Lineage-specific functions of the homeodomain transcription factor *Pitx2* in eye development

by

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*"If we knew what we were doing,
it would not be called research,
would it?"*
-Albert Einstein

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ARS   Axenfeld-Rieger Syndrome

ASD   anterior segment dysgenesis

BA   branchial arch

ChIP   chromatin immunoprecipitation

DMD   Duchenne's Muscular Dystrophy

EOM   extraocular muscle

H&E   hematoxylin and eosin

IOP   intraocular pressure

MIF   multiply innervated fiber

MRF   muscle regulatory factor, includes *Myf5, Myod1, Myog*, and *Mrf4*

Pitx2-mko   *Pitx2* mesoderm specific knockout mouse

Pitx2-ncko   *Pitx2* neural crest specific knockout mouse

POM   periocular mesenchyme

RPE   retinal pigmented epithelium

SIF   singly innervated fiber

TUNEL   terminal dUTP nick end labeling

Abstract

Numerous inductive patterning events occur in eye development. The periocular mesenchyme plays a role in these processes by patterning the optic stalk, corneal epithelium, and retinal pigmented epithelium during eye development, in addition to contributing cells to many non-neural tissues in the eye. The periocular mesenchyme includes cells from two embryonic lineages, the neural crest and mesoderm, which each form distinct cell types.

The homeodomain transcription factor *Pitx2* is required for normal eye development in both mice and humans, and mutations can lead to early onset glaucoma in humans. *Pitx2* is expressed in both the neural crest and mesoderm lineages of the periocular mesenchyme, but the mechanisms of its function in each lineage were not known. To test the hypothesis that *Pitx2* has unique functions in each lineage during eye development, lineage-specific knockout mice of *Pitx2* in the neural crest and mesoderm were created using the Cre-lox system. *Pitx2* in the neural crest is cell-autonomously required for anterior segment development, sclera formation and ocular blood vessel growth. *Pitx2* also has non-cell autonomous functions in the neural crest in optic stalk development and RPE patterning. The defects in optic stalk development and ocular blood vessel growth represent two new potential mechanisms underlying the glaucoma seen in human patients with *PITX2* mutations. In the mesoderm, *Pitx2* is cell autonomously required for extraocular muscle precursor survival and non-cell autonomously required for optic fissure closure. *Pitx2* function is also required in the mesoderm lineage for eyelid closure. *Pitx2*, not *Pax7*, regulates MRF expression in the extraocular muscles, and PITX2 can activate the *Myod1* promoter through a novel sequence. This identifies distinct mechanisms of *Pitx2* function in the two lineages of the periocular mesenchyme in the developing eye. These findings significantly expand the understanding of the functions of *Pitx2* in eye development and its role in human disease.

Chapter 1: Background and introduction

The development of structures related to vision has fascinated scientists since the earliest days of embryology (Darwin, 1859; Spemann, 1901). While embryologists were limited to labeling and transplantation experiments, modern developmental biology has developed an ever-expanding understanding of the genes that control eye development (Jean et al., 1998). These include the transcription factors which specify cell fate, members of the signaling cascades which induce their expression, and their downstream target genes that enable the development of diverse cell types like photoreceptors, lens fibers and corneal stroma cells.

One of the reasons the eye remains a popular model system are the inductive relationships between tissues and cells of different embryonic lineages. The eye receives contributions from the neural ectoderm, the surface ectoderm, and the periocular mesenchyme, which includes contributions from mesoderm and neural crest (Johnston et al., 1979; Le Douarin, 1980, 1982; Le Lievre and Le Douarin, 1975; Noden, 1982). Contact with the neural ectoderm-derived optic vesicle causes the surface ectoderm to form the lens placode, which in turn causes the invagination of the optic vesicle and the lens itself to form the optic cup (Figure 1.1A, B) (Chow and Lang, 2001). Signals from the lens cause the inner layer of the optic cup to develop into the neural retina, while signals from the periocular mesenchyme induce the outer layer to form the retinal pigmented epithelium (Fuhrmann et al., 2000; Hyer et al., 1998; Nguyen and Arnheiter, 2000). Signals from the mesenchyme also promote the proper development of the proximal part of the optic vesicle, the optic stalk, into the optic nerve (Gage et al., 1999). As development proceeds, the inductive events continue. The optic cup induces the specification of the cornea from mesenchyme and the overlying surface ectoderm, and signals from the neural retina cause the cells of the lens vesicle to differentiate as lens

fibers (Figure 1.1C, D) (Coulombre and Coulombre, 1964; Genis-Galvez, 1966; Jean et al., 1998; Piatigorsky, 1981). A functional consequence of these inductive relationships that form the various tissues of the eye is the assurance that the functioning "parts" of a camera eye are properly located with respect to one another, i.e. the light-focusing lens is placed between the light-sensing film of the retina and the window-like cornea.

The Periocular Mesenchyme

While the inductive aspects of retina and lens development have long been known, the inductive functions of the periocular mesenchyme have only recently been discovered (Fuhrmann et al., 2000; Gage et al., 1999). Perhaps for this reason, the study of the periocular mesenchyme in eye development was less advanced until recently. In addition to participating in inductive events in eye development, the periocular mesenchyme contributes to many critical tissues in the anterior segment of the eye. These include the corneal stroma and corneal endothelium, which allow light to enter the eye; the stroma of the iris, which regulates the amount of light; the stroma of the ciliary body, which supports the ciliary epithelium that produces aqueous humor to nourish the avascular cornea and lens; and the trabecular meshwork and Schlemm's canal, which form the outflow pathway by which aqueous humor exits the eye (Figure 1.2). Other parts of the anterior segment include the corneal epithelium and lens, which are derived from the ocular surface ectoderm, and the iris and ciliary body epithelium, which are derived from the neural ectoderm at the tip of the optic cup. The periocular mesenchyme also contributes the cells that form the extraocular muscles, which enable eye movements and thus stereovision; the sclera, the tough white outer coating of the eye, which provides structural shape and an anchoring point for the muscles; and the hyaloid, choroidal, and retinal ocular blood vessels (Figure 1.2).

As noted previously, the periocular mesenchyme contains cells of two different developmental lineages, the neural crest and the mesoderm. Cells from these two lineages often contribute to the same tissues, although they form different cell types within those tissues (Figure 1.2) (Gage et al., 2005). The endothelial cells in the blood vessels of the

choroid and hyaloid are derived from mesoderm, while their pericytes and smooth muscle cells are derived from neural crest. The myocytes of the extraocular muscles are mesoderm, whereas the fascia and tendons are formed from neural crest, as is the sclera. The iris stroma is mesoderm-derived, while the stroma of the ciliary body is neural crest-derived. Schlemm's canal is mesoderm-derived, not surprising given its vessel-like structure and function. The corneal stroma, corneal endothelium, and trabecular meshwork are all composed of primarily neural crest cells, but a small population of interspersed mesoderm-derived cells is also present (Gage et al., 2005). It is unclear whether the neural crest and mesoderm cells of the cornea and trabecular meshwork all carry out the same functions; it has been proposed that the mesoderm derived cells may be the dendritic and Langerhans immune surveillance cells observed in the anterior segment (Gage et al., 2005; Hamrah et al., 2003a; Hamrah et al., 2003b; Hamrah et al., 2002).

Developmentally, the cells of the neural crest and mesoderm begin in distinct locations and both migrate into the eye field where they mix to create the loose periocular mesenchyme (POM), which surrounds the optic cup and stalk. The mesoderm contribution of the POM comes from the most anterior portion of the mesoderm, which is unsegmented and often referred to as pre-somitic; it is a separate population of mesoderm from that which contributes to branchial arch formation (Figure 1.3A). The prechordal and paraxial mesoderm that contribute to the POM are a continuous population of loose mesenchyme located ventral and caudal to the developing optic vesicle and cup from e8.0 to e10.5 (Figure 1.3A, B) (Gage et al., 2005; Noden and Francis-West, 2006). By e11.5, the mesoderm has proliferated and condensed into a morphologically distinct wedge of cells just dorsal, caudal and slightly proximal to the optic cup, while a few cells have migrated into the spaces between the developing retina, lens and ocular surface ectoderm (Figure 1.3C). The neural crest portion of the POM is derived from the cranial neural crest which migrate out of the posterior diencephalon, mesencephalon and metencephalon beginning at e8.5 (5-6 somites) and arrive in the eye field beginning at e10.0 (Creuzet et al., 2005). At this point, the lens vesicle has separated from the overlying ocular surface ectoderm, and the neural crest invade the space between the two

and quickly surround the optic cup and stalk by e10.5 (Figure 1.1C, 1.3C). By e12.5, the mesoderm and neural crest portions of the POM are extensively co-mingled and difficult to distinguish morphologically (Gage et al., 2005).

The morphogenesis of the periocular mesenchyme continues as it proliferates; between e12.5 and e13.5, the 3-5 cell thick layer of mesenchyme between the lens and the surface ectoderm begins to condense. By e13.5 individual extraocular muscle primordia are visible. At e14.5, the mesenchyme cells closest to the lens begin to flatten and form the corneal endothelium, which becomes separate from the lens, creating the anterior chamber (Figure 1.1E). Mesenchymal cells migrate into the angle that is created between the corneal endothelium and the anterior edge of the optic cup, known as the iridocorneal angle. Posterior to this region, the cells surrounding the developing retinal pigmented epithelium (RPE) begin to condense in a layer 2-4 cells thick that will form the sclera and choroid vasculature. At e15.5, the anterior edge of the optic cup begins to flatten and elongate to form the iris; it is colonized by mesenchyme that forms the iris stroma, which becomes detached from the cornea by e16.5 (Figure 1.1F). The stroma of the adjacent ciliary body is also formed from mesenchyme at this time. Proliferation of the corneal stroma levels off by e16.5 and these cells begin to adopt a lamellar appearance (Cvekl and Tamm, 2004; Gould et al., 2004).

Further differentiation and formation of structures within the anterior segment derived from the POM continues after birth; mesenchyme in the iridocorneal angle condenses to form the trabecular meshwork beginning at postnatal day P4. Schlemm's canal appears at P12 and the remodeling of these structures is fully complete at P35 (Gould et al., 2004). The corneal stroma increases in thickness by excreting substantial amounts of extracellular matrix proteins until P10, and the corneal endothelium closes its intracellular spaces to keep the corneal stroma dehydrated and laminated. Descemet's membrane, a basement membrane, is formed by the corneal endothelium (Zieske, 2004). The sclera also undergoes significant remodeling of its collagen-rich extracellular matrix for up to two months after birth (Zhou et al., 2006).

Gene Expression and Molecular Markers

      Much research has been done to identify the genes that control these morphogenetic events in the differentiation of the periocular mesenchyme and the molecular markers that define various stages. The homeodomain transcription factor *Pitx2* is expressed in the mesoderm that contributes to the periocular mesenchyme at very early stages, before the neural crest enters the eye field (Figure 1.3B) (Gage et al., 2005). As the neural crest cells migrate into the eye field, they activate numerous transcription factor genes including *Pitx2*, *Foxc1, Foxc2*, and *Lmx1b*, all of which are required in mice for normal development of mesenchyme derived structures (Gage et al., 1999; Kitamura et al., 1999; Kume et al., 1998; Lu et al., 1999; Pressman et al., 2000; Semina et al., 1996; Smith et al., 2000; Winnier et al., 1997). Transcription factor AP-2β (*Tfap2b*) is expressed in the developing neural crest, lens and surface ectoderm beginning at e10.5, but is not required for normal eye development (Moser et al., 1997; West-Mays et al., 1999). *Eya2* is expressed in the neural crest and mesoderm portions of the periocular mesenchyme beginning at e11.5, but mice lacking *Eya2* function reportedly have no visible ocular phenotype (Grifone et al., 2007; Xu et al., 1997). *Pitx1* is expressed in the presumptive corneal stroma and extraocular muscles beginning at e11.5, and is rapidly down-regulated by e14.5, but mice lacking *Pitx1* function have no ocular phenotype (Adam Diehl, personal communication).

      As the cells of the largely neural crest-derived corneal stroma differentiate they activate expression of the proteoglycan *keratocan* beginning at e13.5 (Liu et al., 1998). The expression of type I collagen at e13.5 and type II collagen at e14.5 are further indicators of differentiation in the corneal stroma and sclera (Dakubo et al., 2008; Savontaus et al., 1997). The transcription factors *Pitx2*, *Foxc1*, and *Foxc2*, are all downregulated in the central corneal stroma by e16.5, while *Lmx1b* remains on through adulthood (Pressman et al., 2000). Although the corneal epithelium is not derived from the periocular mesenchyme, it does receive signals from the mesenchyme that influence its patterning (Gage et al., 2008). Corneal epithelium is marked by the expression of *Pax6* and its differentiation is indicated by the expression of cytokeratins 12 and 14; CK12 is

cornea specific, while CK14 is expressed throughout the ocular surface ectoderm (Zhang et al., 2005).

In the developing extraocular muscles, *Pitx2* is expressed prior to e8.5, while *Pitx1* is activated at e11.5 (Diehl et al., 2006). Many other transcription factors that are also expressed in other skeletal muscles are found in the extraocular muscles, including *Pax7, Myf5, MyoD,* and *Myogenin* (Mootoosamy and Dietrich, 2002). The timing of the expression of these factors has been well characterized in chick, but not in mammals (Mootoosamy and Dietrich, 2002; Noden and Francis-West, 2006; Noden et al., 1999). Developmental myosin heavy chain expression, which marks differentiated muscles, appears at e13.5 (Diehl et al., 2006). Expression of the transcription factor *Lmx1b* can be seen in the extraocular muscles at e14.5; it is unclear when its expression begins in the mesodermal lineage (Pressman et al., 2000).

If these morphogenetic and differentiation events do not occur properly in the periocular mesenchyme, a variety of ocular diseases result. Diseases involving the extraocular muscles are discussed later, but one of the most common conditions associated with deficits in periocular mesenchyme development is a constellation of developmental eye defects known as anterior segment dysgenesis (ASD). ASD includes defects such as corneal opacity, adhesions of the iris to the cornea, hypoplastic iris and defects of the outflow tract leading to early onset glaucoma. The great majority of genes currently identified to cause ASD encode transcription factors that are expressed in the periocular mesenchyme during development. Transcription factors can activate programs leading to cell fate specification and differentiation, so they are often the targets of inductive signals (Faber et al., 2001; Wawersik et al., 1999). They can also activate the expression of signaling molecules and their receptors and inhibitors, thus causing or preventing other inductive events (Gage et al., 2008). For these reasons, the study of transcription factors has been an area of great interest in developmental biology.

Homeodomain Transcription Factor *Pitx2*

One of the most important transcription factors regulating the development of the periocular mesenchyme is the homeodomain transcription factor gene *Pitx2*. The *Pitx2* gene consists of six exons and is transcribed in three different isoforms; *Pitx2a* and *Pitx2b* are generated by alternative splicing, while *Pitx2c* originates from an alternative transcriptional start site. All three isoforms include exons 5 and 6, which encode the homeodomain and C-terminus, so they bind the same DNA sequences, but they differ in their N-termini (Cox et al., 2002; Gage and Camper, 1997; Semina et al., 1996). The N-terminus of *Pitx2a* includes exons 1 and 2, while *Pitx2b* includes exons 1, 2, and 3, and *Pitx2c* includes exon 4 (Gage and Camper, 1997; Semina et al., 1996). All three isoforms are expressed in the developing periocular mesenchyme (Kitamura et al., 1999; Liu et al., 2001; Zhou et al., 2009).

Of the transcription factors required for normal development of the periocular mesenchyme, *Pitx2* is the only one that is expressed in both the neural crest and mesodermal lineages. It is expressed in the neural crest cells that contribute to the corneal stroma and endothelium, the iridocorneal angle, the sclera, the pericytes of the blood vessels, and the fascia of the extraocular muscles. In the mesoderm, *Pitx2* is expressed in the cells that form the myocytes of the extraocular muscles, Schlemm's canal, and the small cohort that contributes to the corneal stroma, corneal endothelium and trabecular meshwork (Gage et al., 2005). It is also expressed in the eyelid mesenchyme beginning at e12.5 (ALZ, unpublished data).

Like many transcription factors, the expression of *Pitx2* is tightly regulated in time and space. *Pitx2* is expressed in the prechordal and paraxial mesoderm prior to e8.5, and it remains on throughout the development of the extraocular muscles that form from it (Figure 1.3B, C, E). It is downregulated postnatally in some muscle fibers, but it remains on in the satellite cells, the muscle stem cells. In the neural crest, it is activated at e10.0 as the migrating neural crest cells enter the eye field, in response to signals from the optic cup (Figure 1.3C) (Gage et al., 2005; Matt et al., 2005; Molotkov et al., 2006).

Pitx2 remains on in the developing sclera, corneal endothelium, and iridocorneal angle just prior to birth (e18.5), but it is downregulated in the corneal stroma beginning at e14.5, where it continues to be expressed at low levels (Gage et al., 2008).

Axenfeld-Rieger Syndrome

PITX2 was initially identified as an important regulator of eye development when mutations in it were discovered as the cause of an autosomal dominant human disorder called Axenfeld-Rieger Syndrome (Semina et al., 1996). Axenfeld-Rieger Syndrome (ARS) is a constellation of developmental abnormalities that includes the dysgenesis of the anterior segment of the eye, dental hypoplasia including small, malformed or absent teeth, mild craniofacial dsymorphism and failure of the involution of the periumbilical skin. Rare heart, pituitary and limb defects have also been reported. The eye phenotype includes adhesions of the iris to the cornea and trabecular meshwork, iris stromal hypoplasia, corectopia (misplaced pupil), polycoria (multiple pupils), and posterior embryotoxon. Posterior embryotoxon refers to a prominent, anteriorly displaced Schwalbe's line, which represents the intersection of the cornea, sclera and trabecular meshwork (Amendt et al., 2000; Sampaolesi et al., 2009). This additional trabecular meshwork tissue occurs to some extent in up to 15% of normal eyes and alone is not associated with an increased risk of glaucoma (Burian et al., 1955). Approximately 50% of patients with ARS develop early onset glaucoma, possibly due to defects in the outflow tract that lead to increased eye pressure (Shields, 1983). Recently, a case report described an individual with ARS and strabismus (cross eyes), with an abnormal insertion position for one of the extraocular muscles, the superior oblique, although the underlying genetic cause was not known in this case (Park et al., 2009). When the eye phenotype is present alone, the disease is often referred to as Axenfeld-Rieger anomaly or malformation, although the absence of systemic defects is relatively rare in patients with *PITX2* mutations (Strungaru et al., 2007). Since the identification of *PITX2* as a cause of anterior segment dysgenesis, it has also been identified as a cause of iridogonio-dysgenesis (iris hypoplasia with defects of the outflow tract) (Alward et al., 1998), Peters Anomaly (anterior segment dysgenesis with opaque cornea) (Doward et al., 1999), and

ring dermoid of the cornea (abnormal growths and projections of the limbus or sclera into the cornea) (Xia et al., 2004).

Mutations in other genes can also cause Axenfeld-Rieger anomaly. Mutations in the forkhead transcription factor *FOXC1*, located at chromosome 6p25, cause identical eye phenotypes and rarely deafness, heart anomalies, tooth defects, and umbilical abnormalities (Gould et al., 1997; Mears et al., 1998; Mirzayans et al., 2000; Nishimura et al., 1998). Duplications of *FOXC1*, as well as mutations that reduce FOXC1 activity, can cause Axenfeld-Rieger anomaly; the patients with duplications tend to have more severe forms of the disease (Lehmann et al., 2000; Strungaru et al., 2007). This indicates that anterior segment development is also very sensitive to *FOXC1* dose.

A third, uncloned locus for ARS has been identified at 13q14, which includes *FOXO1A* in the critical region (Phillips et al., 1996). *Foxo1a* is expressed in the periocular mesenchyme of developing zebrafish and is a direct target of *Foxc1* (Berry et al., 2008). These three loci likely do not account for all genetic causes of ARS; there may even be other genes in the 6p25 region, such as *TFAP2A* (AP-2α), which cause ARS (Alward, 2000; Davies et al., 1999).

