutants; however, *Pitx3* mutant lack the expression of Foxe3 (Fig. 3.19 and [95]). Similar results have also been reported in the Zebrafish using morpholino approach [217], where expression of Pitx3 is detected in the lens of *Foxe3* knockdown Zebrafish but no expression of *Foxe3* was observed in *Pitx3* knockdown Zebrafish. Therefore, it can be concluded that *Foxe3* is the most downstream transcription factor among the *Pax6*, *Pitx3* and *Foxe3*, causing the persistent lens stalk phenotype. However, in *aphakia* not only Foxe3 is reduced but the expression of *Ap-2α* is also decreased at exactly the point when lens vesicle separates from the surface ectoderm. As *Ap-2α* is expressed in *Foxe3* null mutant (Fig. 3.41), it is expected that either Foxe3 or these two factors contribute to the lens stalk phenotype in *aphakia*.

Another feature of the lens vesicle in *aphakia* is that its lumen is filled with cells contrary to the wild-type conditions. This seems to be the result of loss of cell-cell contact caused by the reduced expression of E- and N-cadherin. However, reduction in the expression of *Tube1* also contribute to this phenotypic feature, which has an important role in determining the orientation of spindle fibers, symmetry of cell division and organization of dividing cells [181,182].

One of the interesting finding at this stage is the expression of γ-crystallin. In the wild-type lens, it is expressed in the terminally differentiating fiber cells and has so far been reported as early as E12.5 [218]; however, in the present study, I have detected its expression at E11.5 (Fig. 3.28), indicating that γ-crystallin expression starts at early stages of lens differentiation (probably, immediately after cell cycle withdrawal). Even more interestingly, in *aphakia* lens expression of γ-crystallin is observed one day earlier than the wild-type lenses (at E10.5). These result are supported by the previos findings [97], where earlier expression of γ-crystallin (at E11.5) has been observed in Pitx3-GFP mice but the authors did not investigation its expression even earlier. These evidences suggest that Pitx3 has an inhibitory action on the expression of γ-

crystallin either directly or indirectly and in the absence of Pitx3 lens cell exit the cell cycle earlier and try to enter in differentiation phase.

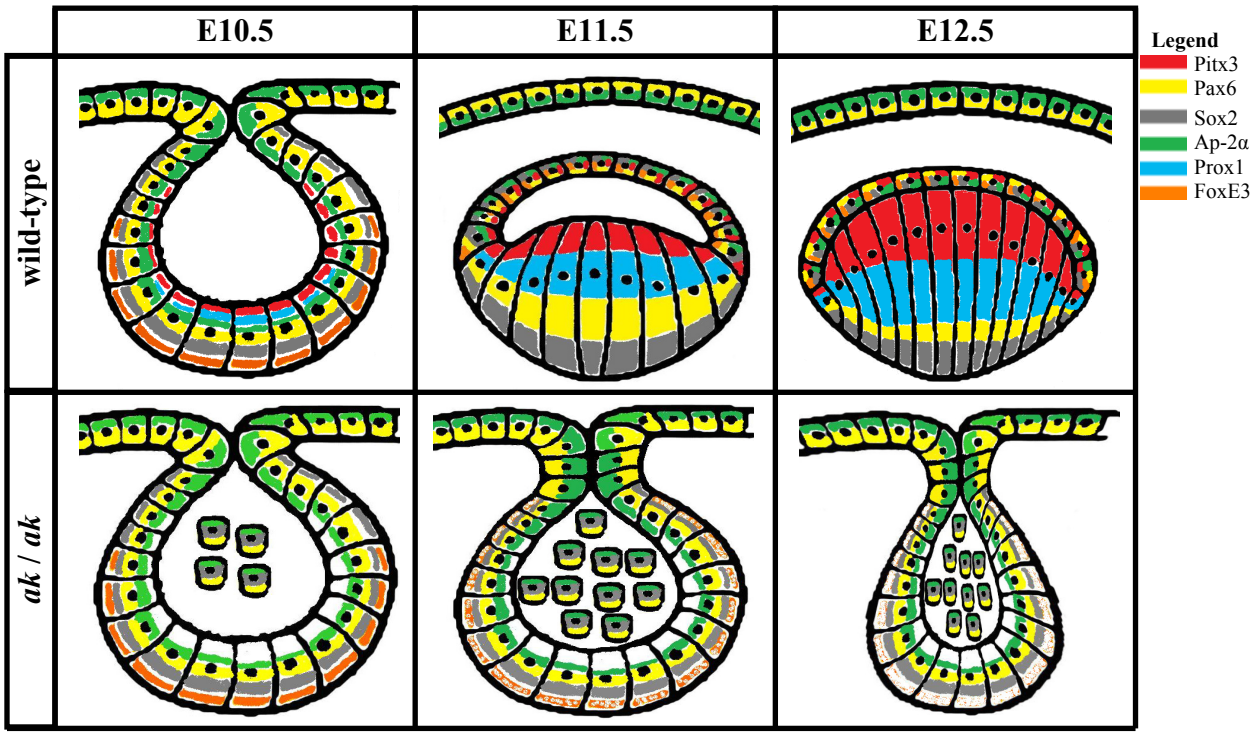
Following the lens vesicle formation, development of the lens is arrested in *aphakia*. Lens cells that are present in the posterior half of the vesicle have shown impaired differentiation. During the normal lens development cells in the posterior half elongate and differentiate into primary lens fibers. However, in *aphakia*, although these cells try to undergo differentiation as indicated by the expression of γ-crystallin but fail to elongate and form the lens fibers. They continue expressing E-cadherin, which is an epithelial marker and suppressed in the differentiating cells under normal circumstances. Such persistent misexpression of E-cadherin has also been observed in *Prox1* knockout mice [92] and also in the lens that show ectopic expression of Ap-2α. These lines of evidences provide further proof of impaired Ap-2α and Prox1 expression in *aphakia*. Loss of Prox1 expression in *aphakia* results in aberrant expression of cell cycle inhibitors [95] causing the failure of posterior lens vesicle cells to exit from the cell cycle and enter the differentiation phase. However, earlier and consistent expression of γ-crystallin in these cells pull them into the differentiation phase but they fails to elongate and to differentiate terminally into primary fiber cells due to the loss of Prox1 expression. Possibly, they undergo apoptosis as in *Prox1* knockout mice [92] forming the rudimentary lens vesicle, which latter disappeared resulting in microphthalmia in *aphakia* mice.