Genes have been identified that cause other forms of anterior segment dysgenesis with phenotypes similar to Axenfeld-Rieger anomaly. Many of these genes are expressed only in the surface ectoderm and/or neural ectoderm, further emphasizing the signaling relationships between the periocular mesenchyme and surface and neural ectoderm and their requirement for normal development. Patients with mutations in *LMX1B*, which is expressed in the developing periocular mesenchyme, have nail-patella syndrome and approximately one-third develop early onset glaucoma, which indicates possible outflow pathway defects, a more mild phenotype than the ASD seen in *Lmx1b* mutant mice (Lichter et al., 1997; Mimiwati et al., 2006; Pressman et al., 2000; Vollrath et al., 1998). Mutations in the transcription factor *PAX6,* which is expressed in both the surface and neural ectoderm, cause aniridia, Peters' anomaly and iris hypoplasia (Prosser and van Heyningen, 1998). Similarly, mutations in *CYP1B1*, a cytochrome P450-related enzyme

expressed in the neonatal corneal and ciliary epithelia, cause Peters' anomaly and congenital glaucoma (Bejjani et al., 2002; Doshi et al., 2006; Stoilov et al., 1997; Vincent et al., 2006; Vincent et al., 2001). Mutations in two transcription factor genes expressed in the lens epithelium, *PITX3* and *FOXE3*, cause anterior segment mesenchymal dysgenesis, which includes corneal opacity, cataracts, and lens-cornea and iris-cornea adhesions (Semina et al., 2001; Semina et al., 1998; Summers et al., 2008). Mutations in *JAG1*, a signaling molecule in the Notch pathway that is expressed in the developing iris epithelium, cause Alagille syndrome, which includes posterior embryotoxon and iris abnormalities. (Bao and Cepko, 1997; Hingorani et al., 1999; Li et al., 1997; Oda et al., 1997)

As noted, patients with anterior segment dysgenesis have greatly increased risk for developing glaucoma, and often develop it much earlier in life than other patients (Strungaru et al., 2007). Glaucoma is a disease in which vision loss occurs due to optic nerve damage and retinal ganglion cell death; the visual information from the retina cannot be transmitted to the brain. The causes of glaucoma are complex and not well understood, but the developmental glaucomas associated with ASD represent an excellent entrez into studying this disease. In general, there are two classes of glaucoma: hypertensive glaucoma in which elevated intraocular pressure (IOP) causes damage to the optic nerve, and normal-tension glaucoma in which IOP is normal but optic nerve damage still occurs, possibly due to vascular defects (Araie et al., 1994). IOP is determined by the rate at which the ciliary body produces aqueous humor and the rate it exits the eye through the trabecular meshwork and Schlemm's canal (outflow pathway). Elevated IOP can occur without ever causing optic nerve damage; this fact and the existence of normal tension glaucoma indicate that there are other factors that cause glaucoma besides elevated IOP (Grodum et al., 2005). In many cases of hypertensive glaucoma, the reasons for the increase in IOP are completely unknown, which is why the study of developmental glaucoma can provide insight. In these patients, elevated IOP is likely caused by developmental defects in the outflow pathway that partially inhibit the aqueous humor from exiting the eye, however, this may not be the whole story.

In a review of 126 Axenfeld-Rieger patients with genetic changes in *PITX2* or *FOXC1*, Strungaru et al. found that those with glaucoma frequently showed no improvement in response to either surgery (even multiple surgeries) to open the outflow pathway or medication to reduce aqueous humor production. If the glaucoma in these patients was due entirely to developmental defects of the outflow pathway, these interventions should be very successful, suggesting that like in adult-onset glaucoma, additional causative factors exist. The authors propose that these may include progressive degeneration of the outflow pathway, aqueous humor production that does not respond to medical/surgical interventions, extremely sensitive optic nerve cells, increased fibrosis in response to surgery, and/or the involvement of other modifier genes. The study also noted that patients with *PITX2* mutations had glaucoma that was more resistant to intervention and worse visual outcomes than patients with *FOXC1* mutations (Strungaru et al., 2007).

Mutations in *PITX2*

Dozens of unique genetic defects in the *PITX2* gene have been reported to cause anterior segment dysgenesis (Amendt et al., 2000; Lines et al., 2004). Splice-site, frameshift and nonsense mutations have been found throughout the gene, while missense mutations are usually restricted to the homeodomain region (Espinoza et al., 2002; Kozlowski and Walter, 2000; Lines et al., 2004; Lines et al., 2002; Perveen et al., 2000). Microdeletions of the 4q25 region including *PITX2* have also been reported (Lines et al., 2004). The association between *PITX2* and ARS was originally made using two families with balanced translocations in which the actual breakpoints are 5-65 KB from the coding region of the gene, indicating that distant enhancers are required to drive normal *PITX2* expression (Semina et al., 1996; Trembath et al., 2004). The vast majority of the mutations identified in *PITX2* are null mutations; they create protein products which are truncated, unstable, unable to translocate to the nucleus, bind DNA, or activate transcription (Amendt et al., 2000; Footz et al., 2009; Lines et al., 2004). Since the eye diseases caused by genetic changes in *PITX2* are dominant, this suggests that they are caused by haploinsufficiency and eye development is sensitive to reduced levels of PITX2 expression.

Two other types of mutations in *PITX2* have been described. One group described a missense mutation in the homeodomain that resulted in a V45L change that caused the protein to have slightly reduced DNA-binding capabilities, but a massive increase in transactivation (Priston et al., 2001). Another group identified a K88E change in the homeodomain that caused the protein to have dominant negative interactions with wildtype PITX2 protein (Saadi et al., 2003; Saadi et al., 2001). Other groups have had difficulty replicating these findings with the V45L and K88E mutations with other cell lines and promoters, suggesting that these effects may be very specific to certain conditions (ALZ unpublished observation, Min Qian & Michael Walter, personal communication). The possibility that a hyperactive form of PITX2 could cause the same disease as a null mutation suggests that the eye is exquisitely sensitive to PITX2 dose. To some degree, genotype-phenotype correlations have been identified based on the particular mutation a patient carries. Missense mutations in the homeodomain that reduce, but do not eliminate DNA binding and transactivation are associated with iris hypoplasia and iridogoniodysgenesis, while missense mutations that produce transcriptionally dead proteins are associated with ARS (Kozlowski and Walter, 2000). However, the same frameshift mutation was found to cause Axenfeld-Rieger syndrome in one family and Axenfeld-Rieger anomaly in another, so modifying factors may play a role (Amendt et al., 2000).

Mouse Models of *Pitx2* Dysfunction

Analysis of Axenfeld-Reiger syndrome and other eye diseases caused by mutations in *PITX2* has shown that this transcription factor has important functions in eye development and also the pathogenesis of glaucoma. The use of mouse models to study the function of *Pitx2* can provide much insight, and as such, numerous mouse models have been created (Gage et al., 1999; Holmberg et al., 2004; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Suh et al., 2002). Most of these mouse models are various types of null alleles. Mice that lack *Pitx2* function have defects in many other tissues besides the eye, tooth and umbilicus, but surprisingly, mice heterozygous for null mutations in *Pitx2* have mild eye defects, such as corectopia and polycoria, at low penetrance (Gage et

al., 1999). Heterozygotes also have reduced central corneal thickness (Asai-Coakwell et al., 2006). The heterozygous phenotype has not been examined on a variety of genetic backgrounds, so the low penetrance could be due to the effects of modifying loci, but it could also be that mouse ocular development is less sensitive to haploinsufficiency of *Pitx2*.

The ocular defects seen in mice homozygous null for *Pitx2* affect tissues in which *Pitx2* is expressed, as well as tissues in which it is not. Cell autonomous defects include the absence of extraocular muscles, the agenesis of the sclera and corneal endoderm, thickening of the presumptive corneal stroma, failure of the anterior chamber to form, and reduced ocular vasculature. Many of the affected tissues receive contributions from both the mesoderm and neural crest. Non-cell autonomous defects include the thickening of the developing optic nerve, reduced pigmentation in the RPE, and retinal coloboma (the latter at reduced penetrance) (Evans and Gage, 2005; Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). It should be noted that *Foxc1* expression is unchanged, indicating that *Pitx2* does not regulate this gene. Unfortunately, *Pitx2$^{null/null}$* mice die at e14.5, so further analysis of later structures, such as the trabecular meshwork and Schlemm's canal, has not been possible. Examination of mice with a reduced function allele of *Pitx2* also showed that it is required for normal eyelid development and closure (Figure 1.4C, D)(Adam Diehl, personal communication).

Non-ocular defects found in the *Pitx2$^{null/null}$* mice include pituitary agenesis, tooth agenesis, agenesis of the facial muscles, abnormal jaw development, severe heart defects, right isomerization of the lungs, turning defects, failure of the ventral body wall to close, and the absence of certain nuclei in the brain (Dong et al., 2006; Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Martin et al., 2004; Shih et al., 2007a). Targeted knockouts and stem cell studies have identified roles for *Pitx2* in vascular smooth muscle differentiation and formation of the stem cell niche in bone marrow (Kieusseian et al., 2006; Shang et al., 2008). In lower vertebrates, *Pitx2* homologues play key roles in left-right patterning, but the *Pitx2^{null}* mice have relatively mild laterality defects by comparison, despite the asymmetric expression of the gene in

the trunk mesoderm (Ryan et al., 1998). Situs inversus and other laterality defects have also never been reported for patients with *PITX2* mutations. This suggests that *Pitx2* has a lesser role in left-right asymmetry in mammals or that other factors are compensating for its loss.

To understand how a transcriptionally hyperactive mutation of *PITX2* might cause ARS, a transgenic mouse model of *Pitx2* overexpression was created. The keratocan promoter was used to drive overexpression of either wildtype or mutant *PITX2A* in the cornea beginning at e13.5. Adult mice carrying the wildtype transgene had cloudy corneas with a disrupted collagen matrix, abnormal iridocorneal angles and progressive retinal degeneration. Some also had severely hypertrophic cornea and iris with iridocorneal attachment, while the mice overexpressing mutant *PITX2* had no phenotype (Holmberg et al., 2004). These data indicate that downregulation of *Pitx2* is required for normal cornea development and increased activation of *Pitx2* targets in the iridocorneal angle has similar deleterious effects as reduced activation, so the expression level of *Pitx2* must be tightly regulated.

To better understand the functions of decreased *Pitx2* dose, an allelic series was created using the $Pitx2^{null}$ loss of function allele and the $Pitx2^{neo}$ hypomorphic allele, which produces approximately 40% as much protein as a single wildtype allele due to inefficient splicing (Suh et al., 2002). The $Pitx2^{+/neo}$ (70% wildtype dose PITX2) eyes are indistinguishable from wildtype, but in the $Pitx2^{+/null}$ mice (50%) the extraocular muscles are affected; the two oblique muscles are absent and the four rectus muscles are reduced in size (Figure 1.4A, 1.5B, C). In the $Pitx2^{neo/neo}$ (40%) mice the extraocular muscles are even more reduced and the optic cup is rotated ventrally. At only 20% of wildtype PITX2 dose in the $Pitx2^{neo/null}$ eyes, extraocular muscles are completely absent and the eyelids fail to close (Figure 1.4). The shape of the optic cup is slightly elongated, possibly because the sclera is thin. However, anterior segment development and optic nerve development are largely normal at e14.5, the latest timepoint examined histologically (Figure 1.4). There are no gross defects of the body wall or turning defects (Adam Diehl, personal communication). These data suggest that, with the exception of extraocular

muscle development, a small dose of *Pitx2* is sufficient to induce the major morphogenetic events in mouse eye development.

Other Mouse Models of Anterior Segment Dysgenesis

There are interesting similarities and differences between the ocular phenotype of the *Pitx2* mutant mice and mice carrying defects in other transcription factor genes expressed in the periocular mesenchyme. Mice homozygous null for *Foxc1* have agenesis of the corneal endothelium and thick dysmorphic, vascularized corneal stroma that is attached to the lens because the anterior chamber fails to form. The iris is hypoplastic with irregular shaped pupils, there is a lack of cells in the presumptive trabecular meshwork at e18.5, and the eyelids fail to close. However, the level of *Pitx2* expression is unchanged, indicating it is not transcriptionally regulated by *Foxc1*. The mice also have non-cell autonomous defects; the corneal epithelium is thick and dysmorphic and overall, the mutant eyes are smaller than wildtype. The mutant mice also have hydrocephalus and multiple skeletal abnormalities that cause them to die at birth (Kidson et al., 1999; Kume et al., 1998). Adult mice heterozygous for *Foxc1* also have ocular phenotypes, the severity of which depends on genetic background. Clinical defects included misplaced and irregularly shaped pupils, posterior embryotoxon, iridocorneal adhesions, and increasing corneal opacity with age (Hong et al., 1999; Smith et al., 2000). Upon histological analysis, most mice were observed to have small or absent Schlemm's canal; hypoplastic, compressed, or absent trabecular meshwork; abnormal vascularization; and hypoplastic ciliary body, iris stroma, and iris epithelium (Smith et al., 2000).

Recently, the PITX2 and FOXC1 proteins were shown to physically interact, with PITX2 inhibiting the ability of FOXC1 to activate target gene transcription in the subset of cells in the developing anterior segment in which the two proteins are co-expressed. The precise balance between the competing activities of PITX2 and FOXC1 may explain why eye development is sensitive to the dosage of both proteins; reduced PITX2 function leads to both reduction in expression of its target genes and increased expression of FOXC1 target genes, while additional copies of *FOXC1* may increase FOXC1 levels

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enough to overcome the inhibition by PITX2 in some cells (Berry et al., 2006).

Foxc2 encodes a second forkhead-related transcription factor that is highly related to *Foxc1* and has an analogous expression pattern. Despite the demonstration that heterozygous or homozygous *Foxc2[null]* mice have analogous anterior segment phenotypes to the corresponding *Foxc1* mice, no *FOXC2* mutations in association with anterior segment defects or glaucoma have been reported (Smith et al., 2000; Winnier et al., 1997). *Foxc2[null]* heterozygous mice also have distichiasis, an ectopic extra row of eyelashes (Kriederman et al., 2003). Heterozygous *FOXC2* mutations have been identified in patients with Lymphedema-Distichiasis Syndrome (Fang et al., 2000). Mice that were double heterozygous for *Foxc1* and *Foxc2* mutations have similar defects to the single mutants, but with more severe defects in the iris stroma and ciliary body, plus corneal vascularization and open eyelids at birth (Smith et al., 2000).

Lmx1b knockout mice lack a corneal endothelium and anterior chamber and have dysmorphic corneal stroma, similar to *Foxc1* or *Foxc2* deficient mice. *Lmx1b-/-* homozygotes also exhibit corneal revascularization, microphthalmia, iris hypoplasia with irregular pupillary openings, and ciliary body hypoplasia, although their extraocular muscles appear normal. In contrast to *Foxc1* and *Foxc2* heterozygotes, eye development in *Lmx1+/-* animals is normal (Pressman et al., 2000).

None of these phenotypes are as severe as the *Pitx2* knockout mice, especially in the effects on non-mesenchymal tissues, but they all have defects in corneal endothelial formation and thus anterior chamber formation. The *Foxc1* heterozygous mice provide a better model for Axenfeld-Rieger anomaly than the *Pitx2* heterozygotes, as they display all the clinical hallmarks with high penetrance. The *Foxc1^{+/-}* clinical phenotype was almost completely penetrant (20/21) on the C57BL/6J background, but not seen on the 129 background, although histological sections revealed outflow pathway defects in all mice. The *Pitx2^{+/-}* mice that were examined were mixed 129/SJL and C57BL/6J, and defects were observed in approximately 10% of mice upon clinical examination. It might be worthwhile to examine the *Pitx2^{+/-}* mice on a pure C57BL/6J background with both

clinical and histological tools.

Signaling Pathways in Mouse Models of ASD

Numerous mice with mutations in signaling pathway genes also have anterior segment dysgenesis, some of which regulate *Pitx2* expression in the eye. One such signaling pathway is the retinoic acid signaling pathway. Mice which lack the enzymes that produce retinoic acid, as well as mice with no retinoic acid receptors in the neural crest, phenocopy the neural crest-specific knockout of *Pitx2* (see Chapter 2 for details). These mice also lose expression of *Foxc1* (Evans and Gage, 2005; Matt et al., 2005; Matt et al., 2008; Molotkov et al., 2006).

The canonical Wnt pathway also seems to play a role in the activation of *Pitx2* expression during eye development, possibly as part of a feedback loop. PITX2 activates the expression of *Dkk2*, a secreted inhibitor of canonical Wnt signaling, in the neural crest at e11.5, and mice lacking *Dkk2* fail to down-regulate *Pitx2* expression in the corneal stroma at e16.5. *Dkk2* mutant mice have conjunctivalization of the cornea with abnormal blood vessel growth, iridocorneal adhesions, and hypomorphic eyelids that fail to close (Gage et al., 2008). Wnt signaling has also been proposed to stabilize *Pitx2* mRNA, preventing its rapid turnover and allowing its translation (Briata et al., 2003). Recent evidence indicates the canonical Wnt effector protein β-catenin may be required for the maintenance of *Pitx2* expression in the neural crest at e11.5 (ALZ & Philip Gage, unpublished observations). In the developing pituitary, the Wnt responsive transcription factor LEF1 binds a conserved, required site in a pituitary *Pitx2* enhancer, indicating that canonical Wnt signaling can activate the expression of *Pitx2* in other tissues (Ai et al., 2007). Physical interaction with β-catenin has been proposed to functionally activate PITX2-dependent transcription, possibly by relieving inhibition mediated by the chromatin associated protein HMG-17 (Amen et al., 2008; Amen et al., 2007; Vadlamudi et al., 2005).

Another signaling pathway that plays a role in the activation of *Pitx2* is the G-

coupled protein receptor, *Gpr48*. *Gpr48* acts through adenylate cyclase to increase intracellular cyclic AMP (cAMP), which activates the CREB transcription factor. During eye development, it is expressed in the mesenchyme of the developing anterior segment as early as e12.5, and later expression is seen in the lens epithelium and tips of the optic cup (presumptive iris and ciliary body). CREB can bind and activate the *Pitx2* promoter and mice mutant for *Gpr48* have significantly reduced *Pitx2* expression at e12.5. These mice also have reduced eye size, severe iris hypoplasia, iridocorneal adhesions, corneal opacity and vascularization, abnormalities of the trabecular meshwork and Schlemm's canal, and cataracts. Unlike other mice with anterior segment defects, the corneal endothelium appears unaffected (Weng et al., 2008).

The TFG-β signaling pathway may also have a role in the activation of *Pitx2*, but the evidence is incomplete. *TGFβ2* is expressed in the developing lens and knockout mice have a very similar eye phenotype to neural crest specific knockout mice for the *Tgfbr2* receptor, indicating that its primary signaling target is the neural crest. These mice have thin, hypoblastic corneas with no endothelium or stromal lamination, persistent hyaloid vasculature and hyperblastic retinas (Ittner et al., 2005; Sanford et al., 1997). The neural crest specific knockout lacks *Pitx2* expression at e15, but the cornea is already severely hypocellular at this point, so the cells that normally express *Pitx2* may be absent or reduced in number (Ittner et al., 2005). Examination of these mice at earlier timepoints would provide more insight into whether TGF-β signaling is required for the activation or maintenance of *Pitx2* expression or the survival and/or proliferation of the neural crest. The overexpression of a related molecule, TGF-β1 in the lens beginning at e12.5 causes the opposite effect. These mice have severely hypertrophic corneas due to increased proliferation and absence of the vitreal space between the retina and lens. Their corneal stroma is disorganized, unlaminated and vascularized and they lack a corneal epithelium and iris stroma (Flugel-Koch et al., 2002). The expression of *Pitx2* was not examined in these mice, which also might help determine if it is regulated by TGF-β signaling. Other TGF-β family members, activin and TGF-α, are required for eyelid closure (Berkowitz et al., 1996; Luetteke et al., 1993; Vassalli et al., 1994; Xia and Kao, 2004).

Recent work has shown that *Indian Hedgehog* (*Ihh*), an activator of the hedgehog signaling pathway, plays a role in regulating *Pitx2* expression in the more proximal part of the periocular mesenchyme which surrounds the optic nerve and optic cup. *Ihh* is expressed in the developing choroid vasculature, and the hedgehog target gene *Gli1* is expressed in the adjacent POM surrounding the optic cup. In *Ihh^{null}* mice, *Gli1* expression is lost and *Pitx2* expression is reduced in the posterior mesenchyme but not the anterior segment at e12, and completely absent in the posterior by e13.5. However this regulation is not direct, because inhibiting or activating hedgehog signaling in e12 whole eye explants cultured for 48 hours does not affect *Pitx2* expression. The *Ihh* mutant mice also have extensive loss of the sclera, probably due to defects in differentiation, which results in misshapen eyes. The choroid vasculature is reduced, and there are patchy defects in RPE pigmentation associated with the abnormal choroid and sclera (Dakubo et al., 2008). This emphasizes the importance of *Pitx2* in scleral development. The identified signaling pathways that have been proposed to activate *Pitx2* expression are all acting on the neural crest lineage, based on the location and fate of the cells affected, as well as lineage specific analysis. No activators of the *Pitx2* expression in the mesodermal portion of the periocular mesenchyme have been proposed.

Other signaling pathways are critical for anterior segment development, but apparently do not affect *Pitx2* expression. One such pathway is BMP4; in early eye development, it is expressed in the optic vesicle and ventral mesenchyme, and is later localized to the dorsal retina (Behesti et al., 2006). Beginning at e14.5, *Bmp4* is restricted to the ciliary body, iris stroma, and RPE, where it is expressed through adulthood (Chang et al., 2001). Mice heterozygous for a *Bmp4* null allele have Schlemm's canal and trabecular meshwork defects, which cause a glaucoma phenotype. They also have iridocorneal adhesions, irregular shaped pupils and thinning, opacity and/or vascularization of the peripheral cornea. The involvement of anterior segment tissues that do not express *Bmp4* suggests that BMP4 is secreted in the aqueous humor by the ciliary body and thus signals long range to the affected tissues, but the molecular targets for such signaling were not investigated (Chang et al., 2001). Mice expressing very low levels of *Bmp4* are anophthalmic or severely microphthalmic, and human patients with *BMP4*

mutations also have this severe eye phenotype, suggesting that BMP4 also has very important functions in the early formation of the eye (Bakrania et al., 2008; Goldman et al., 2006).

Functions of *Pitx2* in Other Organs

In addition to its functions in eye development, the functions of *Pitx2* have been studied in the development of many other tissues, some of which may be generalized functions of *Pitx2* in organogenesis. One of the best studied organs is the pituitary; there *Pitx2* has been shown to play roles in the formation and survival of the organ primordia, cell fate specification through the activation of other transcription factors, and differentiation by activating genes expressed by terminally differentiated cells (Charles et al., 2005; Quentien et al., 2002a; Quentien et al., 2002b; Suh et al., 2002; Tremblay et al., 2000). It plays a similar role in activating transcription factor expression in many other tissues. In the pituitary and in other organs, some of these target genes require certain transcriptional co-factors and the three isoforms of *Pitx2* differ in their abilities to regulate certain target genes (Amendt et al., 1998; Cox et al., 2002; Quentien et al., 2002a; Quentien et al., 2002b; Schubert et al., 2004; Suh et al., 2002; Toro et al., 2004; Tremblay et al., 2000). The *Pitx2c* isoform in particular has been proposed to be the dominant isoform in left-right patterning (Essner et al., 2000; Liu et al., 2001; Schweickert et al., 2000; Yu et al., 2001).

Pitx2 has also been proposed to regulate members of various signaling pathways, including the Wnt pathway in the eye and pituitary, and FGF8 and BMP4 in the developing tooth (Gage et al., 2008; Liu et al., 2003; Lu et al., 1999; Vadlamudi et al., 2005). Although *Pitx2* has been shown to act primarily as a transcriptional activator, it has been demonstrated to inhibit the expression of the osteogenic transcription factor *Osterix* in cultured muscle cells (Hayashi et al., 2008). The function of *Pitx2* in inducing histone modifications has not been well studied, but it has been shown to increase histone acetlyation in smooth muscle differentiation (Shang et al., 2008). *Pitx2* has also been shown to play a role in inhibiting cell death and promoting proliferation by the activation

of cyclins (Charles et al., 2005; Kioussi et al., 2002; Rodriguez-Leon et al., 2008). It has been reported to regulate cell-cell adhesions and the remodeling of the extracellular matrix during the process of gut looping (Kurpios et al., 2008). *Pitx2* is also required for normal cell migration in the developing brain, heart and branchial arches, although it is unclear whether *Pitx2* is playing a role in cell movements or chemotaxis (Liu et al., 2002; Liu et al., 2003; Skidmore et al., 2008). *Pitx2* has been proposed to activate a large variety of cellular functions and it may be influencing any or all of these functions in eye development.

Transcriptional Targets of *Pitx2*

Since *Pitx2* encodes a transcription factor, the majority of its functions are presumed to involve binding DNA and activating (or inhibiting) transcription. The homeodomain of *Pitx2* has functional homology to that of the Drosophila homeobox gene *bicoid*; it has the characteristic lysine residue at the ninth amino acid position in the third helix (Hanes and Brent, 1989; Semina et al., 1996). Other mammalian *bicoid*-class transcription factors include *Pitx1*, *Pitx3*, *Otx1*, and *Otx2* (Semina et al., 1996; Simeone et al., 1993). PITX2 can bind the characteristic bicoid binding site TAATCC and activate transcription (Amendt et al., 1998; Driever and Nusslein-Volhard, 1989). Until recently, *Pitx2* target genes had been identified by searching the proximal promoters of likely candidates for *bicoid*-like sites. The only proven PITX2 binding sites have identified in this manner and the TRANSFAC matrix used in the computational analysis is based on these sites and is therefore biased towards sites with high homology to the bicoid site (Amendt et al., 1998; Hjalt et al., 2001; Vadlamudi et al., 2005). Recently, a large scale, non-biased screen for mammalian homeodomain transcription factor binding sites was conducted. While it identified TAATCC as the ideal binding site for PITX2, it identified many other binding sites. These were used create a new matrix that will facilitate more unbiased identification of potential binding sites (Berger et al., 2008). A complicating factor in the *in silico* identification of binding sites is the fact that PITX2 has been shown to cooperate with other transcription factors, but how this affects its binding site and/or specificity is unknown (Amendt et al., 1998; Schubert et al., 2004; Toro et al., 2004).

Until recently, only two direct transcriptional targets of PITX2 had been identified in the eye. *Plod-1* and *Plod2* are both procollagen lysyl hyroxylases that are important for collagen cross-linking. Collagen fibrils are found in large numbers in the cornea and sclera and *Plod-1* and *Plod2* are expressed in the embryonic eye. The genes were identified from a library of sequences obtained from chromatin precipitation of PITX2 and e14 mouse head DNA. PITX2 can bind the bicoid-like sequences in the proximal promoters of *Plod-1* and *Plod-2* both *in vitro* and *in vivo* and it can activate transcription (Hjalt et al., 2001). In humans, mutations in *PLOD-1* and *PLOD-2* cause Ehlers-Danlos syndrome and Bruck Syndrome respectively, both of which cause primarily skeletal defects, but abnormalities of the cornea and sclera are commonly reported (Durham, 1953; Salavoura et al., 2006; Sharma and Anand, 1964; van der Slot et al., 2003). These phenotypes are consistent with potential regulation by *PITX2* in the eye.

A newly identified transcriptional target of *Pitx2* in the eye is a secreted inhibitor of Wnt/β-catenin signaling, *Dkk2*. *Dkk2* is expressed in the periocular mesenchyme beginning at e11.5, and is lost in *Pitx2* global and neural crest-specific knockout mice. PITX2 binds the *Dkk2* promoter *in vivo* and can trans-activate it *in vitro* (Gage et al., 2008). Although the PITX2-responsive sequence(s) in the *Dkk2* promoter have yet to be identified, they are not homologous to bicoid sites (Philip Gage, Min Qian, and Chen Kuang, personal communication). Mice with a loss of *Dkk2* function have eyelid closure defects and conjunctivalization and vascularization of the cornea (Gage et al., 2008). Humans with mutations in *Dkk2* have not yet been identified.

Extraocular Muscles

The extraocular muscles are an additional tissue derived from the periocular mesenchyme that is critical for vision and requires *Pitx2* for its development. The extraocular muscles (EOMs) move the eyes within the orbit, enabling a whole new array of visual functions; tracking objects, reading text, and seeing in three dimensions (stereovision). In humans there are six extraocular muscles: four rectus muscles that move the eye side-to-side and up and down, and two oblique muscles that enhance rotational motion (Figure 1.3D, E). These muscles are innervated by the cranial nerves;

the superior, inferior, and medial rectus muscles, as well as the inferior oblique, are innervated by cranial nerve III, the superior oblique by cranial nerve IV, and the lateral rectus by cranial nerve VI. Mice have an additional extraocular muscle, the retractor bulbus, which serves to retract the globe deeper into the orbit as part of a protective reflex (Noden and Francis-West, 2006). The presence of extraocular muscles is conserved through all vertebrates, gnathasomes (lamprey), and even cephalopods (octopus) (Budelmann and Young, 1993; Fritzsch et al., 1990). Some species have added accessory EOMs or adapted them to serve other purposes. These include some cold-water fish that have adapted the lateral rectus as a heat generating tissue, and frogs, which have co-opted the retractor bulbi to aid in swallowing (Block, 1994; Levine et al., 2004).

The demands of ocular motility are extreme and the extraocular muscles have evolved to meet them. Reflexive oculomotor control is required to stabilize an image on the retina to prevent blur and double-vision, plus higher vertebrates have added visual targeting (saccade) and vergence movements, so the neural system controlling ocular movements is complex. In saccadic eye movements, the neuronal input to the EOMs is substantial and rapid. The EOMs respond by moving the eye at speeds of up to 600˚/s. Because ocular movements occur almost constantly, and even during sleep, the EOMs must be extremely fatigue resistant as well. To achieve these functions, EOMs have used the full array of traits available to adult skeletal muscle and borrowed some from cardiac and embryonic skeletal muscle (Spencer and Porter, 2006).

Even the connective tissue of the eye orbit facilitates the functions of the extraocular muscles. The extraocular muscles function as part of a pulley system with the orbital connective tissue to mediate eye movements. Fibroelastic sleeves, or pulleys, anchor the EOMs to a fixed point in the orbit, acting as an inflection point to guide the movement of the globe (Clark et al., 1997; Demer et al., 1995; Kono et al., 2002; Porter et al., 1996). The recently proposed "active pulley hypothesis" suggests that the smooth muscle found in the orbit may activate movements of the pulleys that impact EOM forces on the globe, helping to make the neural inputs to initiate movement independent of the initial eye position (Clark et al., 2000; Demer et al., 2000). The mechanisms of activation

for this smooth muscle and its functions are still unknown, and the hypothesis remains controversial (Demer et al., 1997).

Extraocular Muscle Fibers Are Unique

The extraocular muscles are highly specialized in order to meet unique functional demands. The multinucleated muscle fibers found in EOMs do not fit into any of the classifications used for other skeletal muscles. They are also polarized; there are different fiber types on the side of the muscle facing the eye and optic nerve (global) than on the side facing the orbit (orbital). The fibers on the orbital side are much smaller in diameter than those on the global side. The orbital layer fibers have more mitochondria, more extensive microvasculature and have higher levels of oxidative enzyme activity, reflecting their fatigue resistance (Spencer and Porter, 2006). The global layer fibers retain expression of developmental markers like embryonic myosin heavy chain (Myh3), NCAM and acetylcholine receptor γ (Brueckner et al., 1996; Kaminski et al., 1996; McLoon and Wirtschafter, 1996).

There are two categories of muscle fibers in the extraocular muscles: singly innervated fibers (SIFs) and multiply innervated fibers (MIFs). SIF fiber types are similar to other skeletal muscle fast-twitch fiber types, but they contain very little glycogen, while the MIF fiber types are atypical compared to other slow fibers. MIF fibers are innervated by a separate population of motor neurons than SIF fibers, raising the possibility that these nerves might have special properties as well (Buttner-Ennever et al., 2001). There are six fiber types: two orbital (one SIF, one MIF) and four global (three SIF, one MIF). The orbital SIF type makes up 80% of the orbital layer. It has small myofibrils containing Mhy3 and Mhy13 with large numbers of mitochondria surrounded by extensive sarcoplasmic reticulum, and high lipid content compared to skeletal fibers. The orbital MIF type has structural variation along its length corresponding to the multiple sites of innervation—the center is moderately fast-twitch, while the distal and proximal ends exhibit characteristics of slowly contracting fibers. This type is unlike any other muscle fiber in the body and it is not known how its unique characteristics affect its

function. The three global SIF types, red, intermediate, and pale, differ in terms of their myosin heavy chain expression, fiber size, sarcoplasmic reticulum content, fatigue-resistance and mitochondrial number and organization. The global MIF fiber type has very large myofibrils containing slow and cardiac myosin heavy chain isoforms, very few mitochondria, and scant sarcoplasmic reticulum. It exhibits a slow, graded, non-propagating contraction unlike any other skeletal muscle fiber type. The four global fiber types are found in approximately equal proportions (Spencer and Porter, 2006).

In order to carry out the rapid, highly precise movements needed for proper visual function, the motor units, the number of myofibers innervated by a single motor neuron, are very small in EOMs as compared to other skeletal muscles. They have very short contraction and relaxation times compared to other fast skeletal muscles and require less nerve stimulation (Spencer and Porter, 2006). This speed is enabled by faster calcium transients due to the extensive sarcoplasmic reticulum and novel calcium reuptake mechanisms, as well as differences in contractile kinetics due to the EOM-specific myosin heavy chain isoform *Myh13* (Asmussen and Gaunitz, 1981; Briggs et al., 1988; Jacoby and Ko, 1993; Kjellgren et al., 2003; Shrager et al., 2000). These adaptations are possible in part because the globe of the eye is a small, unchanging load, so the EOMs can further specialize compared to other skeletal muscles since the force needed is constant (Spencer and Porter, 2006).

Given their specialization, it is unsurprising that the extraocular muscles have distinct gene expression profiles from other skeletal muscles, even from other specialized muscles like the masticatory muscles. The EOMs express virtually all forms of myosin heavy chain: adult skeletal (*Myh1*, *2*, *4*, *7*), developing (*Myh3*, *8*), cardiac (*Mhy6*) and EOM specific (*Myh13*) (Spencer and Porter, 2006). The analysis of the differentially expressed genes has identified key differences in muscle biology, including transcriptional regulation, sarcomeric organization, metabolism and immune response. For example, it was identified that EOMs do not depend on glycogen as an energy source, as was previously found by histochemical analysis, probably because the EOMs have such high glucose demands that storage as glycogen is inefficient (Khanna et al.,

2003). Similarly, transcriptional profiling showed that EOMs lacked M-line proteins that organize the myofilaments and link the contractile apparatus to the cytoskeleton. Analysis confirmed the loss of the M-line in EOMs, which represents another functional adaptation to the unique demands of ocular motion (Andrade et al., 2003; Porter et al., 2003a).

Extraocular Muscles in Disease

Dysfunction of the extraocular muscles can result in blurred or double vision (Kaminski et al., 2002). Numerous diseases, both developmental and adult-onset, include extraocular muscle pathologies. The most common, strabismus, colloquially known as "cross-eyes", occurs in up to 4% of children (Gronlund et al., 2006). Strabismus is a generally developmental defect that occurs when the six ocular muscles are not equally balanced in strength, causing the eye to turn in one direction. This misalignment prevents the eyes from focusing on a single point, so binocular vision cannot be achieved. If left untreated, the brain will ignore the input from the misaligned eye and blindness can occur. Causes for the imbalance include neural, innervation and muscular defects, but it indicates that EOM development must be tightly regulated to ensure equal strength of the EOMs. Strabismus can also be caused by abnormalities of the pulley system (Oh et al., 2002). More severe defects in EOM innervation result in the Congenital Craniofacial Dysinnervation Disorders (or CCDDs), which cause a default downward gaze or absence of vertical or horizontal eye movements. These disorders occur when the cranial nerves fail to properly innervate the EOMs; in the absence of nerve input the EOMs develop abnormally, often with fibrosis, immobility and aberrant innervation (Engle, 2006; Spencer and Porter, 2006). Mice lacking EOM innervation form normal extraocular primordia up through e14.5, but in the later phases of development the primary fibers degenerate and secondary fibers fail to form, which may help explain this phenotype (Porter and Baker, 1997). Rarely, congenital absence of one or more of the extraocular muscles has been reported (Astle et al., 2003; Chan and Demer, 1999; Drummond and Keech, 1989; Greenberg and Pollard, 1998; Hart et al., 2005; Kolling, 1999; Mather and Saunders, 1987; Taylor and Kraft, 1997).

Adult-onset diseases involving the extraocular muscles are often autoimmune in nature. In myasthenia gravis, an autoimmune disease that attacks neuromuscular junctions, the extraocular muscles are often the first muscles affected (Spencer and Porter, 2006). This may be due to the fact that EOMs express a different acetylcholine receptor isoform than other skeletal muscles (Kaminski et al., 1996; Kaminski and Ruff, 1997; Missias et al., 1996). Graves disease, an autoimmune-induced form of hyperthyroidism, also causes extraocular muscular dysfunction. The EOMs become enlarged due to the abnormal accumulation of glycosaminoglycans in the connective tissue of the orbit. The EOM fibers are normal, but the swelling causes difficulty in eye movements and causes the eye to protrude from the orbit (Porter et al., 1995). It is now thought that the eye phenotype is primarily autoimmune in nature, rather than simply a response to the increased levels of circulating thyroid hormone (Kaminski et al., 2002; Porter et al., 1995). Recently, EOMs were found to express lower levels of genes that inhibit the complement pathway of the immune system, which may partially explain their susceptibility to diseases like myasthenia gravis and Graves disease (Kaminski et al., 2002; Porter et al., 2001). Extraocular muscle is also a primary tissue affected in some mitochondrial myopathies such as chronic progressive external ophthalmopathy and Kearns-Sayre syndrome, in which mitochondrial dysfunction leads to muscle weakness. The EOMs may be sensitive to mitochondrial defects due to their reliance on oxidative energy metabolism, which produces large amount of reactive oxygen species. Although the EOMs express large amounts of antioxidants, it may not be enough to last a lifetime, allowing damaged mitochondria to accumulate in old age (Spencer and Porter, 2006).

Interestingly, the extraocular muscles are spared in many forms of muscular dystrophy, including the most common form, Duchenne's Muscular Dystrophy (DMD), which results from mutations in the cytoskeletal protein, dystrophin. DMD is characterized by disruption of the dystrophin-glycoprotein complex, which links the muscle fiber cytoskeleton to its plasma membrane, the sarcolemma. This disruption allows calcium to leak into the cells, causing changes in calcium homeostasis that eventually lead to muscle damage, followed by repair by satellite cells, the muscle stem cells. Eventually, the satellite cell population is exhausted by the damage and repair

cycle, and inflexible scar tissue takes the place of muscle. Proposed mechanisms for EOM sparing include a larger population of satellite cells, enhanced calcium homeostasis, increased protection from reactive oxygen species and higher levels of proteins like utrophin, which can compensate for dystrophin (Andrade et al., 2000; Porter, 1998).