Another important feature of the lens development is to maintain the proliferating anterior lens epithelium which is a source of secondary lens fibers cells. But in *aphakia* the proliferation in lens is severely impaired as shown by the BrdU incorporation assay. This reduced mitotic activity is due to diminished expression of Foxe3, which is an important factor in maintaining the pluripotency of the lens epithelial cells [84,85]. However, other mechanisms could possibly exist to block the expression of γ-crystallin and maintain the pluripotency of lens epithelial cell. One of them could be Sox2, which has the proliferative role and considered as marker of stem cell [219]. Altered expression of Sox2 in *aphakia* (down-regulated at least there is a trend and pattern is clearly changed) result in the ectopic expression of γ-crystallin in the lens, as studies have shown that Sox2 is not expressed in the lens fiber cells [49] therefore, could be important for maintaining the characteristic lens epithelium by inhibited the γ-crystallin in the anterior lens epithelium.

As the lens development is arrested in *aphakia* mice but the rudimentary lens exists till late embryonic stages. Altough all the *aphakia* lens cells express γ-crystallin indicating that they are differentiated cells but they also persistently express genes (summerized in Fig. 4.1) that are considered as epithelial markers such as, Ap-2α and E-cadherin [75,198,200,220], making the identity of these cells controversial. Additionally, expression of N-cadherin which is present in

the lens vesicle and absent in the lens epithelial cells [73] has misexpression in *aphakia*. However, expression pattern of other genes related to the lens development provide important hints in determining the identity of these cells present in the rudimentary lens. Consistent expression of Pax6 and Sox2 in the *aphakia* lens (see Fig. 4.1), which is restricted to the lens epithelial cells during the normal lens development, indicates that these cells have impaired programming. Lack of Pitx3 expression results in the loss of not only proliferative ability of the cells but also differentiation. The cells in the *aphakia* lens stuck in the phase between differentiation and proliferation; failing to express the early differentiation markers like, Prox1 [92,221-223]; blocking the expression of epithelial markers like, Ap-2α and E-cadherin [75,220,224,225] in the posterior lens and differentiation markers like γ-crystallin in the lens epithelium. Persistent expression of these factors in the *aphakia* rudimentary lens indicates that they are epithelial cells but lack the ability to proliferate thus indicating dual role of Pitx3 in lens development by regulating various downstream targets.

A)



B)

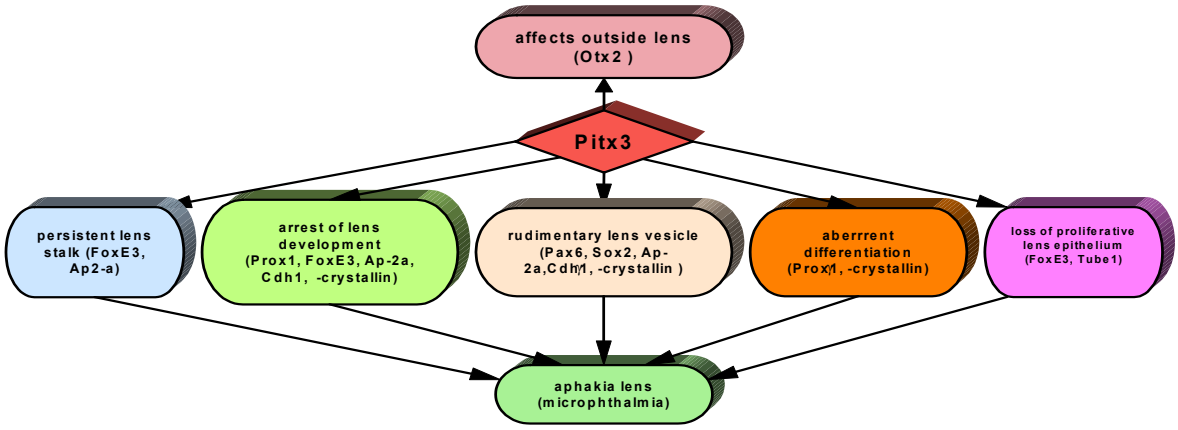


Figure 4.1: Summary of the morphological and molecular events taking place in *aphakia* lens. Expression of different transcription factors are impaired in the *aphakia* lenses (A). Different colors represent the spatial expression of the transcription factors (see legend); however, they should not be confused with sub-cellular localization. Note the absence of Prox1 and reduced expression of Foxe3 in *aphakia* as a result of lack of Pitx3. Missexpression of Pax6, Sox2 and Ap-2a is also very prominent. Defects in these molecular factors are responsible for the morphological features of these mutants (B). Molecular factors that are involved in different processes and are affected in *aphakia* are mentioned in parentheses.

4.2 Molecular targets of Pitx3

There are growing number of evidences that Pitx3 directly regulates the expression of genes involved in the maintenance of dopaminergic neurons as discussed earlier. In the present study I focused on the role of Pitx3 in the ocular lens development and its interaction with the regulatory factors influencing lens development.

a) Pax6

Pax6 being the master controlling gene is a crucial factor for ocular development. I have shown that *aphakia* mice have an increased Pax6 level at an earlier stage of lens development, where its expression is important for the invagination of lens vesicle. Development of lens is highly sensitive to the level of Pax6 dosage. Over-expression of Pax6 causes reduced proliferation as a result of arrest in cell cycle [226] and may contribute to the reduced mitotic activity observed in the *apahkia* mice. However, mice heterozygous for Pax6 also show the morphological features similar to *aphakia*, with persistent lens stalk. But this feature seems to have different molecular aspects discussed in the previous sections, as the *aphakia* mice do not show reduced level of Pax6 comparable to the *Pax6* heterozygous ($Aey18^{+/-}$) (Fig. 3.37). These *Pax6* heterozygous mutants posses the detectable level of Pitx3 expression but is reduced in *Pax6* heterozygous knockout mice [215], pointing towards complex regulatory interactions between these two factors: positive regulation of Pax6 by Pitx3 and inhibitory action of Pitx3 on the expression of Pax6 [180].

To understand this regulatory mechanism, I analyzed the double mutant embryos generated by Doris Muenster (Muenster, 2005) by crossing $ak^{+/-}$ with $Pax6^{+/-}$ ($Aey11^{+/-}$). Double homozygous mice did not developed eyes. However, $Aey11^{+/-}$ / $ak^{-/-}$ embryos show predominantly *aphakia* phenotype. The most striking feature of this mutant is the presence of pinched neuro-retina at the ventral side of the eye that show higher expression of Pax6.

Pax6 has a very complex spatiotemporal expression confined to the developing eye, spinal cord, developing cortex of the central nervous system and endocrine pancrease. In addition, usage of three different promoters, P0, P1 and Pα further add to the complexity (Fig. 4.2). However, *Pax6* loci are phylogenetically conserved between human, mouse, zebrafish and fugu. Moreover, different tissue specific enhancers present on the 5'- and 3'-end of the promoter have been identified. An ectodermal enhacer element 'EE' present at the 5'-end of the most proximal promoter (P0) is responsible for its expression in the lens, surface ectoderm and cornea. These cis-regulatory elements are conserved between the species and contain the binding sites for Six3, Pax2, Sox, Oct and Pax6 as well. Additionally, lens specific expression of Six3 can rescue the

haploinsufficiency of Pax6 [227] but the expression of Pax2, a transcription factor involved in eye development [228], is normal in *aphakia* although Six3 is changed in accordance with Pax6 [168].

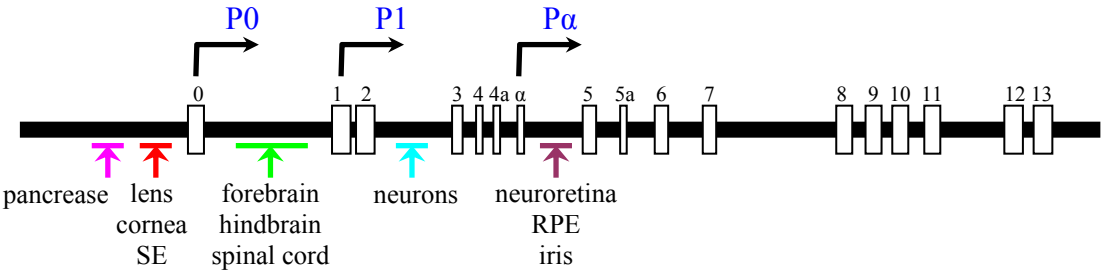


Figure 4.2: Structure and enhancers of *Pax6* gene in mouse. Different promoters for Pax6 are mentined on the top, while the enhancers with their expression domains are mentioned below the sequence line [38,39,229,230]. Abbreviations; SE, surface ectoderm; RPE, retinal pigment epithelium.

The response of *Pax6* proximal (P0) promoter to Pitx3 in the reporter assay in this study is contrary to the previous results from the *in vitro* assay (Muenster, 2005). This contradiction could be because of the different reporter vector backbone and experimental conditions. However, contrary result in response to the *Pitx3* promoter by Pax6 points towards the replication of these finding in different cell lines without the endogenous expression of Pax6. Furthermore, the distinct phenotypic features and an increased expression of Pax6 in the *Aey11*$^{+/-}$ / *ak* $^{-/-}$ embryos indicates the involvement of some signalling cascade at least in the eye, as Pitx3 is not expressed in retina and the *Pax6* retinal enhancers are different than those in the lens; present on the 5'side of the Pα promoter. Although the co-operative role of enhancers and presence of novel enhancers can not be ignored but the involvement of sonic hedgehog (Shh) signalling in the regulation of *Pax6* in this context is important. Shh and Bmp4 signalling act antagonistically to establish the dorso-ventral axis of the retina repressing each other [231]. Expression of Bpm4 is not changed in *aphakia* (at least at E11.5) but not measured in *Aey11*$^{+/-}$ / *ak*$^{-/-}$ mutants. However, the specific affect in the ventral optic cup in this double mutant seems to be affected by Shh due to its involvement in the formation of this ocular region [232].