Animal models of muscular dystrophy like the *Mdx* mouse have been invaluable in evaluating these hypotheses. The extraocular muscles of *Mdx* mice do not degenerate and they have no muscle fibers with central nuclei, a hallmark of recently regenerated muscle, indicating that the large satellite cell population plays no part in EOM sparing (Karpati et al., 1988; Porter et al., 1995). The *Mdx* mice also have reduced levels of the antioxidant nitric oxide synthase in the EOMs as well as other skeletal muscles, which makes the reactive oxygen species protection hypothesis less attractive (Kaminski and Andrade, 2001; Porter et al., 2003b; Wehling et al., 1998). Increased levels of utrophin do seem to play a role; in dystrophin-utrophin double-knockout mice, the EOMs do show degeneration, but only in 3 of the 6 fiber types, indicating that other protective mechanisms are at work (Porter et al., 1998). Calcium homeostasis and improved sarcolemmal integrity may play a role. In *Mdx* mice, EOM fibers with disrupted dystrophin-glycoprotein complexes did not have alterations in their sarcolemma or calcium levels, unlike affected muscles (Porter et al., 2003b). Genome-wide transcription profiling of EOMs from wildtype and *Mdx* mice showed almost no differences in expression, indicating that the protective mechanisms used by EOMs are constitutive properties, not adaptations (Porter et al., 2003b). Interestingly, the extraocular muscles are specifically affected in oculopharyngeal muscular dystrophy, which is caused by mutations in PABPN1, a poly-A binding protein that is a component of filamentous nuclear inclusions. The mechanisms of disease pathogenesis are unclear, but it seems fundamentally different from other forms of muscular dystrophy, which are generally caused by defects in specialized muscle proteins like those of the dystrophin-glycoprotein complex (Brais, 2009; Spencer and Porter, 2006).

Skeletal Muscle Development

With all of the differences between extraocular muscles and other skeletal muscles, it is not unexpected that their development is unique as well. In order to understand how EOM development is unique, it is helpful to first understand the development of the other skeletal muscle populations. The muscles and tendons of the trunk and limbs are derived from somites, regularly spaced and sized epithelialized condensations of paraxial mesoderm that form on either side of the neural tube during development. The homeobox transcription factor *Pax3* is expressed in the paraxial mesoderm prior to somite formation, as well as the newly formed somites. The homologous gene, *Pax7*, is also expressed in part of the epithelial somite (Buckingham and Relaix, 2007). PAX3 and PAX7 are equivalent in the majority of their functions in myogenesis (Relaix et al., 2006; Relaix et al., 2004). The cells of the somites then delaminate and begin the process of cell fate determination; cells that enter non-myogenic lineages quickly down-regulate the expression of *Pax3*/*Pax7*.

Cells commit to the myogenic lineage by activating a group of basic helix-loop-helix transcription factors known as the muscle regulatory factors (MRFs) because they can convert other cell types to a skeletal muscle phenotype (Braun et al., 1989a; Braun et al., 1989b; Edmondson and Olson, 1989; Thayer et al., 1989; Weintraub et al., 1989). The MRFs are 4 homologous genes that were generated by two separate duplication events over the course of evolution. *Myf5* lies just upstream of *Mrf4* (*Myf6*), while their respective homologues, *MyoD* (*Myod1*) and *Myogenin*, have moved to different chromosomes (Atchley et al., 1994; Atsushi and Rudnicki, 2002). The MRFs have complex and highly overlapping functions and expression patterns. They are co-expressed in some, but not all cells, knockout mice for any single gene generally have subtle muscle phenotypes, and many of them can activate each other's expression (Bryson-Richardson and Currie, 2008). *Myf5*, *MyoD*, and to a lesser extent, *Mrf4*, are required for myogenic specification, i.e. commitment to the muscle lineage. *Myogenin*, *Mrf4*, and *MyoD* are subsequently required for differentiation, i.e. the expression of genes required for muscle cell function, such as the myosins. The expression of *Myogenin*

marks the transition of cells from proliferating myoblasts to myocytes, which are post-mitotic mononuclear cells that express skeletal muscle specific markers. As differentiation proceeds, the myocytes will fuse to form multinucleated myotubes, which coalesce to form myofibers (Noden and Francis-West, 2006).

PAX3/PAX7 can directly activate the expression of *Myf5* and *MyoD* (Bajard et al., 2006; Hu et al., 2008), although cells generally downregulate *Pax3*/*Pax7* expression once they activate the myogenic program (Relaix et al., 2005). *Pax3*/*Pax7* are not absolutely required for the expression of *Myf5* and *MyoD*, but they do drive the majority of the expression (Buckingham et al., 2006; Relaix et al., 2005). *Pax3*/*Pax7* are also required for the specification and function of satellite cells, the stem cells found in mature muscle, but only *Pax7* is able to ensure satellite cell survival (Kuang et al., 2006; Relaix et al., 2006). *Pax3*/*Pax7* are generally required for muscle precursor survival and the vast majority of proliferating muscle precursors express PAX3/PAX7 rather than the MRFs (Kuang et al., 2006; Relaix et al., 2006).

Cranial Muscle Development

The muscles of the head are derived from the paraxial and prechordal mesoderm, similar to other skeletal muscles, although the mesoderm is unsegmented. The MRFs *Myf5*, *MyoD*, and *Myogenin* also function as activators of myogenic specification and differentiation, although *Mrf4* is absent in the head (Haldar et al., 2008; Noden and Francis-West, 2006). Transplantation experiments in chick have also shown that grafts of early, presomitic trunk mesoderm or newly formed somites that are transplanted into the head can contribute to normal EOM and branchiomeric muscles with normal gene expression. (Borue and Noden, 2004). The converse grafting experiment moving cranial mesoderm into the trunk found that cranial mesoderm can contribute to trunk but not limb muscles (Noden and Francis-West, 2006). There are, however, two major differences between cranial and somitic myogenesis. The first is that the signals and transcription factors upstream of the MRFs are distinct between the two muscle populations. The

second is that the tendons of the cranial muscles are derived from the neural crest lineage, and neural crest-mesoderm interactions influence the development of the cranial muscles.

There are many lines of evidence that indicate the activation of the myogenic program is different in head versus trunk muscles. The signals activating myogenesis differ: *Sonic Hedgehog* and canonical Wnt signaling are critical for trunk myogenesis, but *Shh* is dispensable in the head and Wnt signaling actually represses cranial myogenesis (Borycki et al., 2000; Kruger et al., 2001; McDermott et al., 2005; Munsterberg et al., 1995; Tajbakhsh et al., 1998; Teillet et al., 1998; Tzahor et al., 2003). The transcription factors that activate myogenesis are also different between the head and the trunk. While the *Pax* genes are critical MRF activators in trunk muscle development, *Pax3* is not expressed in the head, and *Pax7* is not expressed in the branchial arches until after the MRFs (Horst et al., 2006). Head muscles are reported to form normally in the *Pax7* knockout mice, although a detailed examination was not performed (Relaix et al., 2004). In the absence of *Myf5*, *MyoD* expression is delayed in the trunk, but not the head, further indicating the MRFs are activated by different mechanisms (Tajbakhsh et al., 1998; Tajbakhsh et al., 1997). Consistent with this, separate trunk and branchial arch enhancer regions have been identified for *Myf5* and *MyoD*, although no EOM specific enhancers have been identified (Hadchouel et al., 2003; Kucharczuk et al., 1999; Summerbell et al., 2000).

Several genes that regulate branchial arch (BA) myogenesis have been identified. *Tbx1*, *Musculin* (*MyoR*), and *Tcf21* (*Capsulin*) are required for myogenesis upstream of the MRFs in the branchial arches (Grenier et al., 2009; Kelly et al., 2004; Lu et al., 2002; Robb et al., 1998). *Tbx1* has been proposed to regulate *Myf5* and *MyoD* expression directly (Dastjerdi et al., 2007; Grifone et al., 2008; Kelly et al., 2004). However, none of these genes are necessary for EOM development, although *Tbx1* and *Musculin* are expressed, indicating that the program for extraocular muscle development is distinct (Grenier et al., 2009; Kelly et al., 2004; Lu et al., 2002; Robb et al., 1998). No direct regulators of MRF expression have been identified for the EOMs. *Pax7* has been proposed to serve this function in the extraocular muscle primordia, but a functional role

has not been demonstrated (Mootoosamy and Dietrich, 2002). *Pitx2* is required for the formation of the extraocular muscles, as well as the muscles of the first branchial arch, in a dose-dependant manner, and it has also been proposed to activate the MRFs directly (Diehl et al., 2006; Dong et al., 2006; Gage et al., 1999; Kitamura et al., 1999; Liu et al., 2003; Lu et al., 1999; Shih et al., 2007a). The genes which regulate MRF expression in the branchiomeric and extraocular muscles are clearly distinct from those of somitic muscles, but more work needs to be done to functionally demonstrate MRF activation.

The other major difference between cranial and trunk muscles are the mechanisms underlying the formation of tendons and connective tissue. In the trunk, tendons are formed from the somitic mesoderm and require the transcription factor *Scleraxis*. In the head, tendons are neural crest derived, and although *Scleraxis* expression is found in the extraocular and branchiomeric tendons beginning at e12.5, it is not required for their formation (Grenier et al., 2009; Grifone et al., 2008; Murchison et al., 2007; Pryce et al., 2007). Expression of *Scleraxis* initiates in the absence of branchiomeric muscle, but is not maintained, indicating that signals from the developing muscle are required for neural crest cells to form tendon (Grenier et al., 2009).

The neural crest cells also pattern the cranial muscle primordia during development, which does not occur in trunk myogenesis. In the branchial arches, the neural crest cells surround the developing mesoderm, and mouse mutants with neural crest migration defects have marked defects in the myogenic specification of the branchiomeric muscles (Rinon et al., 2007). The extraocular muscles do not establish contact with the neural crest until relatively later than the branchial arches, at approximately e10.5 in mouse (Gage et al., 2005; Noden and Trainor, 2005). Mice with defects in neural crest development have varying phenotypes. In *Twist1* mutant mice, most of the periocular neural crest migrate to the correct location and these mice have no extraocular muscle phenotype (Rinon et al., 2007). Mice with constitutively activated β-catenin in the neural crest have defects in the migration of portions of the periocular neural crest and these mice lack expression of *Myf5*, *MyoD*, and *Myogenin* in the extraocular muscle primordia (Rinon et al., 2007). The number of extraocular muscle

precursor cells expressing *Pitx2* is reduced in these mice, suggesting the neural crest may influence mesoderm proliferation or survival (ALZ, unpublished observations). However, it is unclear whether this phenotype is caused by reduced interactions of the mesoderm with the neural crest or abnormal interactions with the neural crest cells that are present, due to their activated canonical Wnt signaling. These findings underscore the importance of neural crest-mesoderm interactions in EOM development.

Pitx2 in Skeletal Muscle Development

Pitx2 is expressed in the developing extraocular muscles as well as the branchial arches and somites. It is expressed in almost all somite-derived musculature during development (Shih et al., 2007b). While *Pitx2* is expressed prior to markers of muscle specification in the EOM and BA muscle precursors, it lags behind the expression of *Pax3* and the MRFs in the somites (L'Honore et al., 2007; Shih et al., 2007b). In the somitic muscles, PITX2 expression co-localizes with PAX3, PAX7, MYOD, and MYOG, as well as proliferation markers BrdU and Ki67, but less with differentiation markers MF20 and α-actin, indicating it marks muscle precursors and progenitors (L'Honore et al., 2007). *In vitro* culture experiments with limb-derived myoblasts indicate that PITX2 promotes proliferation by regulating cyclin activity and may inhibit terminal differentiation (Kioussi et al., 2002; Martinez-Fernandez et al., 2006). While *Pitx2* is required for the formation of extraocular muscles and the mastication muscles of the first branchial arch, a function in the development of the trunk and limb muscles has not been described. The lack of a somitic muscle phenotype in the *Pitx2^{null}* mice may be due to compensation by the other *Pitx* genes, *Pitx1* and *Pitx3*, which have overlapping expression patterns in the skeletal muscle (L'Honore et al., 2007; Lanctot et al., 1997; Shang et al., 1997).

Functions of *Pitx2* in cranial myogenesis

In branchial arch development, only the 1st branchial arch requires *Pitx2* expression for it development, even though *Pitx2* is expressed in arches I-IV (Dong et al.,

2006; Lu et al., 1999; Shih et al., 2007a, b). *Pitx1* and *Pitx3* are expressed in the 1st arch, but are apparently unable to compensate for the loss of *Pitx2* (L'Honore et al., 2007; Lanctot et al., 1997). Expression of the branchiomeric muscle specification genes *Tbx1* and *Musculin* were shown to be lost at e9.5 (Dong et al., 2006; Shih et al., 2007a). In the reverse of their relationship in cardiac development, PITX2 was shown to directly activate *Tbx1* expression (Shih et al., 2007a). At the same time, massive cell death occurs in the mesodermal core of the first arch beginning at e9.5, such that it is almost completely gone by e10.5 (Dong et al., 2006; Shih et al., 2007a). Myogenesis is not observed and mesoderm specific deletions of *Pitx2* demonstrate that the defect is cell autonomous (Dong et al., 2006). The requirement for *Pitx2* is also dose sensitive; *Pitx2^{neo/null}* mice with approximately 20% normal PITX2 levels have reduced *Myogenin* expression in the first branchial arch and no masseter muscles (Dong et al., 2006).

Extraocular muscle formation is also dependent on *Pitx2* dose. EOMs are reduced in *Pitx2^{+/-}* mice with 50% wildtype *Pitx2* dose, and completely absent in *Pitx2^{neo/null}* mice with 20% dose. The expression of the MRFs and other muscle transcription factors is also dependent on *Pitx2* dose. *Myf5*, *MyoD*, and *Myogenin* are reduced to 10%-20% of their wildtype expression level in *Pitx2* heterozygotes and are effectively absent in *Pitx2* null mice by qRT-PCR (Figure 1.5). *Musculin, Smyd1* and *Csrp3* are other muscle transcription factors affected by loss of *Pitx2*. *Pitx2* was proposed to be a direct regulator of these muscle transcription factors, including the MRFs. The expression of myosin heavy chain is also *Pitx2* dose dependent, as are many other muscle proteins, indicating differentiation is reduced as well as the muscle size. No changes were seen in cell death or proliferation in the EOMs at e12.5 and e14.5 (Diehl et al., 2006). In *Pitx2^{null}* mice labeled with a *Myf5-Cre*, it was observed that *Myf5*-labeled EOM precursor cells were no longer present in their normal location, just dorsal to the eye, while an abnormal mass of *Myf5*-labeled cells was later seen in the frontonasal region. It is unclear if these cells represent a mislocalization of the EOM primordia (Dong et al., 2006). The expression of *Pitx2* was disrupted in the neural crest as well as the mesoderm in all of these experiments, so it is unclear what role *Pitx2* expression in the neural crest cells might have on the developing extraocular muscle primordia.

In addition to *Pitx2*, the only genes demonstrated to regulate extraocular muscle myogenesis are the MRFs, *Myf5* and *MyoD*, which are redundantly required for myoblast specification. The double knockout mice lack any differentiated muscles in the head or trunk (Kablar et al., 2003; Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). *Six1/Six4* double knockout mice have EOMs that are present but reduced in size, indicating they are not required (Grifone et al., 2005). *Eya2* and *Dach2* are transcription factors expressed in the developing EOM primordia, but mutant mice for both are reported to have no eye phenotype, although whether the EOMs were examined was not specifically mentioned (Davis et al., 2008; Davis et al., 2001; Grifone et al., 2007). The absence of other genetic regulators besides the MRFs further emphasizes the critical role of *Pitx2* in extraocular myogenesis.

Pitx2 expression in the extraocular muscles is maintained through adulthood in both myonuclei and satellite cells in all EOM fiber types (Zhou et al., 2009). Other myogenic transcription factors are not expressed in differentiated muscle fibers, and *Pitx2* expression is absent from other muscles in adulthood, which further indicates it may have unique functions in the extraocular muscles (L'Honore et al., 2007; Relaix et al., 2006). Recently, an adult skeletal muscle knockout of *Pitx2* was reported, in which *Pitx2* expression was removed beginning at post-natal day zero. The EOMs of these mice display reduced fiber size, and increased force, contractile speed and fatigability. Many forms of myosin heavy chain were reduced in expression levels, particularly the extraocular muscle specific isoform, *Myh13*, which may explain the changes in contractile properties. Expression levels of *Myf5*, *MyoD*, and *Myogenin* were all highly down-regulated at all timepoints examined, while expression of the myosin proteins progressively decreased over time, leading the authors to propose that the MRFs are direct targets of PITX2, while the myosins are indirect targets (Zhou et al., 2009).

Thesis Aims

Pitx2 is an essential regulator of both anterior segment and extraocular muscle development in the eye, but the mechanisms underlying its function were not well

understood. *Pitx2* plays a variety of roles in many cell types and it was unclear whether *Pitx2* was involved in the regulation of proliferation, cell survival, cell fate specification or other processes in eye development. This issue is further complicated by the fact that *Pitx2* is expressed in two embryonic lineages in the eye, the mesoderm and the neural crest, which both make contributions to the anterior segment and extraocular muscles. It was unknown whether *Pitx2* has different functions in the neural crest and mesoderm, due to the unique mix of endogenous transcription factors in each lineage and their differential ability to respond to extracellular signals. It was also unknown how *Pitx2* expression in one lineage affects the function of the other lineage, particularly in the interactions between neural crest and mesoderm during extraocular muscle development. *Pitx2* was proposed to activate myogenesis in extraocular muscle development, but the underlying mechanism of this function was unclear.