b) Ap-2α

Ap-2α is a critical transcription factor regulating the expression of various genes involved in proliferation and differentiation [233,234]. Its expression starts around E8.5 during embryogenesis and is regulated spatially and temporally [64]. Various cis-regulatory elements have been observed in different studies using *in vivo* and *in vitro* approaches to demonstrate their importance for the regulation of *Ap-2α* expression. A 140 bp enhancer element, present between -1279 and -1139 bp of human *AP-2α* gene has been identified using trophoblast cells [235]. In

addition to this, Creaser et al. [236] has identified a minimal promoter ~100 bp upstream of the transcription start site containing various binding sites but does not contain a TATA box.

A conserved octamer element is considered as critical for the basal expression of *Ap-2α*. However, this minimal promoter is not sufficient for the tissue specific expression. Zhang and Williams [237] analyzed 45kb sequence including 20kb 5'-upstream region of human DNA to identify the tissue specific regulatory sequence of *Ap-2α*. They have observed various regulatory elements throughout the gene for tissue specific expression including the one in the 5th intron which is required of the limb and facial expression; however, they failed to identify the regulatory region for the neural crest and eye. These lines of evidences indicate tissue specific regulatory network for the expression of *Ap-2α*. In the present study I have identified a regulatory element of ~200 bp in the mouse *Ap-2a* gene between -500 and -698 bp that is responsive to the transcription factor Pitx3.

This finding is supported by the evidence of reduced expression of *Ap-2α* as measured by RT-qPCR and the morphological changes observed in the Pitx3-deficient *aphakia* mice (that show persistent lens stalk). However, the down-regulation of *Ap-2α* only at E10.5 indicates that Pitx3 influences the expression *Ap-2α* in the lens at this stage, where lens vesicle separates from the surface ectoderm. These evidences point toward the role of other trans-acting elements in the regulation of *Ap-2α* at different stages of lens development but the role of Pitx3 could not be compensated by other factors at lens vesicle separation stage in *aphakia* mice.

c) E-Cadherin

E-cadherin is one of the important and well-studied cadherins involved in cell-cell interaction. Its expression starts during embryonic development at 2-cell stage and continued in the epithelial cells at latter stages. Various cis-regulatory elements, E-box, CCAAT-box and CpG islands upstream of *Cdh1* (E-cadherin) are responsible for its expression and have been shown to possess tissue specific preferences. In the lens, Ap-2α has a highly overlapping expression pattern with E-cadherin and regulates the expression *Cdh1* by binding to the E-box1 region. In the present study, I have pointed towards two regulatory elements in the upstream region of this gene that are responsive to Pitx3, with almost 65% of the reporter luciferase activity coming from proximal promoter. As this proximal promoter has also the binding site for the Ap-2α, it can be speculated that in the lens both Pitx3 and Ap-2α may have a co-operative role in maintaining the expression of E-cadherin. This hypothesis is supported by the evidence of remnant expression (almost 42%) of *Cdh1* in the lens conditional Ap-2α mutant that have persistent Pitx3 activity [72] and the reduced expression of E-cadherin in *aphakia*. But we can not ignore the possibility that this difference in expression of *Cdh1* is accounted by the reduced

expression of Ap-2α a day earlier. However, further experiments are necessary to find out the *in vivo* role of Pitx3 in the regulation of E-cadherin.

The reduced amount of E-cadherin results in the loss of cell-cell contact in *aphakia* lenses and allows the cells to move freely, resulting in the lumen of the lens vesicle filled with the cells. But this loss in cell-cell contact is important for the terminal differentiation of fiber cells [78] in the posterior part of the lens, where the expression of E-cadherin is diminished during differentiation in the wild-type lens contrary to the *aphakia*. In the *aphakia* lens, ectopic expression of *Cdh1* point towards one of the reason of failure in the terminal differentiation of lens cells.

d) Foxe3

Foxe3 controls the proliferation during lens development [84,85]. In *aphakia* lens, reduced proliferation has been observed around E9.75, a time-point where the expression of Foxe3 starts [95]. This finding is in line with the reduced expression of Foxe3 at this stage observed in *aphakia* in the current study. Furthermore, its expression is lost in the lenses of *aphakia* embryos following day E12.5, clearly indicating that Pitx3 is necessary for the maintenance of its expression, in line with previous findings [95,97]. In the present study, I have shown that an evolutionary conserved 5'-upstream region (-3,517 bp / -3,234 bp) of the *Foxe3* gene contains a putative Pitx3 binding site. This region is highly responsive to Pitx3 in the luciferase reporter assay and provides an evidence that it can directly regulate the expression of *Foxe3*. This spatio-temporal regulation of *Foxe3* expression by Pitx3 is responsible for the normal development of lens and proliferation deficits in *aphakia*. However, these two genes do not interact synergistically as observed in the compound heterozygous mice. But the expression of Foxe3 earlier than Pitx3 during lens development, as observed by Medina et al [95] points towards additional trans-regulatory elements that are necessary for the expression of *Foxe3*. Those include Pax6, which has highly conserved overlapping expression pattern; mice lacking Pax6 show highly diminished expression of *Foxe3* [214]. Contrary to these, *Foxe3* mutants show conserved Pax6 expression indicating that Foxe3 is downstream of Pax6. However, some studies indicate that Pax6 indirectly regulate the expression of *Foxe3*, involving other factors downstream of Pax6, such as *Mab21l1* [238,239]. Targeted disruption of *Mab21l1* in mice results in severely impaired expression of *Foxe3*. However, it is still not clear if Mab21l1 has a direct impact on the regulation of *Foxe3* or involve some other factors, possibly Pitx3: as the *Pax6* heterozygous mice also show reduced expression of Pitx3 [215,240]. Investigation of Pitx3 expression in *Mab21l1* mutant mice will help to develop this regulatory cascade.

Additionally, *Sip1* has known to regulate the expression of *Foxe3* involving Smad8 [218]; an important mediator of Bmp4 signalling. A role of Pitx3 in modulating BMP4 signalling has not

yet been established and the expression of Bmp4 itself is not altered in *aphakia* mice (at least at E11.5) as measured by RT-qPCR. However, Bmp4 signalling is influenced and involves many players and may act independent of the Pitx3 in regulating the expression of *Foxe3*. So we can speculate that during the placodal phase of lens development, expression of *Foxe3* is influenced by Bmp4 signalling but at latter stages of lens development involvement of Pitx3 is compulsory to maintain its expression.

e) Tube1

Reduced proliferation in *aphakia* is not only as a result of impaired *Foxe3* expression, as proliferation in *aphakia* is more severely affected compared to the *Foxe3* mutant [85,95]. There are possibly some additional factors influencing mitotic activity in this mutant, which could either be upstream or independent of Foxe3. Identification of *Tube1* as a novel downstream target of Pitx3 has made it easy to point towards one of these factors. ε-tubulin is not only important for the orientation of spindle fibers during M-phase, but also involved in the duplication of centrioles [182], a pre-requisite for mitotic activity. However, this gene has not so far been implicated in any pathological phenotype. Further, due to the lack of availability of any animal mutant, its role in organogenesis and morphological features has not been studied. But the generation of mouse mutant for this gene is under progress and so far only the ES-cell lines are available form EUCOMM (European Conditional Mouse Mutagenesis program). Availbility of these mouse mutants will help to explore the role of this gene in different processes and pathological features.

In the present study, I have identified a cis-regulatory region present on the 5'-upstream of *Tube1* (-1,288 / -1,862 bp). This regulatory region is not only highly responsive to Pitx3 but also Ap-2α. Based on these findings, we can not ignore the possibility of co-operative role of these factors in the regulation of *Tube1*.

f) Prox1

Expression of *Prox1* starts at E9.5 [92] in the developing eye around the same time when the expression of Pitx3 is observed [94,95]. In the present study, I have shown that their expression pattern completely overlaps in the wild-type lens during all stages of its development. A close observation has shown that the expression of Prox1 follows the expression of Pitx3 indicating some molecular interaction between these factors. This notion is further strengthened by the absence of the *Prox1* in *aphakia* mice. Analysis of cis-regulatory elements using bioinformatics approaches has shown an evolutionary conserved region present ~10,000 bp upstream of the *Prox1* transcription start site and is responsive to Pitx3 *in vitro*. This region contains a conserved

site that is known to be the binding site of Pitx3. This binding site has also been observed in the upstream sequence of TH and DAT and has been verified for the binding of Pitx3 using *in vivo* (ChIP) and *in vitro* approaches [178,179]. Additionally, proximal *Prox1* promoter did not responded to the presence of Pitx3 *in vitro* as expected because of the lack of any putative binding site; strengthening the finding that the former distal region is really important for the regulation by Pitx3. Further, I also considered the possibilities of indirect regulation of *Prox1* by Pitx3 involving other factors that are down-regulated in *aphakia,* like, Foxe3. However, unchanged expression of Prox1 in *Foxe3* mutant mice clearly shows that the expression of *Prox1* is independent of Foxe3 and is regulated directly by Pitx3 at least in the lens.

4.3 Impact of Pitx3 in the eye beyond the lens

In addition to the malformation caused by the absence of Pitx3 in the lens, *aphakia* mice show some additional defects in the eye. I have shown for the very first time that the expression of *Otx2* is decreased in the RPE of *aphakia* eye, a tissue where Pitx3 is not expressed. However, we do not know the exact time point when this reduction in expression starts in *aphakia*, as in the wild-type mouse expression of *Otx2* starts in the whole optic vesicle [241] but latter it is restricted to its dorsal portion, representing the presumptive RPE [242]. Otx2 is important for specification and differentiation of RPE [243,244] and is required for the normal ocular development, as its disruption result in microphthalmia [243].

The decrease in *Otx2* is not throughout the RPE but rather account for the loss of Otx2 around the anterior margins of RPE indicating its gradient regulation. This regulation pattern may involve some signalling cascades, as many pathways have cross talks between the lens and retina during the normal development of eye. Wnt/β-catenin signalling seems important in this context as it is active in RPE and is responsible for the regulation of *Otx2* in these cells [244]. Canonical Wnt-signalling is also functional in the lens epithelium during early fiber differentiation [129], which is abolished in *aphakia*. This loss of lens epithelium in *aphakia* and consequently Wnt-signalling may have some impact in the surrounding tissues including the presumptive ciliary epithelium and iris causing the down-regulation of Otx2 in these cells.