To determine the lineage specific functions of Pitx2, we created both neural crest and mesoderm specific knockout mice using the Cre-lox system. In addition to identifying the mechanism of *Pitx2* function in the two lineages, we hoped to uncover new and potentially later functions in eye development, if the mice survived longer. Furthermore, these experiments allowed us to examine the role of *Pitx2* in the interactions between neural crest and mesoderm, especially during extraocular muscle development. In order to identify the upstream activator(s) of myogenesis in extraocular muscle development, we examined the functions of two proposed regulators of MRF expression, *Pax7* and *Pitx2*.
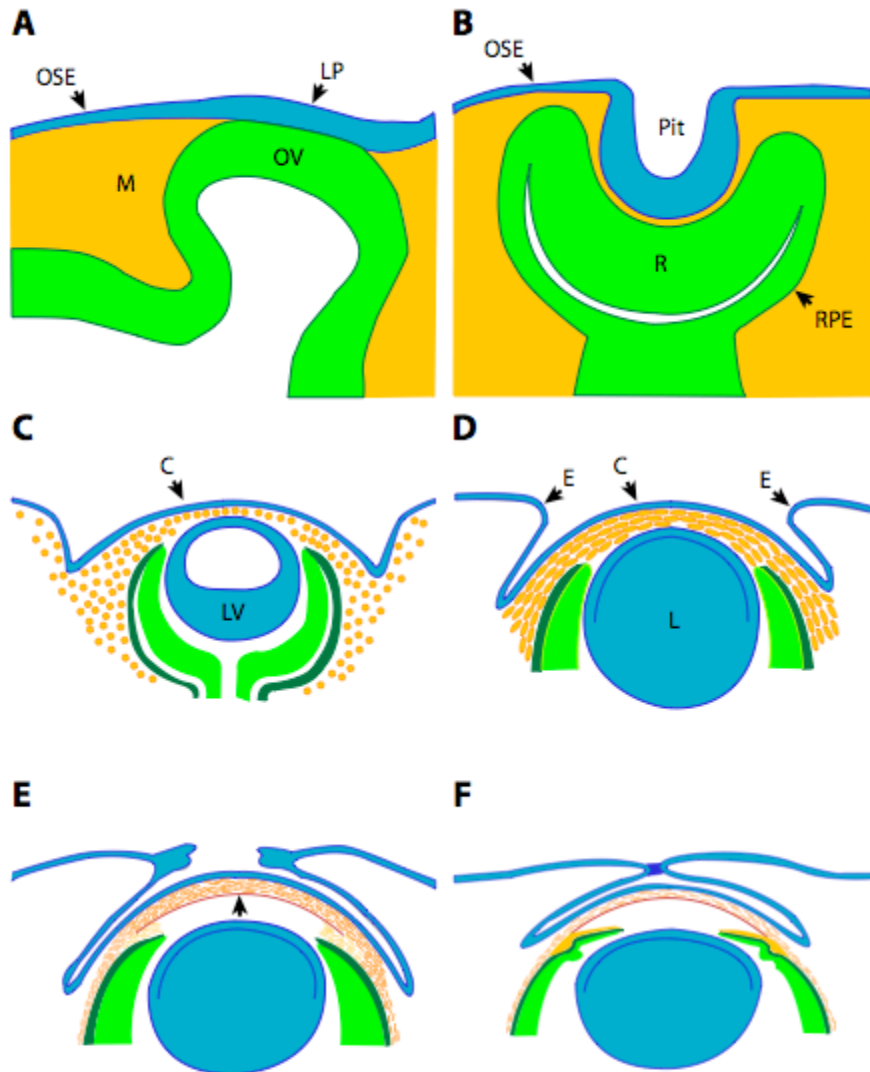
Figure 1.1: Morphogenesis of the developing eye. The eye develops from the ocular surface ectoderm (blue), neural ectoderm (green), and the periocular mesenchyme (orange). (**A**) At e9.5 the optic vesicle induces the formation of the lens placode from the ocular surface ectoderm. The cranial mesoderm is already present adjacent to the optic vesicle (**B**) At e10.0, the lens pit and optic vesicle invaginate to form the optic cup. The outer layer of the optic cup will form the RPE, while the inner layer forms the retina. At this point, neural crest cells begin to invade the eye field. (**C**) By e11.5, the lens vesicle has separated from the surface ectoderm, and a thin layer of loose mesenchymal cells has invaded the space between the lens and surface ectoderm. (**D**) At e13.5, signals from the lens induce the compaction of the mesenchymal cells that will form the corneal stroma and endothelim. The outgrowth of the eyelid primordia begins. (**E**) At e14.5, the corneal endothelium has formed (arrow), which separates the mesenchyme from the lens, creating the anterior chamber. (**F**) By e16.5, the iris has been colonized by mesenchymal stroma and the eyelids fuse closed. Key: C, cornea; E, eyelid; L, lens; LP, lens placode; M, mesoderm; OSE, ocular surface ectoderm; OV, optic vesicle; Pit, lens pit; R, retina; RPE, retinal pigmented epithelium. Adapted from Gage & Zacharias, 2009.
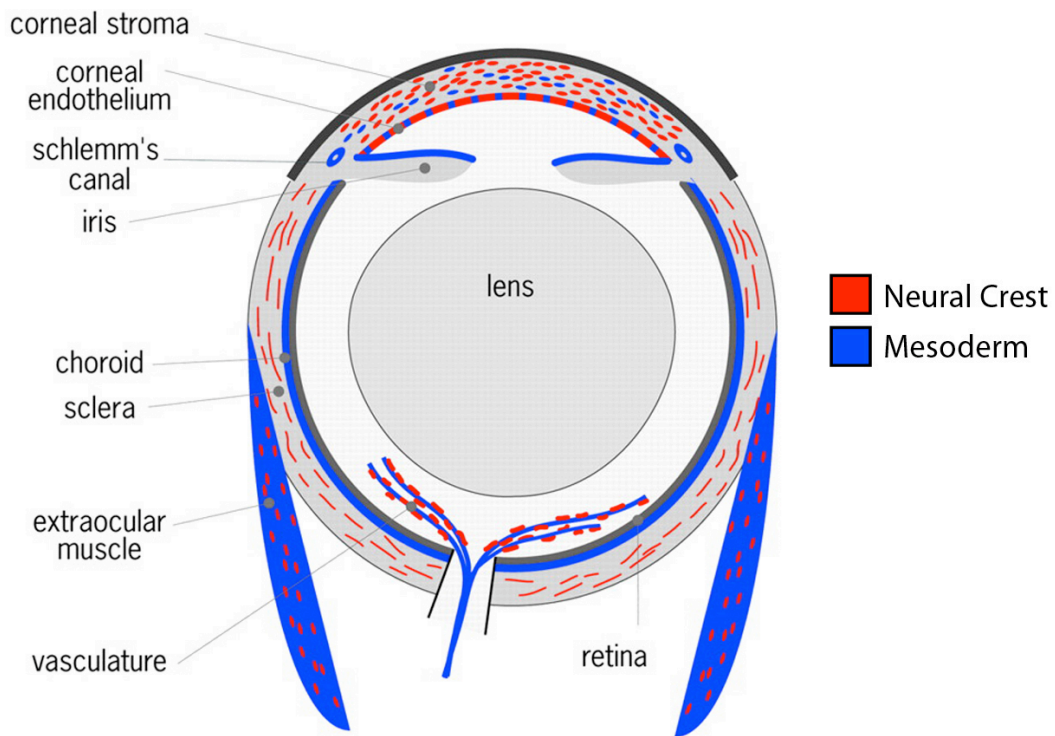
Figure 1.2: Fate map of the adult mouse eye. A structural diagram of the adult mouse eye showing critical features. Cells type derived from the neural crest lineage are shown in red. Cell types derived from the mesoderm lineage are shown in blue. Many tissues receive contributions from both cell types. Adapted from Gage et al., 2005.
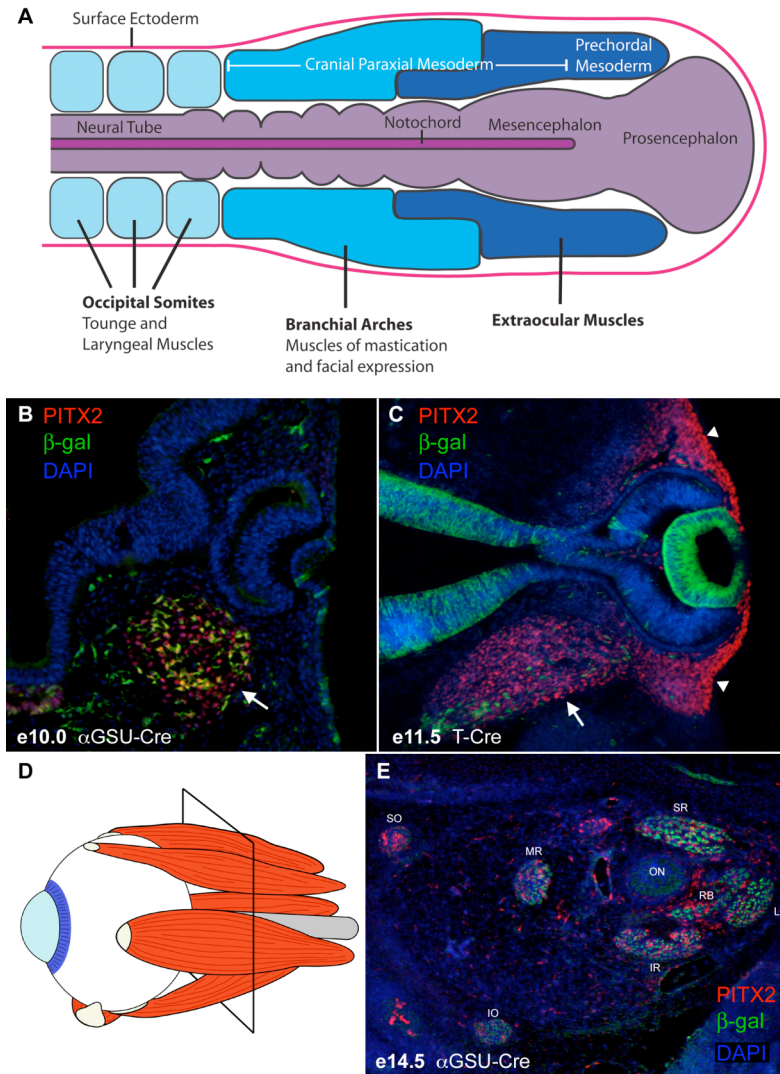
Figure 1.3: Morphogenetic movements of extraocular muscle precursor cells during development. The extraocular muscles derive from the most anterior portion of the mesoderm, based on fate mapping experiments performed in chick by Evans and Noden, 2006 (**A**). The extraocular muscles form from unsegmented prechordal and paraxial mesoderm. The more posterior cranial mesoderm cells invade the branchial arches at e9.0, while the extraocular precursors remain in the eye field (**B**). A loose collection of β-gal-labeled mesoderm cells (arrow) express PITX2 at e10.0, prior to the invasion of the neural crest cells (**B**). β-gal-labeled mesoderm cells expressing PITX2 (arrow) have condensed into a wedge at e11.5 (**C**). PITX2 is also expressed by the neural crest cells surrounding the optic cup (**C**, arrowheads). Ectopic transgene expression is seen in the lens, retina, and optic stalk. Later in development, the precursors separate into individual muscles, shown in red in the diagram (**D**). The optic nerve is shown in grey, and the plane of section seen in (**E**) is indicated. A sagittal section at e14.5 shows all seven extraocular muscles, which express PITX2 (**E**). SO, superior oblique; IO, inferior oblique; MR, medial rectus; IR, inferior oblique; SR, superior rectus; LR, lateral rectus; RB, retractor bulbus; ON, optic nerve. Embryos in A and C were genetically labeled with *αGSU-Cre*, B was labeled with *T-Cre*, see Chapter 3 for details.
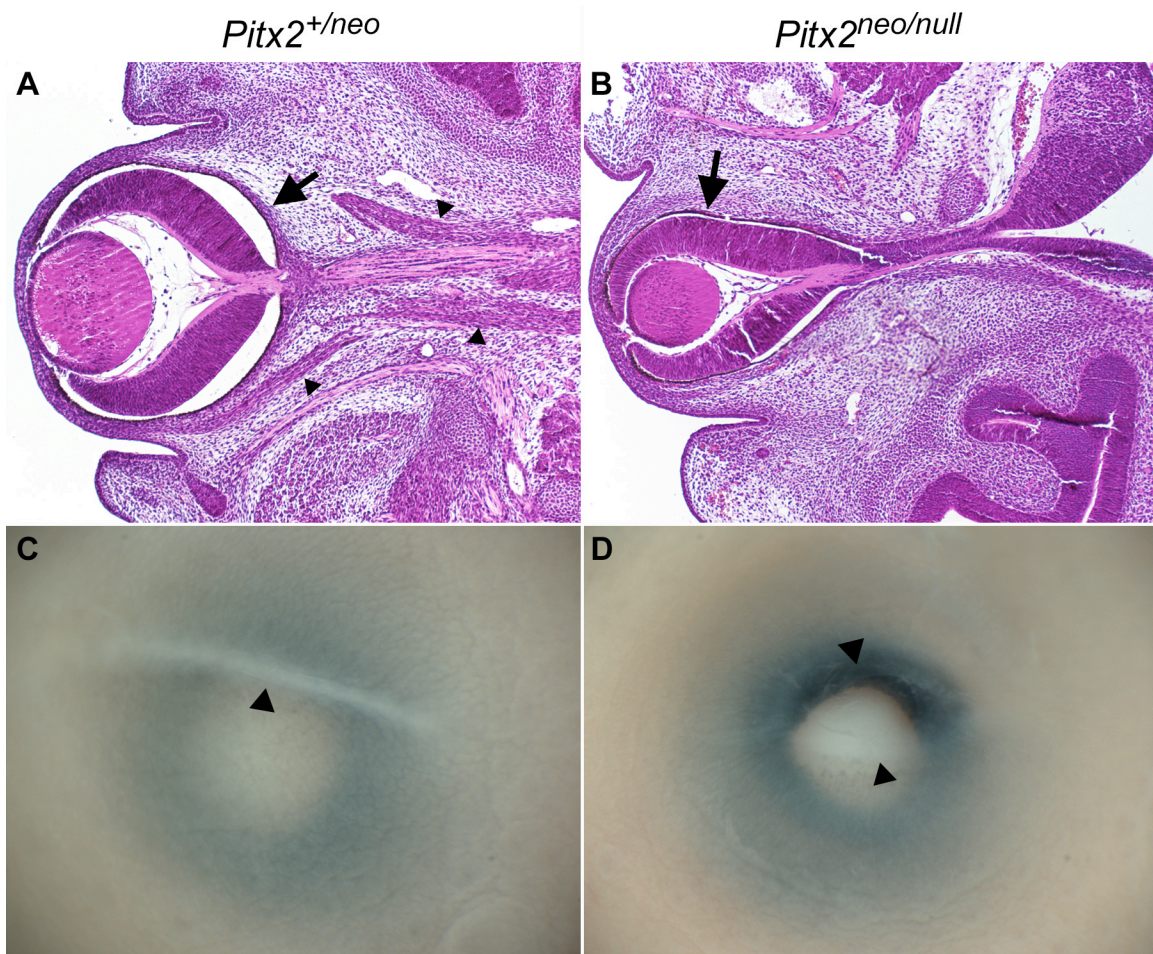
Figure 1.4: Ocular phenotype in mice with reduced *Pitx2* dose. At e14.5, mouse embryos with one copy of a reduced function *Pitx2^neo^* allele have a normal eye phenotype, with normal sclera formation (arrow) and extraocular muscles (arrowheads). (**A**). *Pitx2^neo/null^* embryos, which have approximately 20% of the *Pitx2* dose of a wildtype embryo, have absent extraocular muscles, and defects in sclera formation (arrow) which may cause the observed distortion in eye shape (**B**). At e16.5, the eyelids of a *Pitx2^+/neo^* embryo have closed normally and fused shut (arrowhead) (**C**). The eyelids of a *Pitx2^neo/null^* embryo remain open (arrowheads) (**D**). The open eyelids phenotype also occurs in *Pitx2^neo/neo^* embryos, which have approximately 40% wildtype *Pitx2* dose (data not shown). Adapted from the work of Adam Diehl.
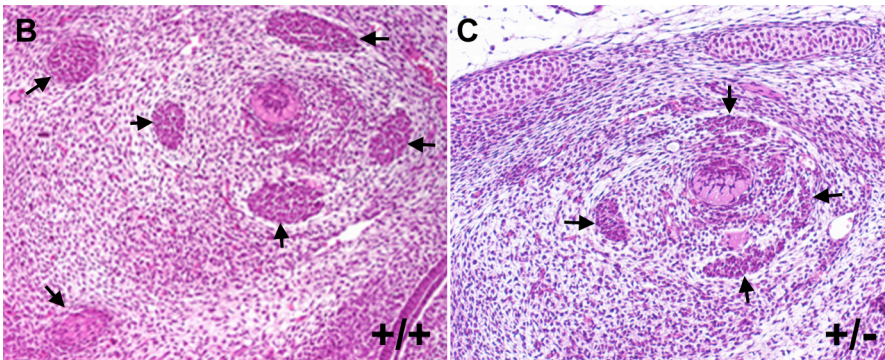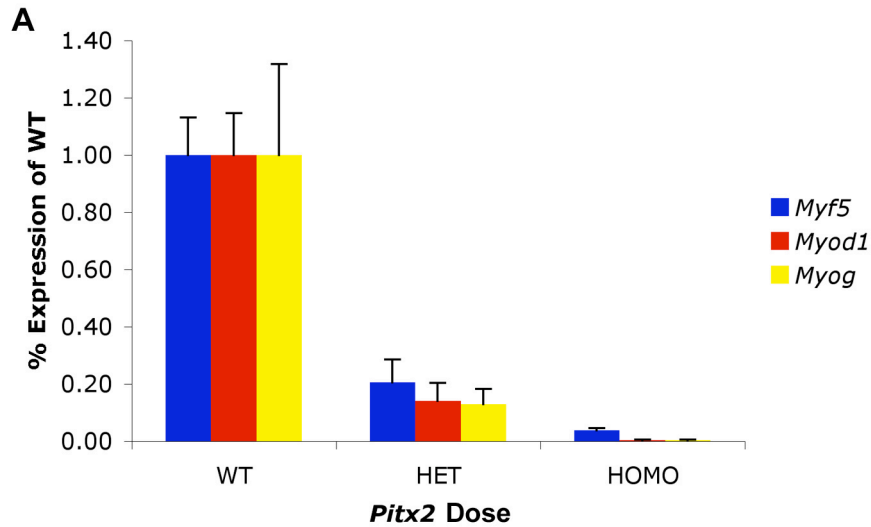
Figure 1.5: MRF expression is severely affected in the extraocular muscles of
Pitx2^null heterozygous embryos. The expression levels of the muscle regulatory factors
(MRFs) _Myf5_, _Myod1_, and _Myogenin_ are reduced to 10-20% of wildtype levels in _Pitx2^{+/-}_
heterozygous eye primordia, by quantitative RT-PCR (**A**). The reduction in MRF levels is
more severe than the reduction in the number of muscle cells (arrows) in the heterozotes
(**C**). In the _Pitx2_ heterozygotes (**C**), the two oblique muscles are absent and the four
rectus muscles are reduced in size (arrows), as compared to a wildtype embryo (**B**).
Adapted from Diehl et al., 2006.

Chapter 2: Expression of the homeobox gene *Pitx2* in neural crest is required for optic stalk and ocular anterior segment development.

Introduction

Glaucoma is a constellation of disease processes that result in vision loss due changes in the optic nerve head and accompanying death of retinal ganglion cells. Glaucoma often occurs in association with elevated intraocular pressure (IOP) due to physical or functional changes within the anterior segment of the eye, but its relationship to IOP is not absolute. The most common form of glaucoma in the United States is primary open angle glaucoma, in which elevated IOP is associated with optic nerve damage. In contrast, the most common form in Japan is normal tension glaucoma, in which IOP is unchanged, but optic nerve damage is associated with defects in blood flow (Araie et al., 1994). Additionally, elevated IOP does not always lead to glaucoma (Grodum et al., 2005). The underlying molecular mechanisms leading to initiation and progression of most forms of glaucoma are largely unknown.

Developmental glaucomas provide a unique entry into studying the disease; patients generally develop more severe glaucoma at younger ages, which facilitates the mapping of gene loci. In cases in which genetic mutations are identified, the study of animal models is possible. Heterozygous mutations in the human homeobox gene, *PITX2*, underlie Axenfeld-Rieger Syndrome (ARS) in the subset of patients mapping to chromosome 4q25 (Semina et al., 1996). This autosomal-dominant condition results in developmental defects within the ocular anterior segment in structures derived from the periocular mesenchyme, including the cornea, iris and outflow tract (Alward et al., 1998; Kulak et al., 1998; Noden, 1982; Semina et al., 1996; Shields, 1983). In addition, 50% of

affected individuals develop early onset glaucoma. *PITX2* encodes a homeodomain transcription factor that regulates expression of downstream target genes (Amendt et al., 1998; Charles et al., 2005; Cox et al., 2002; Ganga et al., 2003; Green et al., 2001; Hjalt et al., 2001; Suh et al., 2002; Vadlamudi et al., 2005). Interestingly, both gain- and loss-of-function mutations in PITX2 have been functionally identified (Brooks et al., 2004; Kozlowski and Walter, 2000; Perveen et al., 2000; Priston et al., 2001; Saadi et al., 2003; Saadi et al., 2001). Murine *Pitx2* is expressed throughout the periocular mesenchyme, including not only the structures of the anterior segment but also the sclera, ocular vasculature, and extraocular muscles (Gage et al., 2005; Semina et al., 1996). It is widely assumed that glaucoma in these patients is the result of elevated IOP due to the anterior segment defects (Shields, 1983). However, the underlying molecular mechanisms and the reason(s) why only 50% of affected individuals develop glaucoma are not known. Collectively, these observations suggest that more complex factors than simply elevated IOP contribute to the etiology of glaucoma in affected patients.

Animal models of glaucoma have been very powerful in identifying its underlying causes (Levkovitch-Verbin, 2004). Previously, an animal model of ARS was created by targeted deletion of *Pitx2* in mice in order to further analyze the functions of this important regulatory gene (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). These global knockout mice die at e14.5 of heart defects, but this model has revealed several important roles for *Pitx2* in early eye development. The eye defects include the agenesis of the corneal endothelium and stroma and loss of extraocular muscles, which derive from periocular mesenchyme (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). Additionally, development of the optic nerve from neural ectoderm is abnormal, despite the fact that *Pitx2* is not expressed there (Gage et al., 1999). These results suggest that *Pitx2* has both intrinsic and extrinsic roles in eye development.