Wnt-signalling and Otx2 are also important for the neurogenesis in the midbrain [245] and in determining the fate of dopaminergic progenitors [246]. But the expression of Otx2 in the midbrain of *aphakia* mice has not yet been studied, which may help to establish some important genetic regulatory loop in the development and maintenance of dopaminergic neurons. Expression of Pitx3 in *Otx2* mutant is not known; however, *in silico* analysis of the *Pitx3* promoter spanning the proximal deletion in *aphakia* show strong binding site for Otx2, suggesting some interaction between these factors.

4.4 Interaction between various genes during lens development

In the lens, cells are organized in three different zones; an anterior epithelium comprises of undifferentiated single layer of cells, an equatorial zone where the cells are actively dividing and differentiation zone which is the posterior part of lens for primary fiber cells and transitional zone for the secondary fiber cells. These secondary fiber cells are then terminally differentiated in the center of the lens loosing the cellular organelles. During this lens cellular differentiation process various factors interact with each other including transcription factors, cell signalling cascades and structural genes.

Previous studies using various approaches (mutant analysis, overexpression, gene knockout and knockdown etc) enabled us to draw a model of regulatory cascades to understand how different transcription factors interact with each other for the development of a transparent lens. However, there are still many links missing and with the advancement of technology and novel regulatory elements not only new links are being explored but also refinement of the existing links are taking place.

In the current study I have used *in vivo* and *in vitro* approaches to refine this networking between transcription factors and their targets. I have not only used *apahakia* mice to place Pitx3 in this regulatory cascade but also used other mouse mutants including, *Foxe3* and two *Pax6* alleles to look for the interaction between other factors. Additionally, use of *Pax6* and *Pitx3* double mutant also helped to explore their co-operative role. The interaction among various important lens determinents explored in this study and discussed in all the previous sections is summerized in a model along with the supported citations wherever applied (Fig. 4.3).

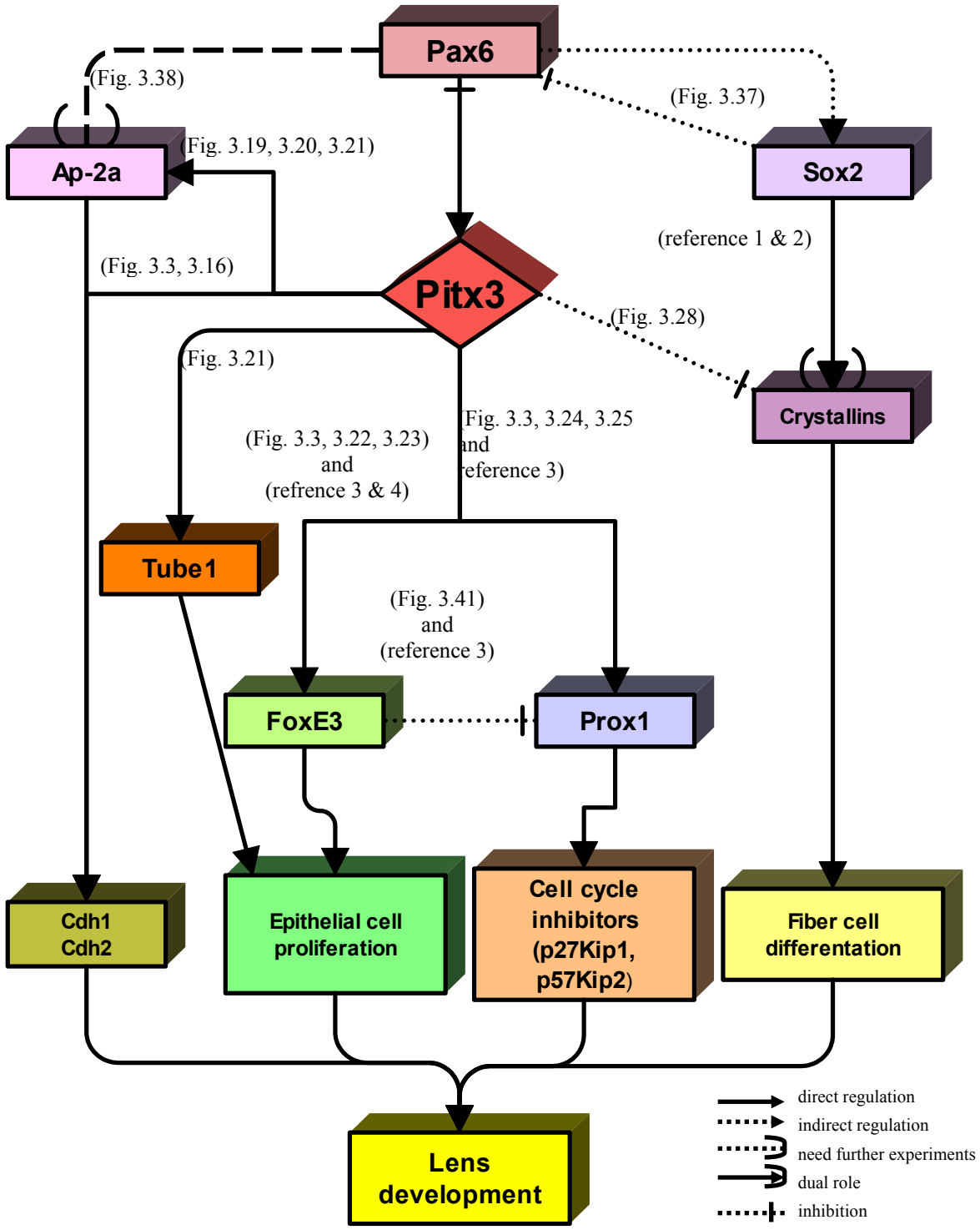


Figure 4.3: Gene regulatory network in the developing lens on the basis of present study. The references are given in parenthesis along with the supporting evidences from literature. Reference are; 1 [247], 2 [49], 3 [95], 4 [97].

4.5 Future perspectives

As the molecular targets of Pitx3 in the lens and brain are different but somehow they are related to similar mechanisms including development and maintenance of cells. Ocular lens is an excellent model organ to study all the developmental mechanisms including proliferation, migration, differentiation and survival. Pitx3 is expressed in all the cells of lens during the whole life except fully mature fiber cells in the adult that express mainly crystallins. Although some of the targets influencing these processes are identified in the present study but there is still a lot more to do.

An area that needs further attention is the role of ε-tubulin in the pathogenesis of *aphakia*. Although, I have shown that Pitx3 can directly regulate the expression of *Tube1* (ε-tubulin) but its role *in vivo* is awaiting ratification. I have already prepared three different siRNA clones for this gene in Lenti-loxP vector. *In utero* injection of these siRNAs during different developmental stages (e.g. E9.5, E10.5 and E11.5) will help to understand the exact role of this gene during lens development. Additionally, transfection of these siRNA in cultured cells will help to determine the role of ε-tubulin in spindle orientation and thus symmetry of cell division by using already published methods [248-251]. Moreover, the co-operative role of Pitx3 and Ap-2α in the regulation of *Tube1* as pointed out in the present study can further be confirmed by using co-immunoprecipitation (Co-IP)

The developmental defects caused by Pitx3 in *aphakia* can partially be recovered by retinoic acid at least in the brain [252]. Interestingly, one of the candidate genes (*Ap-2α*) found downstream of Pitx3 in this study is responsive to retinoic acid. It will be worthy to look for the impact of retinoic acid treatment on the lens development in *aphakia* and to study the feature of this mutant in a less complex context, especially the persistent lens stalk. If the decrease in Ap-2α is compensated by retinoic acid, it will help to figure out if the failure in the separation of lens vesicle is caused by Ap-2α or Foxe3 in *aphakia* mice.

One crucial fact about Pitx3 is that its expression domains and importantly its functional impact is different than other members of his family (Pitx1 and Pitx2). These functional differences are not only because of spatial and temporal variations of their expression but also could be due to the variations in N- and C-terminal domains of this protein as the homeodomain region is identical among this protein family. This idea is supported by the fact that all the mutations found so far in the *Pitx3*, either in humans or in mice (e.g., *eyeless*) are present in the C-terminus affecting the OAR motif (in most of the cases); a trans-regulatory domain. This trans-regulatory domain (also known as trans-activation domain, TAD) is likely the key for the varied functional impact of Pitx3 by interacting with different other factors. These novel interacting binding partners can be identified using mass spectometry (MS) and will help to get an inside of the

regulatory mechanism of Pitx3. This information can be used to manipulate and decipher the role of this transcription factor in various domains and to further develop the strategies to overcome the congenital and acquired deficits caused by Pitx3, e.g., Parkinson.

Finally, use of further techniques including ChIP-chip and single cell transcriptome analysis can help us not only to find out new targets of Pitx3 but also in understanding the complex regulatory networks in the ocular development and generation of dopaminergic neurons. Taking out the cell populations from different areas, e.g., anterior proliferating epithelium, posterior differentiating part and the equatorial region of developing lens (as Pitx3 is expressed in all the cells in the developing lens) by using laser capture microdissection (LCM) will allow us to explore their comparative transcriptome. This will help us to understand how Pitx3 plays differential role in the regulation of proliferation, differential and cell survival which is somehow its common feature in lens and brain although the targets are different.

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Appendix

Preparation of solutions

Agar plates	LB agar 400 ml thawed the LB agar in microwarve and put that at 60^{o}C for 1 hour and then added X-gal 800 µl IPTG 80 µl Ampicillin 400 µl Mixed that well and added 20 ml to each petri dishes and stored at 4^{o}C.
Agarose gel	Dissolved the gel in TBE buffer by heating in microwave and then poured in gel caster using suitable comb.
Alkaline Phosphate buffer (make fresh)	1 ml 5M NaCl 2.5 ml 1M $MgCl_2$ 50 µl Tween 20 5 ml 1M Tris-Cl(pH9.5) 2mM Levamisol (100 µl of 1M) Added water upto 50 ml.
Blocking Solution (for Immunostaining)	Normal donkey Serum 3% Tween 20 0.25% Added PBS to the required volume.
Blocking stock solutions (for *in situ*)	Blocking reagents was dissolved in MAB to a final concentration of 10% (w/v) with shaking and heating on a heating block, autoclaved and added 0.1% Tween 20 afterward. Stored as aliquots at -20^{o}C subsequently.
Borate buffer	Boric Acid 6.183 g / l Dissolve in H_2O and adjusted pH to 8.5.
Citric acid	1 M in DEPC water
Heparin	100 mg / ml Heparin in DEPC water.
Hybe Buffer	5 ml deionized formamide 2.5 ml 20x SSC 5 µl heparin solution 10 µl Tween 20 2.05 ml DEPC H2O Adjusted the pH to 6.0 with 1 M Citric acid (ca. 450 µl / 50 ml).