Fate mapping experiments in chick provided the first evidence that both neural crest and mesoderm precursors contribute to the periocular mesenchyme (Johnston et al., 1979; Le Douarin, 1980, 1982; Le Lievre and Le Douarin, 1975; Noden, 1982). The optic

cup, optic stalk, and lens interact with both precursor pools early in eye development. We recently used binary transgenic systems to establish that neural crest and mesoderm fates in mice are similar to those in chick but there is a greater contribution of mesoderm to anterior segment structures, including the corneal endothelium and stroma. Interestingly, *Pitx2* is expressed in both neural crest and mesoderm precursors beginning early in eye development (Gage et al., 2005). Collectively, these data indicate that the ocular defects of the global *Pitx2* knockout mice could arise from a requirement for *Pitx2* function in neural crest or mesoderm, or both. The neural crest precursors are of particular interest because it has been suggested that deficiencies in neural crest function underlie many human anterior segment disorders (Kupfer and Kaiser-Kupfer, 1978).

To determine the role of *Pitx2* in the neural crest lineage of the ocular mesenchyme, we created a neural crest specific knockout of *Pitx2* (*Pitx2-ncko*) by mating mice carrying the *Pitx2^flox* allele to mice carrying a *Cre* transgene driven by the *Wnt1* promoter (Danielian et al., 1998). Studying these mice enabled us to determine the roles of *Pitx2* expression in the neural crest during early eye development. In addition, post-natal survival of *Pitx2-ncko* mice allowed identification of novel requirements for *Pitx2* function in the later stages of eye development. We discovered defects in both the mesenchymal tissues that normally express *Pitx2*, as well as in neural tissues that do not, demonstrating that *Pitx2* expression in the neural crest cells has both intrinsic and extrinsic functions. Based on the data, we propose a new model for development of the optic nerve from the optic stalk and new mechanisms that may contribute to the etiology of glaucoma in ARS.

Materials and Methods

Generation of neural crest-specific *Pitx2* knockout mice: The generation of *Pitx2^flox* mice has been previously described (Gage et al., 1999). Mice carrying the *R26R* Cre-reporter allele (Soriano, 1999) were obtained from the Jackson Laboratories. *Wnt1Cre* mice, which carry a transgene containing a Cre cassette under the control of the *Wnt1* promoter, were obtained from A. McMahon (Danielian et al., 1998). The *Pitx2^flox/flox;R26R/R26R*

parental line was generated by serial mating of *Pitx2^flox^* and *R26R* mice, and their progeny. The *Wnt1Cre; Pitx2^+/-^* parental line was generated by mating *Wnt1Cre* and *Pitx2^null^* mice. All procedures involving mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

The *Wnt1Cre; Pitx2^+/-^* mice were mated to the *Pitx2^flox/flox^; R26R/R26R* mice to generate timed pregnancies. The morning after mating was designated as embryonic day 0.5. Embryos were collected by C-section after euthanasia of the mother. The resulting embryos were genotyped for Cre (Cushman et al., 2000), and *Pitx2* (Gage et al., 1999) using PCR-based methods. Embryos with a *Wnt1Cre; Pitx2^flox/-^* genotype were considered mutant, while *Wnt1Cre;Pitx2^flox/+^* embryos were used as controls.

Histology: All embryos were fixed in 4% paraformaldehyde in PBS, washed, dehydrated, embedded in paraffin, and sectioned at 7 microns. Mounted sections for morphological analysis were dewaxed, rehydrated, and stained with hematoxylin and eosin.

Immunofluorescence: Paraffin sections were dewaxed and treated for antigen retrieval by boiling for 10 minutes in 10 mM citrate buffer (pH6.0). Sections were treated with Image-iT FX signal enhancer (Molecular Probes) for 30 minutes. Immunostaining was performed according to standard protocols. Briefly, sections were stained with antibodies against β-galactosidase (Eppendorf-5prime), PITX2 (gift from T. Hjalt), Pitx1 (gift from J. Drouin), Pax2 (Covance), Pax6 (Covance), AP-2β (Abnova), NG2 (Chemicon), Collagen IV (Biogenesis), myogenin (clone F5D, developed by Woodring Wright and obtained from NICHD/Developmental Studies Hybridoma Bank), developmental myosin heavy chain (Vector) or TUJ1 (BabCo). Treatment with the primary antibody was followed by fluorescent-labeled (Molecular Probes) or biotinylated (Jackson Immuno Research) species-specific secondary antibodies. When biotinylated secondary antibodies were used, signals were detected using tyramide signal amplification kits (Molecular Probes).

Results

Tissue-specific targeting of Pitx2 in neural crest

We previously described generation of a conditional *Pitx2* allele (*Pitx2^flox^*) by introduction of *loxP* sites into introns flanking exon 5, which encodes the homeodomain essential for DNA binding and protein function (Gage et al., 1999). We included the *R26R* Cre reporter allele in our crosses so that we could readily detect cells in which Cre-mediated excision occurred (Soriano, 1999). To obtain neural crest specific *Pitx2* knockout mice (*Pitx2-ncko*), mice homozygous for *Pitx2^flox^* and the Cre reporter allele *R26R^loxP^* (*Pitx2^flox/flox^R26R^loxP/loxP^*mice) were mated to *Pitx2* null heterozygotes carrying the *Wnt1-Cre* transgene. The endogenous *Wnt1* gene is expressed in the midbrain and along the entire length of the dorsal neural tube, which contains the pre-migratory neural crest (Echelard et al., 1994). *Wnt1-Cre* is active by e8.5 in the pre-migratory neural crest (Danielian et al., 1998; Echelard et al., 1994), two days before the expression of *Pitx2* in the ocular neural crest at day e10.5 (Gage and Camper, 1997), thereby allowing sufficient time for efficient conversion of *Pitx2^flox^* to the null allele. *Wnt1-Cre* has also been used successfully to study the role of *β-catenin* in the craniofacial neural crest (Brault et al., 2001). In our current experiments, excision of the homeodomain results in conversion of the fully functional *Pitx2^flox^* to the non-functional *Pitx2^null^* allele in the cells expressing *Wnt1-Cre*, creating pups that are homozygous null for *Pitx2* in the neural crest population, but heterozygous elsewhere. Control and experimental mice were identified by PCR-based genotyping. In order to confirm that expression of *Wnt1-Cre* resulted in

specific and efficient silencing of *Pitx2* in neural crest, sections from control and *Pitx2-ncko* embryo were co-immunostained for β-gal, to detect marked neural crest, and PITX2. In control embryos at e12.5, PITX2 is expressed in both neural crest and mesoderm surrounding the eye primordia (Figure 2.1A, C, E). In contrast, in *Pitx2-ncko* embryos, PITX2 expression is expressed only in mesoderm, even though neural crest cells appear to be present (Figure 2.1B, D, F). These results establish that Cre-mediated excision of *Pitx2* was highly efficient and specific for neural crest in the mutant embryos. In mice that were identified as mutants, twenty of twenty-two mice examined exhibited the consistent mutant phenotype described below. The remaining two mice had one mutant eye and one eye with similar but less severe defects. These observations confirm that the *Pitx2-ncko* phenotype is highly consistent and fully penetrant. In contrast to global *Pitx2* knock out animals, *Pitx2-ncko* mice are viable and survive at least until weaning, suggesting that *Pitx2* function in neural crest is not strictly required for development of the cardiovascular system or other essential organs (data not shown).

Pitx2-ncko mice are clinically anophthalmic

To examine the cumulative phenotype, as well as to gain insight into the role of *Pitx2* in later eye development, *Pitx2-ncko* mice and their control littermates were harvested at e16.5 and e18.5. The eyes of control littermates are visible through the fused eyelids (Figure 2.2A), while the eyes are not visible externally in the *Pitx2-ncko* mutants (Figure 2.2B). Dissection of the mutant head revealed that two eyes are present, but buried within the skull, near the midline, directly beneath the brain (Figure 2.2C, D). The presence of globes that are not externally visible indicates that *Pitx2-ncko* mice are clinically anophthalmic (Ishikawa et al., 1996). Mutant eyes are generally devoid of pigment except for a cone-shaped region at the anterior segment (Figure 2.2 C, D). The lens and retina are present in each mutant eye (Figure 2.2 C-H).

Examination of histological sections revealed that the mutant eyes are attached directly to the ventral hypothalamus rather than connected through an extended optic nerve (Figure 2.2 E, F). Multiple histologically distinct layers are apparent in the retinas

of mutant animals, as are retinal ganglion cells that extend axons that enter the ventral hypothalamus and form a structure resembling an optic chiasm (Figure 2.2H, 2.6J). Lens blebbing is also observed, which is a common feature in many eye mutants (Smith et al., 1997). The control retinal-pigmented epithelium (RPE) is fully pigmented at e16.5 (Figure 2.7E). In contrast, the mutant RPE is devoid of pigment at this stage, but it remains a columnar epithelium (Figure 2.2C, H). The developing anterior segment remains heavily pigmented (Figure 2.2D, H). Both the optic stalk and the RPE arise from the neural ectoderm where *Pitx2* is not expressed (Figure 2.1E; (Gage and Camper, 1997)). These phenotypes indicate that one essential function of *Pitx2* in the neural crest is to regulate expression of extrinsic factors that influence development of the neural ectoderm.

Multiple defects in structures derived partially or totally from neural crest are also apparent in *Pitx2-ncko* eyes. The corneal endothelium and stroma, which receive contributions from neural crest and mesoderm, are absent in mutant eyes (Figure 2.2F, H; (Gage et al., 2005). The sclera surrounding the eye is derived from the neural crest and is completely absent in the mutant eyes (Figure 2.2G, H; (Gage et al., 2005)). Hyaloid blood vessels, composed of a mesoderm-derived endothelial lining and neural crest-derived pericytes, are present but appear hypomorphic relative to wild type eyes (Figure 2.2G, H). Muscle bundles, which contain mesoderm-derived myocytes and neural crest-derived fascia cells, are present adjacent to the dysmorphic anterior segment in *Pitx2-ncko* eyes (Figure 2.2H). We hypothesized these muscles may be the extraocular muscles. Collectively, these results indicate that a second essential function of PITX2 is to regulate expression of intrinsic factors within neural crest precursor cells that are required for their subsequent specification into mature lineages.

Defects in Pitx2-ncko eye primordial begin at e11.5

To identify the origins of the accumulated eye defects, we examined *Pitx2-ncko* mice beginning at e10.5, when *Pitx2* expression is initially activated in the ocular neural crest. Control and mutant mice are indistinguishable at this time point (Figure 2.3A, E). At e10.5, the optic stalk in both control and mutant eyes consists of a 10-12 cell thick neuroblastic cell layer. By e11.5 in control eyes, the optic stalk has begun to extend and thin, reflecting the increased distance between the optic cup and the ventral diencephalon (Figure 2.3B). By e12.5, the wild type stalk consists of a 1-2 cell thick neural epithelium and the distance between optic cup and brain is further increased (Figure 2.3C). In contrast, the optic stalk in *Pitx2-ncko* mice remains a thick, neuroblastic structure throughout and does not extend laterally, but remains very short (Figure 2.3F, G). The mutant eyes remain closely associated with the ventral diencephalon and become directly attached to the diencephalon by e14.5 (Figure 2.5F). There is also increased distance between the optic cup and the surface ectoderm as the head grows rapidly in size during this period (Figure 2.3F, G). We examined markers of cell proliferation (Ki67 and phospho-Histone H3) and apoptosis (TUNEL) but did not find any changes that could account for the optic stalk phenotype (data not shown).

Defects in development of structures derived from periocular mesenchyme are also apparent in *Pitx2-ncko* eyes by e11.5. In wild type eyes, neural crest and mesoderm cells migrate into the anterior segment beneath the surface ectoderm immediately after formation of the lens vesicle (Figure 3B, (Gage et al., 2005)). By e12.5, compaction of the mesenchyme to form the corneal endothelium is evident, and by e16.5 the corneal stroma is also present (Figure 2.3C,D). In *Pitx2-ncko* eyes, mesenchyme is present within the anterior segment by e11.5 (Figure 2.3F). However, the mesenchymal layer is noticeably thickened at this time point and there is no subsequent formation of either the corneal endothelium or stroma layers (Figure 2.3 G, H). Proliferation is not increased in the ocular mesenchyme of *Pitx2-ncko* mice (data not shown). Therefore the increasing thickness of the anterior segment in mutant eyes is not due increased proliferation caused by the loss of *Pitx2* in this tissue, but instead appears to be secondary to displacement of the optic cup.

Several of the structures defective in the *Pitx2-ncko* eye contain neural crest cells that normally express *Pitx2*, so we examined molecular markers to determine how the loss of *Pitx2* affects cell fate. To determine how the loss of *Pitx2* and the displacement of the optic cup affect cornea development, we examined the expression of the transcription factor AP-2β, which is expressed in the neural crest of the developing cornea, but is not required for its development (Moser et al., 1997; West-Mays et al., 1999). We found that AP-2β expression was present but significantly disrupted by the displacement of the eye (Figure 2.4A, B). There are markedly fewer mesenchymal cells expressing AP-2β adjacent to the surface ectoderm, where the presumptive corneal cells are normally located, and there is also a population of AP-2β-postive cells adjacent to the lens and displaced optic cup. While *Pitx2* expression in the neural crest is not absolutely required for AP-2β expression, it influences corneal specification as shown by the reduced number of corneal precursors, but the displacement of the optic cup prevents normal cornea development.

To confirm our initial finding that the mutant vasculature is reduced in the *Pitx2-ncko* eye, we performed immunostaining for Collagen IV, a vascular endothelial marker (Merville et al., 1976). We found that there were fewer hyaloid vessels in the mutant eye (Figure 2.5A, B). While it is unknown whether the hyaloid vasculature forms by vasculogenesis or angiogenesis, in either case, the vessels are initially formed as primitive tubes by endothelial cells of mesodermal origin. Platelet derived growth factor (PDGF) released by the endothelial tubes recruits mural cells, which in turn stabilize the new vessels, possibly by secreting vascular endothelial growth factor (VEGF) or angiopoeitin-1 (Darland et al., 2003; Hellstrom et al., 1999; Hirschi et al., 1998; Lindahl et al., 1997; Nishishita and Lin, 2004). The mural cells of the eye vessels are pericytes, which are neural crest derived (Etchevers et al., 2001; Gage et al., 2005). We hypothesized that the reduced vasculature phenotype seen in the *Pitx2-ncko* could be due to a deficiency in the migration of neural crest-derived pericytes to the primitive endothelial tubes. We assayed this by examining the presence of NG2, a marker for

differentiated pericytes and their precursors (Hughes and Chan-Ling, 2004; Murfee et al., 2005; Ozerdem et al., 2001), and found that pericytes were present by immunofluorescence, although they may not be fully functional (Figure 2.5C, D). Defects in the ability of *Pitx2*-negative pericytes to differentiate and stabilize the developing vessels could account for the reduction in the number of hyaloid blood vessels.

Although extraocular muscles are absent in global *Pitx2* knockout mice, our initial histological examination of the *Pitx2-ncko* eye suggested that extraocular muscles were present, albeit in an abnormal orientation to the optic cup. To confirm, we analyzed the expression of several muscle-related markers during extraocular muscle development. Extraocular muscles were specified normally as indicated by the presence of the transcription factors PITX1 and myogenin via immunofluorescence at e12.5 (Figure 2.6C, D). These populations of cells did not migrate from their original locations in the eye field, despite the displacement of the optic cup towards the midline. By e14.5, the muscles had differentiated, as indicated by the presence of developmental myosin heavy chain immunofluorescence (Figure 2.6G, H).

Loss of Pitx2 in the neural crest lineage results in severe optic nerve defects

The optic nerve defects in the *Pitx2-ncko* mice are striking, and to our knowledge unique, so we pursued the underlying molecular aspects further. Based on the early and severe optic stalk phenotype in the *Pitx2-ncko* mice, we considered the possibility that the optic stalk was never specified properly. To test this hypothesis, we examined the expression of *Pax2*, an early marker for optic stalk specification that is also required for its normal development (Dressler et al., 1990; Otteson et al., 1998; Torres et al., 1996). We found PAX2 protein is expressed in the optic stalk of both control and mutant animals at e12.5, establishing that a lack of initial specification cannot account for the optic stalk defects in *Pitx2-ncko* mice (Figure 2.7A, B). Consistent with previous reports, PAX2 expression in control eyes at e12.5 is limited to the optic stalk and does not enter the optic cup (Figure 2.7A (Otteson et al., 1998; Torres et al., 1996)). In contrast, PAX2 expression in e12.5 *Pitx2-ncko* eyes extends into the posterior presumptive RPE and

outer layer of the optic stalk (Figure 2.7B, D). The expansion of PAX2 into the posterior RPE becomes progressively more extensive until approximately 50% of the RPE is expressing PAX2 by e16.5 (Figure 2.7E, F). We also found that *Vax1*, another homeobox transcription factor required for optic stalk specification, is present and expanded in the same manner as *Pax2* (Figure 2.7E, F; (Bertuzzi et al., 1999; Hallonet et al., 1999; Mui et al., 2005)). Since *Pax2* and *Vax1* are both activated by *Sonic hedgehog* signaling (Macdonald et al., 1995; Take-uchi et al., 2003), we examined whether increased midline *Shh* expression could account for the expanded expression of these genes. We found that the spatial and temporal pattern of *Shh* mRNA expression is unchanged in the mutant mice (Figure 2.7G, H; data not shown).

After the initial specification of the optic stalk by PAX2, the stalk attracts the axons of the retinal ganglion cells (RGCs) while undergoing significant morphogenetic movements (Otteson et al., 1998; Torres et al., 1996). Defects in routing of the RGCs axons are often associated with defects in optic nerve development (Bertuzzi et al., 1999; Hallonet et al., 1999; Mui et al., 2005; Torres et al., 1996). Initial histological examination suggested that RGC axons entered the optic disk in *Pitx2-ncko* eyes (Figure 2.3G, H). We used immunostaining for β-tubulin to confirm that RGC axons exit the eye at the optic disk and subsequently associate with PAX2-expressing cells of the optic stalk at e12.5 (Figure 2.7A-D). PAX2 positive cells of the optic stalk are ultimately fated to delaminate and migrate among the axons in the optic nerve, where they differentiate as astrocytes (Figure 6I; (Mi and Barres, 1999; Torres et al., 1996)). In the mutant at e16.5, only a small percentage of PAX2 positive cells of the stalk have invaded the axons of the optic nerve while the majority remain localized at the periphery (Figure 2.7J). The axons exiting the mutant eyes enter directly into the ventral hypothalamus where they form a primitive optic chiasm (Figure 2.7K, L).

RPE development is disrupted in Pitx2-ncko eyes

Shortly after the optic vesicle invaginates to form the optic cup, signals from the periocular mesenchyme specify the outer layer of the optic cup as the RPE (Fuhrmann et al., 2000). This initial specification appears to occur normally in *Pitx2-ncko* mice, since the mutant RPE is indistinguishable from the control in both morphology and gene expression at e10.5 (Figure 2.8A, B; data not shown). However, the progressive loss of pigment beginning at e12.5 suggests that subsequent expansion or maintenance of the RPE is blocked in the mutant eyes (Figure 2.8C-J). PAX2 is known to inhibit the expression of *Pax6* as the boundary is established between the PAX2-expressing optic stalk and the PAX6-expressing RPE layer (Schwarz et al., 2000). PAX6 is subsequently required to activate expression of the downstream transcription factor *Mitf*, which is required for RPE specification (Baumer et al., 2003; Martinez-Morales et al., 2004; Mochii et al., 1998; Nakayama et al., 1998; Smith et al., 1998). *Otx1* and *Otx2* also encode transcription factors required for RPE specification, but their expression does not depend on *Pax6* (Bovolenta et al., 1997; Martinez-Morales et al., 2001; Takeda et al., 2003). We hypothesized that suppression of RPE-specifying genes, including *Pax6*, *Mitf*, and *Otx2*, may account for the progressive block in RPE differentiation or expansion in *Pitx2-ncko* eyes. At e10.5, both control and mutant presumptive RPE express PAX6, *Mitf* and *Otx2* (Figure 2.8A, B; data not shown). In *Pitx2-ncko* eyes, expression of PAX6 protein is excluded from cells expressing PAX2 (Figure 2.8A-F). As expected, pigment is similarly excluded from cells expressing PAX2 (Figure 2.8C-F). *Mitf* and *Otx2* expression are also lost in the presumptive RPE cells that do not express pigment at e12.5 (Figure 2.8G-J).

Discussion

Sophisticated experimental genetics in mice provides a powerful approach for understanding human development and disease by allowing for precise molecular, cellular, and temporal dissection of gene function. Tissue-specific knockouts can be particularly useful when trying to understand the function of a gene that is expressed in multiple precursor pools in developing organs. In the present study, we used conditional targeting to determine the processes in early eye development that require function of the

homeobox gene, *Pitx2*, in the neural crest. We also identified critical new roles for *Pitx2* in later eye development, which was previously impossible to study due to the embryonic death of *Pitx2* global knockout mice. Furthermore, we propose two potential mechanisms for the early development of glaucoma in human *PITX2* patients and identify a previously unrecognized regulatory pathway in optic nerve development that may have implications for other eye diseases.

Intrinsic requirements for Pitx2 in tissues derived from the periocular mesenchyme

Given the corneal defects seen in Axenfeld-Rieger syndrome patients with *PITX2* mutations (Doward et al., 1999), *Pitx2* was predicted to play an important role in cornea development. The global *Pitx2* knockout mice lack a corneal endothelium and corneal stroma, confirming an essential role for the gene in corneal development (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). Based on our current results, specification of corneal endothelium and stroma requires *Pitx2* function in neural crest precursors, which are the primary contributors to these tissues (Gage et al., 2005). *Pitx2* is also expressed in the subset of mesoderm cells that contribute to the cornea, but this small number of cells is not able to rescue corneal formation in *Pitx2-ncko* mice (Gage et al., 2005). Although the phenotype of global *Pitx2* knockout mice suggested the mutant anterior segment was hypercellular (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999), we are unable to find evidence of increased proliferation in the periocular mesenchyme of *Pitx2* mutant mice (data not shown). Thus, the thickened appearance of the presumptive cornea is likely secondary to the displacement of the optic cup.

Cornea differentiation requires inductive signals from the lens (Coulombre and Coulombre, 1964; Genis-Galvez, 1966), and lens defects result in cornea defects in human disease (Jamieson et al., 2002; Ormestad et al., 2002; Semina et al., 2001; Semina et al., 1998). The ectopic expression of AP-2β next to the displaced lens in the *Pitx2-ncko* eyes provides further proof of this induction. Normally, *Pitx2* expression is activated in neural crest cells as they migrate into the anterior segment (Gage et. al, 2005 and PJG, unpublished results). Based on these observations and our current results, we propose that

Pitx2 is normally a direct downstream effector that is induced in response to signals from lens. Activation of *Pitx2* in neural crest within in the presumptive anterior segment is required to initiate a cascade of genes required for corneal differentiation. In the absence of *Pitx2* expression, neural crest cells are not competent to respond appropriately to the signals from the lens, and corneal differentiation fails to occur. It is also possible that corneal agenesis is secondary to the displacement of the lens in *Pitx2-ncko* mice, but we favor our model of a primary role for *Pitx2* because human *PITX2* patients have corneal defects without eye displacement (Doward et al., 1999). The role of *Pitx2* in cornea formation is an important question that must be answered in the future.

Scleral agenesis is a second example of an intrinsic defect in the *Pitx2-ncko* eye. The sclera, the white outermost coat of the eye, is derived from the neural crest lineage of the periocular mesenchyme, which expresses *Pitx2* (Gage et al., 2005). While signals from the RPE have been shown to induce sclera formation, only part of the RPE is disrupted in the *Pitx2-ncko* eye (Seko et al., 1994). Since the sclera is completely absent in the *Pitx2-ncko* mice, we conclude that *Pitx2* expression is required for neural crest cells to adopt scleral fates. *Pitx2* may confer competence on neural crest cells to respond to the signals from the RPE. Currently, little is known about the development of the sclera; our data provide the first clues about its genetic origins. Recent data about the causes of nanophthalmos (extreme hyperopia) demonstrate that scleral development is involved in controlling the shape of the eye, which is critical given the precise optics required for vision (Sundin et al., 2005).

Other tissues that are derived from the periocular mesenchyme do not show an absolute requirement for *Pitx2* in the neural crest lineage. While the hyaloid vasculature of the *Pitx2-ncko* mice is hypoplastic, the mutant pericyte precursors are still recruited to the primitive endothelial vessels. This suggests that *Pitx2* expression is not strictly required for neural crest cells to adopt the pericyte fate in the eye, as judged by the commonly used marker NG2 (Hughes and Chan-Ling, 2004; Murfee et al., 2005). However, the mutant pericytes may have compromised function that could cause defects in vessel formation and growth. This idea comes from the knowledge that pericytes play

a particularly crucial role in the formation of the vascular plexus of the eye (Klinghoffer et al., 2001; Uemura et al., 2002). We hypothesize that the *Pitx2-null* pericytes do not express signaling factors required to stimulate the proliferation of vascular endothelial cells and induce the remodeling associated with the formation of additional vessels. The absence of *Pitx2* in the neural crest population results in abnormal development of the hyaloid vasculature, so the retinal vasculature, which replaces the hyaloid vasculature after birth, may also be hypoplastic.

Expression of *Pitx2* is not required for the adoption of all neural crest fates in the developing eye. Although we cannot exclude the possibility to subtle defects, the expression of the muscle specification genes, *myogenin* and *Pitx1*, and the differentiated muscle marker, myosin heavy chain, in the muscle primordia indicate muscle formation is relatively normal in *Pitx2-ncko* mice. Neural crest cells are present in the extraocular muscles of mutant eyes (data not shown), where they form the connective fascia and tendons (Gage et al., 2005). Therefore, we conclude that *Pitx2* expression in neural crest cells is not required for extraocular muscle formation. Since extraocular muscles are completely lost in the global *Pitx2^null^* mice, this implies that the expression of *Pitx2* in the mesodermal lineage is required for extraocular muscle formation, a hypothesis that could be tested in mesoderm-specific *Pitx2* knockout mice.

Extrinsic effects on neural ectoderm

Our results have identified a previously unknown role for *Pitx2* function in the neural crest during optic stalk development. One of the ongoing processes of early eye development is the partitioning of the neural ectoderm-derived optic vesicle into the regions that will form the optic stalk, neural retina and RPE by signaling, gene activation and gene repression. In *Pitx2-ncko* mice, early partitioning of the optic vesicle occurs normally but subsequent morphogenesis of the optic stalk is abnormal. This results in a foreshortened optic stalk and eyes that are displaced towards the midline. Although the end phenotype superficially resembles cyclopia, the presence of two eye fields and absence of other midline defects allowed us to rule out this line of inquiry. Since *Pitx2* is

never expressed in the optic stalk, this indicates that the defect in optic stalk development is extrinsic (non-cell autonomous). *Pitx2* expression in the neural crest mesenchyme must be activating an extracellular signal that is required for optic stalk development. The periocular mesenchyme is known to signal other tissues of the eye. For example, an activin-like signal from the mesenchyme is required to define the RPE, and FGF10 and BMP7 cause the development and morphogenesis of the lacrimal gland from the surface ectoderm (Dean et al., 2004; Fuhrmann et al., 2000; Govindarajan et al., 2000; Makarenkova et al., 2000). Our finding that the *Pitx2*-dependent signaling function is localized to the neural crest component of the mesenchyme raises the possibility that it acts as a form of signaling center.

Our current data support a two step model for optic stalk development (Figure 2.9). During the initial formation of the eye beginning at e8.5, Shh diffuses from the midline and activates *Pax2*, *Vax1*, and *Vax2* expression in the optic stalk (Macdonald et al., 1995; Take-uchi et al., 2003). These transcription factors act synergistically to repress *Pax6* and define the optic stalk as separate from the optic cup (Bertuzzi et al., 1999; Hallonet et al., 1999; Mui et al., 2005; Schwarz et al., 2000; Torres et al., 1996). This initial specification of the optic stalk takes place normally in *Pitx2-ncko* mice, as indicated by normal expression of *Shh*, *Pax2* and *Vax1* (Figure 2.6). Later, beginning at e10.5, *Pitx2* expression in the periocular mesenchyme activates signals that cause the morphogenetic extension of the optic stalk. This step fails to occur in *Pitx2-ncko* mice, resulting in a foreshortened optic stalk. To our knowledge, this is the first example of the neural crest population patterning the neuroectoderm from which it is originally derived, which raises the possibility that this process occurs in other neural tube derivatives.

The progressive RPE defects we observed in the *Pitx2-ncko* eye are likely secondary to the defects in optic nerve development and the displacement of the eyes. The close proximity of the mutant eyes to the midline source of Shh is probably sufficient to drive increased *Pax2* expression, which shifts the boundary between *Pax2* and *Pax6* expression distally. The reciprocal changes in *Pax2* and *Pax6* expression are likely to be the cause of the loss of pigment in large portions of the RPE in the *Pitx2-ncko* mice. *Pax6*

initially activates *Mitf*, which is required for normal RPE development and is lost in the unpigmented portions of the RPE (Baumer et al., 2003; Fuhrmann et al., 2000; Martinez-Morales et al., 2004; Nakayama et al., 1998; Nguyen and Arnheiter, 2000). Expression of *Otx2*, another gene required for RPE development that does not depend on *Pax6*, is also absent in the regions of pigment loss. *Mitf* expression may be required for the maintenance of *Otx2* expression, as proposed by Martinez-Morales et al., or *Pax2* may repress *Otx2* expression (Martinez-Morales et al., 2001). Overall, our data is consistent with the role of *Pax2* as a repressor of RPE development proposed by Martinez-Morales et al. (Martinez-Morales et al., 2004).

Implications for human health

In all, our results suggest several new possible mechanisms by which early-onset glaucoma can occur in Axenfeld-Rieger syndrome patients with *PITX2* mutations. It is widely assumed that these patients have anterior segment defects that cause increased intraocular pressure, which leads to the development of glaucoma relatively early in life (Shields, 1983). While we do not discount the role elevated IOP plays in glaucoma, we propose that human *PITX2* patients may have additional developmental eye defects that lead to the accelerated development of glaucoma. Although the human patients clearly do not have the severe optic nerve defects seen in the *Pitx2-ncko* mice, they may have more subtle defects in optic nerve development that render the optic nerve more susceptible to damage. The reduced number of *Pax2*-positive astrocyte precursors that invade the axons of the RGCs in the *Pitx2-ncko* mice is particularly noteworthy. Astrocytes are critical for the maintenance and survival of the RGC axons, and they have been implicated in the pathogenesis of glaucoma (Morgan, 2000; Neufeld and Liu, 2003; Pena et al., 1999). If human *PITX2* patients have reduced numbers of astrocytes or altered astrocyte function, it could make their optic nerves more vulnerable to the effects of other factors like elevated IOP, and lead to the RGC axon damage and death seen in glaucoma.

Reduced ocular blood flow has long been associated with normal tension glaucoma (Geijssen and Greve, 1995), and has also been proposed to contribute to other

forms of glaucoma (Grieshaber and Flammer, 2005; Hayreh, 1994). Retinal blood vessel development cannot be critically assessed in *Pitx2-ncko* mice due the severely dysmorphic ocular growth. However, the clear defect in hyaloid vessel formation implies that normal retinal blood vessel development or function may also depend on PITX2 function in neural crest since the two vasculature systems are developmentally related (Saint-Geniez and D'Amore, 2004). The observed deficiency in astrocytes in *Pitx2-ncko* eyes provides additional evidence for defective retinal vessel development because astrocytes guide retinal vessel formation (Jiang et al., 1994; West et al., 2005). It has not been clear whether defects in ocular blood flow are a cause or effect of glaucoma (Ikram et al., 2005; Mitchell et al., 2005). Our results with *Pitx2-ncko* mice raise the possibility that *PITX2* patients may have fewer ocular vessels, which would be another risk factor for developing glaucoma.

The pronounced internal displacement of the eyes in *Pitx2-ncko* mice is reminiscent of other human eye diseases, including anophthalmia and septo-optic dysplasia, and suggests potential underlying genetic mechanisms in these diseases. Although we cannot formally exclude the possibility, it seems unlikely that mutations in *PITX2* itself will be identified in these conditions since mice globally deficient in *Pitx2* die during development (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). However, genes for the extrinsic signaling factor(s) regulated by *PITX2* in neural crest or the downstream effectors in the neural epithelium are strong candidate genes for these diseases.

Mutations in *SOX2* (Fantes et al., 2003), *SIX6* (Gallardo et al., 1999), *PAX6* (Glaser et al., 1994), *RAX/RX* (Voronina et al., 2004), and *OTX2* (Ragge et al., 2005) have been associated with anophthalmia, but the underlying genetic defects in many cases of anophthalmia remain to be identified. These genes are only expressed in the neural ectoderm and are associated with early defects in formation or survival of the optic vesicle (Grindley et al., 1995; Jean et al., 1999; Kamachi et al., 1998; Martinez-Morales et al., 2001; Mathers et al., 1997). Our work suggests that cases of clinical anophthalmia may be associated with genes expressed in the periocular mesenchyme as well. Mutations

in *HESX1* have been identified in septo-optic dysplasia, a disease which affects the midline structures of the brain and optic nerves (Dattani et al., 1998). Mice with mutations in *Hesx1* have a "buried" eye phenotype which bears similarities to the phenotype described here (Dattani et al., 1998). This suggests that *Hesx1* may be part of the same pathway as *Pitx2*, thus it is possible that mutations in *PITX2* or its downstream targets may underlie some cases of septo-optic dysplasia.

Our findings raise the possibility that these disorders may also result from defects occurring later in eye development and the underlying molecular defects could affect genes expressed in either the neural epithelium or the surrounding neural crest. These findings demonstrate that understanding the underlying genes and pathways is critical to understanding the disorders that result when development goes awry.