LB agar	Bacto-Trypton 10 g / l Bacto-yeast extract 5 g / l NaCl 10g / l Bacto-agar 15 g / l pH 7.0; autoclaved (120°C, 20 min)
LB media	Bacto-Trypton 10 g / l Bacto-yeast extract 5 g / l NaCl 10 g/l Disolved in H_2O adjust pH 7.0 autoclaved (120 °C, 20 min)
Lysis solution (for DNA)	500 µl 1M Tris pH 8.0 1 ml 5M NaCl 1 ml 0.5M EDTA pH 8.0 2.5 ml 10% SDS Added H_2O upto 50 ml.
MAB	11.6 g Maleic Acid (f.c. 0.1 m / l) 8.8 g NaCl (f.c. 0.15mol / l) Add 800 ml water Adjusted the pH to 7.5 with solid NaOH Added H_2O upto 1L.
MABT	MAB+ 0.1%Tween20
MgCl2 (1M)	$MgCl_2$.6 H_2O 60.99 g / l
NTE	100 ml 5M NaCl 10 ml 1 M Tris/HCl (pH 7.0) 10 ml 0.5 M EDTA Added H_2O upto 1L.
NTMT	20 ml 5M NaCl 100 ml 1 M Tris/HCl (pH9.5) 50 ml 1 M $MgCl_2$ 0.1% Tween 20 Added H_2O upto 1L.
PBS	30 ml 5 M NaCl 15 ml 1 M Na-phosphate buffer pH7.3 Added DEPC-H_2O upto 1L.
PBT	PBS with 0.1% Tween20
PBT/Glycine	2 mg / ml glycine in PBT
PFA (4%)	4g PFA 100 ml 1XPBS-DEPC Added few drops 10N NaOH and heated upto 55^{o}C

	untill PFA was dissolved Cool on ice Adjusted pH to 7.0 with HCl (used indicator paper).
PFA 4% / gluteraldehyde 0.2% in PBT	Added 400 µl 25% gluteraldehyde Added to 50 ml PBT Deionized formamide 10 g BioRad Ag501-X8 in 100 ml formamide Stirred for 1 hour Filtered and stored at -80^{o}C.
Phosphate buffer (0.2 M)	Sodium phosphate monobasic 16.56 g / l Sodium phosphate dibasic 65.70 g / l Dissolved in H_2O
ProteinaseK buffer	10 ml Tris-Cl (pH7.0) 1 ml 0.5M EDTA Added H_2O to 500ml.
RIPA	Used DEPC-H_2O, NaCl and EDTA; (do not autoclave afterward) 2.5ml 10% SDS 15ml 5M NaCl 5ml NP40 25ml 10% Deoxycholate 1ml 0.5M EDTA 25ml 1M Tris-HCl pH 8.0 Added DEPC-H_2O to 500ml.
RNase solution	1ml 5M NaCl (25) 100 µl 1M Tris-HCl pH7.5 (2.5) 10 µl Tween 20 (250) 8.89 ml Water (222.25)
RNaseA	Dissolved RNase A at a concentration of 10 µg / µl in 0.01M NaAc (pH5.2) Heated to 100^{o}C for 15 minutes Cool slowly at RT Adjust pH by adding 0.1Vol of 1M Tris-HCl pH 7.4 Stored in aliquots at -20^{o}C
Sodium Citrate Buffer	0.1% Sodium. Citrate and adjust the pH to 6.5
SSC (20x)	175.53g NaCl 88.2g Na-Citrate Dissolved in 800ml DEPC water Adjusted pH with few drops of conc. HCl to pH7.0 Added to 1000ml DEPC water

SSC/FA/Tween20	5 ml 20xSSC 25 ml Formamide 50 µl Tween 20 Added to 50 ml with H_2O
Staining solution	Boehringer BM purple Ap substrate (#1442074) Added 2 mM Levamisole Added 0.1% Tween 20 Centrifuged (do not use the pellet).
TBE (for agarose gel)	Tris Base 108 g Boric acid 55 g EDTA 9.3g Added upto 1000 ml H_2O
TBST	8 g NaCl 0.2 g KCL 25 ml 1M Tris.HCl pH 7.5 10 ml Tween 20 Added to 100 ml H_2O
TBST (10X)	8g NaCl 0.2 g KCl 25 ml 1M Tris-HCl pH7.5 10 ml Tween 20 Added to 100 ml with H_2O
TE (10x)	1 M Tris100 ml / l EDTA 3.72 g / l Added H_2O, adjust pH to 8.0, Treated with DEPC and autoclaved.
Tris-Cl (1M)	Tris base 121.4 g / l Dissolved in H_2O adjusted pH to 7.4 with HCl
tRNA	10 µg / µl in DEPC water Phenolized 2x and stored as aliquots at -20^{o}C.
X-gal	20 mg / ml Prepared in Dimethyl Formamide (DMF).