Acknowledgments
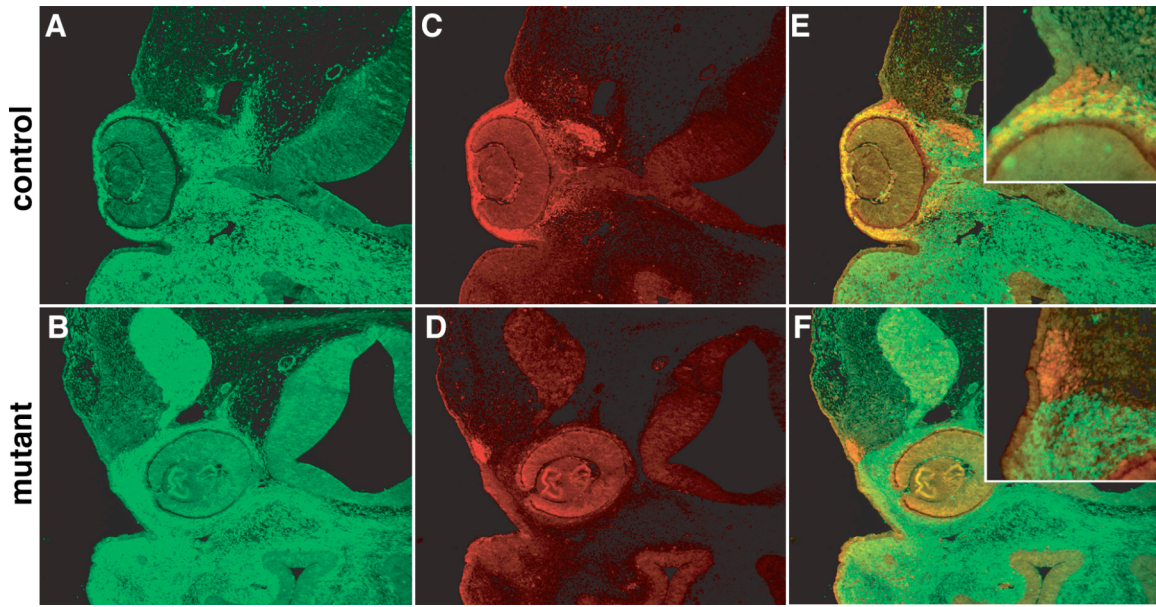
Figure 2.1: Neural crest specific knockout of *Pitx2*. (**A-F**) Double-immunostaining for PITX2 expression in lineage-marked neural crest in e12.5 eye primordia of control (A, C, E,) and *Pitx2-ncko* (B, D, F) embryos. (**A**, **B**) Neural crest, as indicated by β-gal from activated R26R Cre-reporter allele, shown in green. (**C**, **D**) PITX2 protein shown in red. (**G, H**) Merged image shows expression of PITX2 in neural crest (yellow) and mesoderm (red) in the control embryo, while in the mutant embryo PITX2 expression limited to the mesoderm (inset).
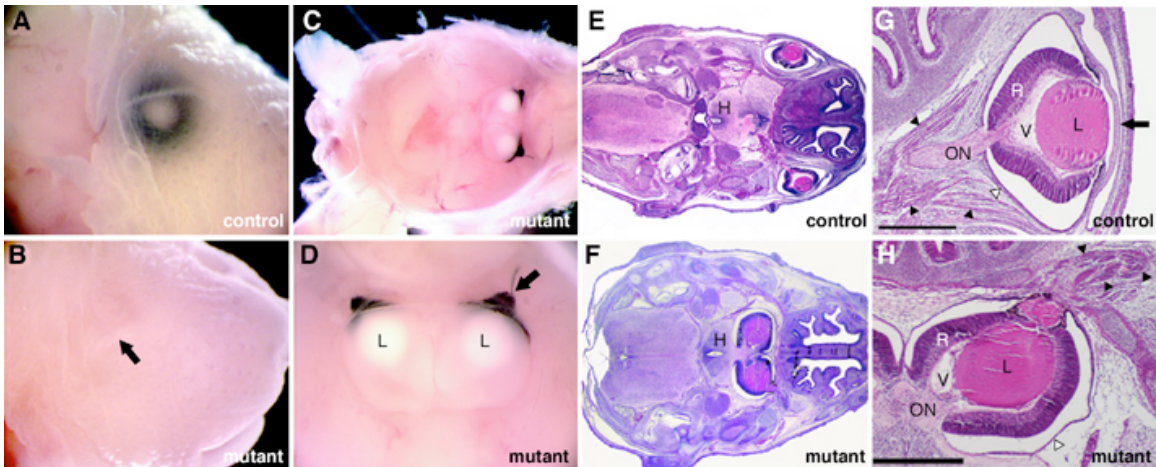
Figure 2.2: Accumulated morphological defects in late gestation *Pitx2-ncko* embryos. (**A**) Control embryo at e16.5. (**B**) Clinical anophthalmia (arrow) in e16.5 *Pitx2-ncko* embryo. (**C, D**) Low and high magnification views of dissected e16.5 *Pitx2-ncko* head. Note the visibility of lens in mutant eyes due to lack of pigment except in the anterior segment (arrow). (**E, F**) Hematoxylin and eosin-stained transverse sections of control and *Pitx2-ncko* heads at e16.5. Note attachment of mutant eyes directly to the ventral hypothalamus. (**G**, **H**) Close up of control and mutant eyes at e16.5. Note that the extraocular muscle condensations (closed arrowheads) are shifted in reference to the optic cup. The cornea (arrow), anterior segment, and sclera (open arrowhead) are absent in the mutant eye. L, lens; H, hypothalamus; R, retina; V, hyaloid vessels; ON, optic nerve.
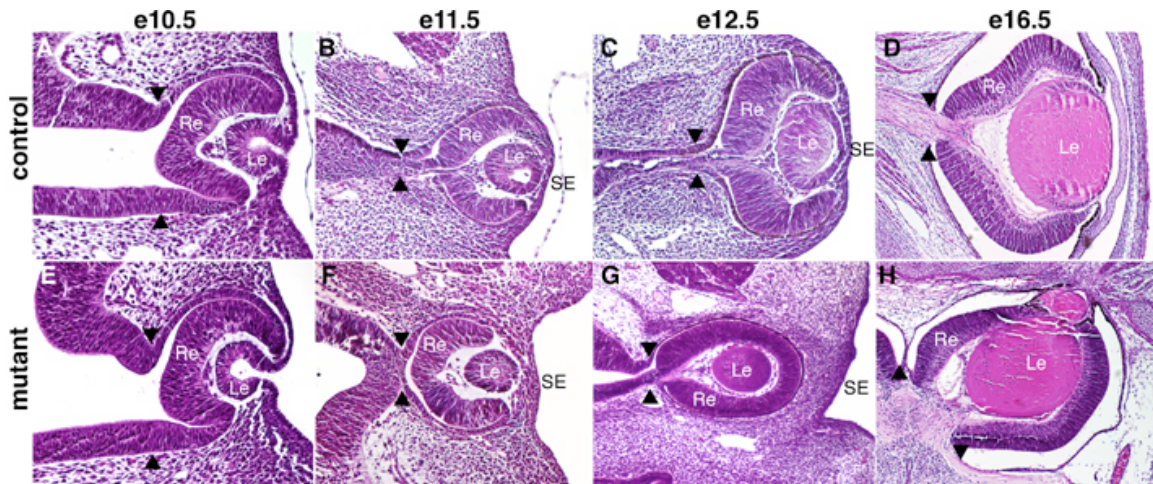
Figure 2.3: Progression of the *Pitx2-ncko* eye phenotype during development. (A, E) Hematoxylin and eosin-stained transverse sections of control and mutant eyes at e10.5, **(B, F)** e11.5, **(C, G)** e12.5, and **(D, H)** e16.5. Note progressive morphogenetic extension of optic stalk in control embryos (arrowheads, A-D) that does not occur in mutant embryos (arrowheads, F-H). The distance between the optic cup and the surface ectoderm also becomes greater over time in the mutant animals. Re, retina; Le, lens; SE, surface ectoderm.
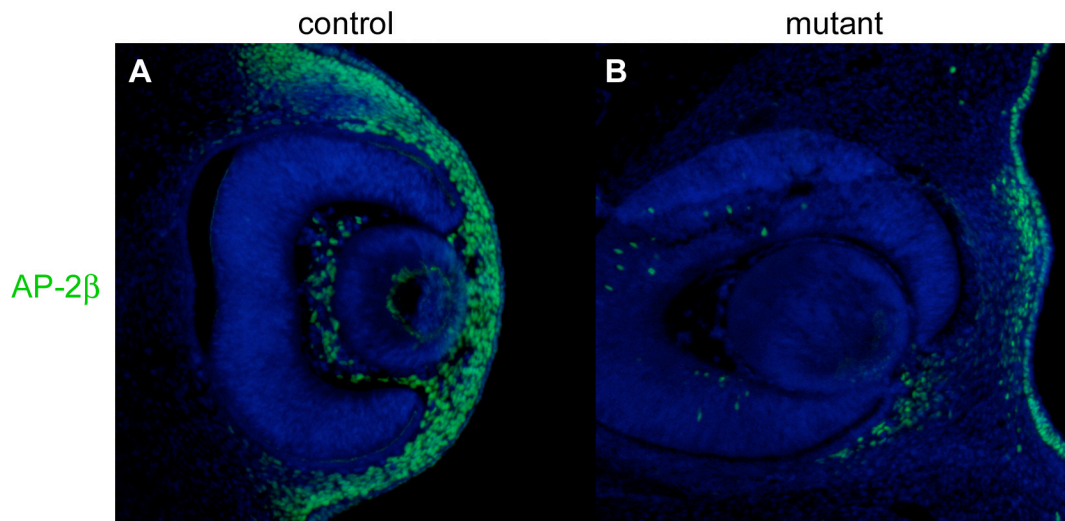
Figure 2.4: Cornea development is disrupted in *Pitx2-ncko* embryos. Expression of the transcription factor AP-2β marks the neural crest cells of the presumptive cornea during development at e12.5(**A)**. Displacement of the optic cup in *Pitx2-ncko* eyes causes a reduction in the number of cells expressing AP-2β adjacent to the surface ectoderm, and induces expression in a separate population of cells adjacent to the optic cup (**B**).
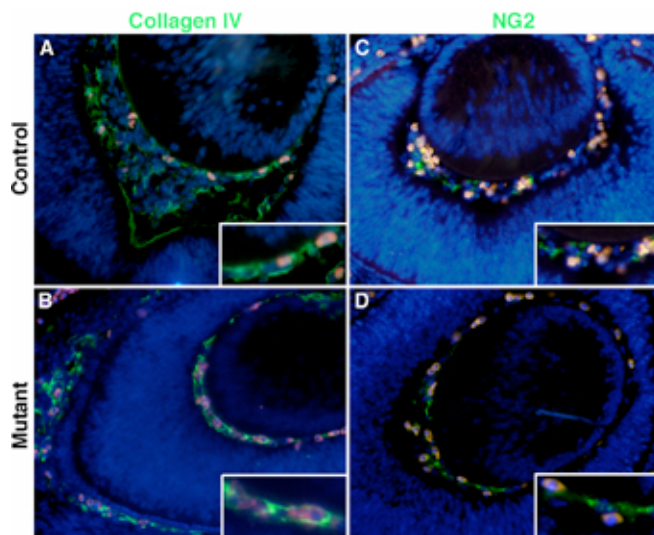
Figure 2.5: Reduction of hyaloid vasculature. (A, B) Immunofluorescence for the blood vessel endothelial cell marker, collagen IV (green), at e12.5 in control and *Pitx2-ncko* eyes. **(C, D)** Immunofluorescence for the pericyte marker, NG2 (green), in control and mutant eyes at e12.5. Insets are representative high magnification views. Red blood cells appear orange due to autofluorescence.
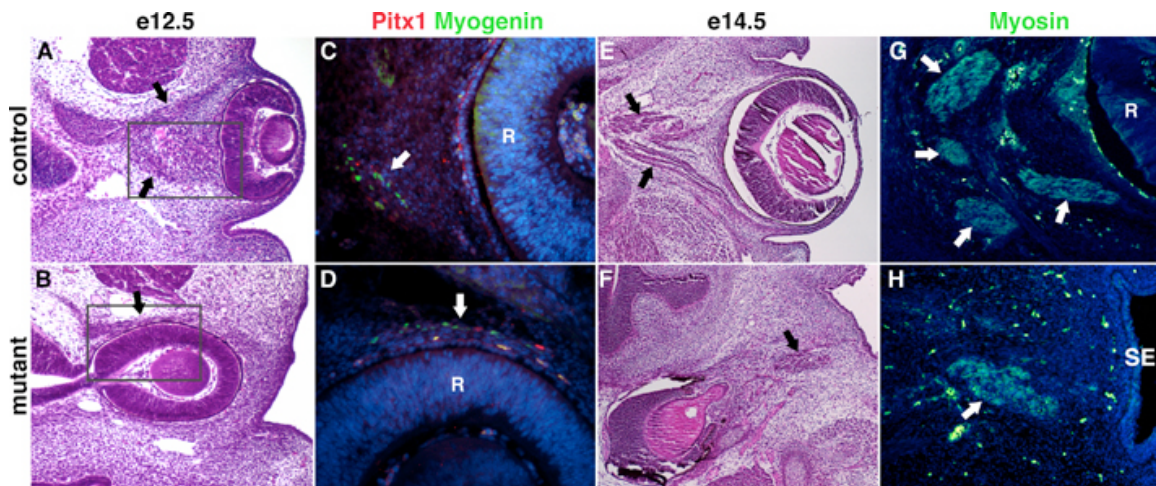
Figure 2.6: Extraocular muscles are present in the normal location. (**A, B**) Extraocular muscles (arrows) are present at e12.5 in both the control and *Pitx2-ncko* eye. Grey boxes indicate the orientation of panels C and D. (**C, D**) Double-immunostaining for PITX1 (red) and Myogenin (green), markers of muscle precursor cells, in e12.5 control and mutant mice. (**E, F**) Extraocular muscles are present at e14.5 (arrows), although their location is changed relative to the eye in the mutant. (**G, H**) Immunofluorescence for the mature muscle fiber marker, developmental myosin heavy chain, at e14.5 in control and mutant mice. R, retina; SE, surface ectoderm.
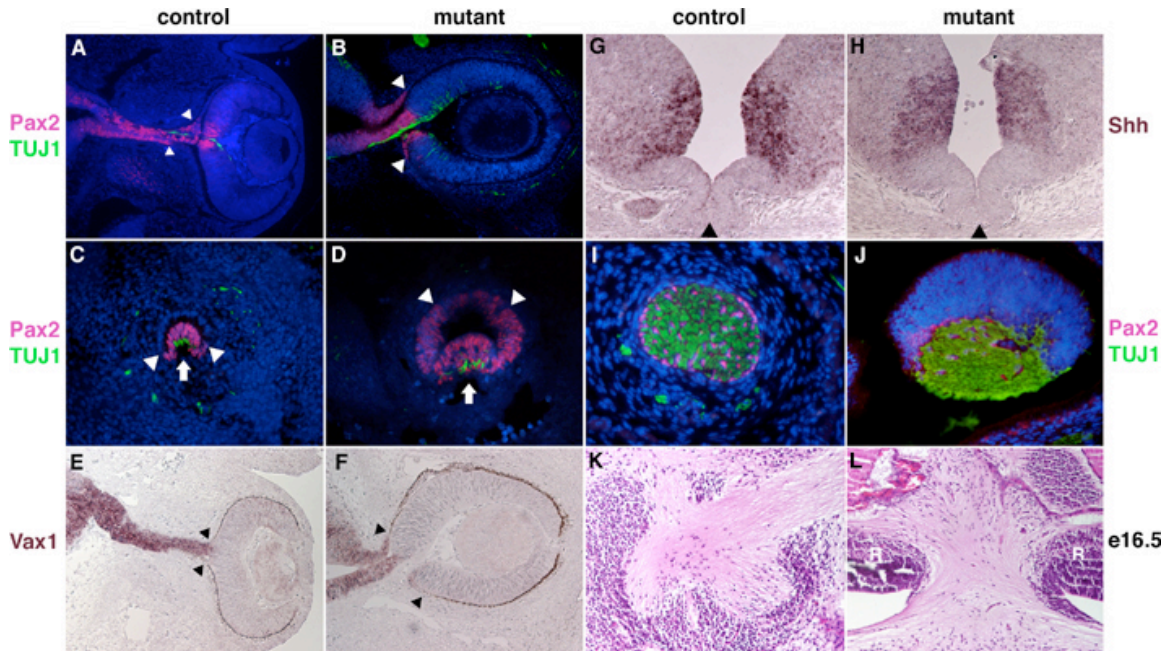
Figure 2.7: Optic stalk specification and morphogenesis. (A, B) Expression of PAX2 protein (red) in control and mutant eyes at e12.5. Staining for β-tubulin labels RGC axons (green). **(C, D)** PAX2 expression (red) and staining for RGC axons (green) in a cross (sagittal) section of the optic stalk at e12.5. White arrowheads denote the limits of PAX2 expression, and a white arrow indicates the optic fissure. **(E, F)** *Vax1* expression (purple) in the control and mutant optic stalk at e12.5. Arrowheads denote the limits of expression. Pigmented RPE appears black. **(G**, **H)** *Sonic Hedgehog* expression in the ventral diencephalon (future hypothalamus) in the control and mutant brain at e12.5. Infundibulum is marked with an arrowhead. **(I, J)** Cross-section of the optic nerve at e16.5, showing the PAX2-expressing astrocyte precursors (red) and the axons (green) in control and mutant mice. The unlabeled blue cells in the mutant are retina. **(K, L)** Transverse H&E sections of e16.5 embryos showing control optic chiasm (K) formed by the axons of the RGCs at the midline near the ventral hypothalamus. In the mutant, a primitive chiasm (L) is formed by the RGC axons as they exit the eyes. R, retina.
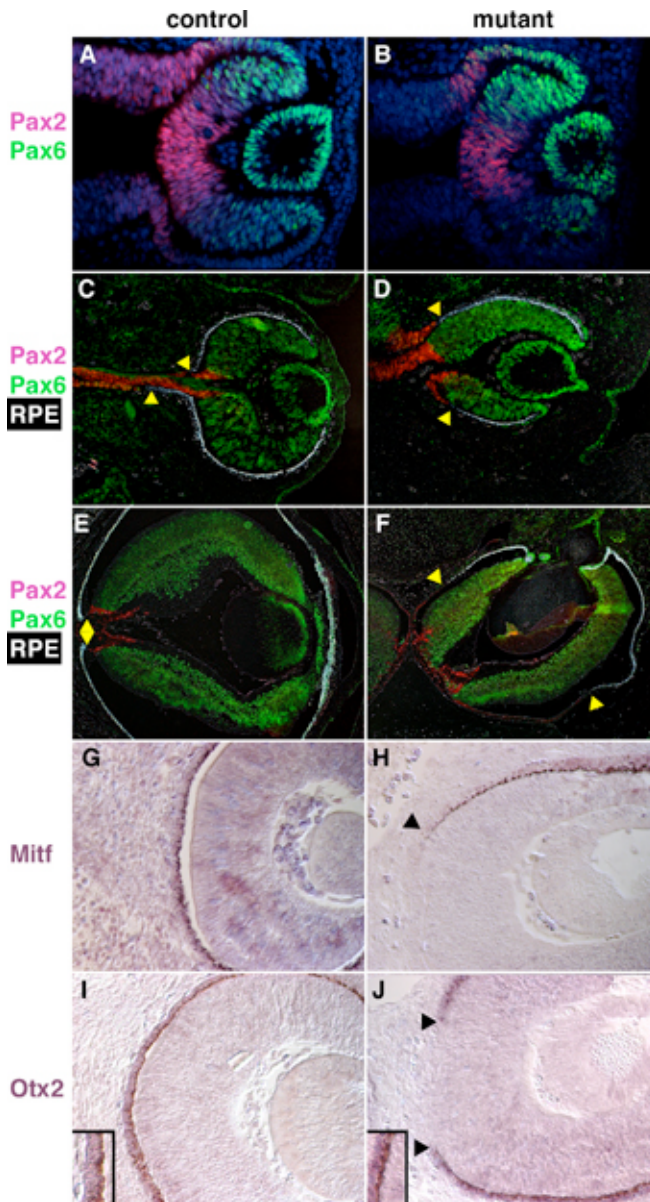
Figure 2.8: Expansion of PAX2 results in RPE specification defects. (**A, B**) Co-immunostaining shows expression pattern of PAX2 (red) and PAX6 (green) at e10.5 in control and *Pitx2-ncko* mice. (**C-F**) Expression of PAX2 (red), PAX6 (green) and pigment (white) at e12.5 (C, D) and e16.5 (E, F) in control and mutant mice. Yellow arrowheads mark the sharp demarcation between PAX2 and pigment expression. To show pigment expression, brightfield images were inverted. (**G-J**) In situ hybridization for *Mitf* (G, H) and *Otx2* (I, J) at e12.5 in control and *Pitx2-ncko* mice. Inset shows the expression of *Otx2* (purple) in cells that express pigment (brown) on their apical surface. Arrowheads indicate the extent of pigment expression in the posterior optic cup.
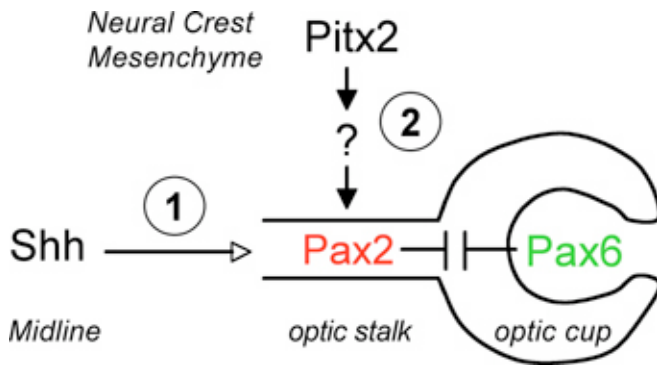
Figure 2.9: Model for *Pitx2* function in optic stalk development. Our work indicates that optic stalk development includes at least two patterning steps. Initially (1), SHH diffusing from the midline activates *Pax2* in the optic stalk, which represses *Pax6* to create a boundary between the optic stalk and optic cup. Later in development (2), *Pitx2* is expressed in the neural crest mesenchyme cells surrounding the optic stalk, where it activates unknown signaling molecule(s) that induces the extension of the optic stalk

Chapter 3: *Pitx2* is required for the survival of extraocular muscle precursors and eyelid closure

Introduction

Cranial mesoderm contributes to the formation of the vasculature and muscles of the head. The unsegmented prechordal and paraxial mesoderm forms the extraocular muscles (EOMs) and contributes to the periocular mesenchyme. The periocular mesenchyme is composed of cells of both neural crest and mesoderm-derived cells, and they combine to form many structures in the eye (Creuzet et al., 2005; Gage et al., 2005; Johnston et al., 1979; Wahl and Noden, 1997). In addition to the myocytes of the extraocular muscles, the mesodermal cells form the endothelial cells of the hyaloid, retinal, and choroidal blood vessels, Schlemm's canal in the outflow tract, and the stroma of the iris. Mesoderm-derived cells are also found in the corneal stroma and endothelium, ciliary body, and trabecular meshwork, but the bulk of these tissues are formed by the neural crest (Gage et al., 2005). In chick, the prechordal mesoderm contributes to the medial, superior, and inferior rectus and inferior oblique muscles, while the superior oblique and the lateral rectus come form the most anterior portion of the paraxial mesoderm (Noden and Francis-West, 2006).

The extraocular muscles that enable eye movements have many unique features that differentiate them from other skeletal muscles. They have unique physiology, fiber types, and gene expression profiles (as reviewed in Porter, 2002; Spencer and Porter, 2006). They are uniquely unaffected by most forms of muscular dystrophy, including Duchenne's, possibly due to their improved calcium homeostasis and higher levels of proteins like utrophin that can compensate in the disrupted dystrophin-glycoprotein complex (Andrade et al., 2000; Karpati et al., 1988; Porter et al., 2003b; Porter et al., 1998). Extraocular muscle function is also disrupted in human disease. Strabismus, or

"cross eyes" is a common condition in which the eye is turned due to an imbalance in extraocular muscle strength. Patients cannot see in three-dimensions because the brain ignores the input from the misaligned eye, and if left untreated, permanent vision loss can result (Gronlund et al., 2006). Although the cause of strabismus can also be neural, the need for the extraocular muscles to be equal in strength indicates that their development must be tightly regulated. Failure of the muscles to be properly innervated can also cause their abnormal development, as is seen in the congenital craniofacial dysinnervation disorders (CCDDs) (reviewed in Engle, 2006). Congenital absence of one or more of the extraocular muscles is rare, but has been reported in the literature (Astle et al., 2003; Chan and Demer, 1999; Drummond and Keech, 1989; Greenberg and Pollard, 1998; Hart et al., 2005; Mather and Saunders, 1987; Taylor and Kraft, 1997).

Given the unique properties of extraocular muscles, it is not surprising that their development is unique as well. The differences in the early steps of trunk and craniofacial myogenesis have been well documented; the trunk muscles develop from somites, whereas the craniofacial muscles develop from unsegmented mesoderm, and transcription factors critical for trunk myogenesis such as *Pax3* and *Mrf4* are not expressed in the head (as reviewed in Noden and Francis-West, 2006). However, the development of the extraocular muscles is different from that of the other craniofacial muscles, which are formed in the branchial arches. *Tbx1*, *Musculin* (*MyoR*), and *Tcf21* (*Capsulin*) are upstream activators of the critical muscle regulatory factors (MRFs), *Myf5*, *MyoD*, and *Myogenin*, in the branchial arches, but are not required for extraocular muscle formation (Brand-Saberi, 2005; Buckingham et al., 2003; Dastjerdi et al., 2007; Grenier et al., 2009; Grifone et al., 2008; Kelly et al., 2004; Lu et al., 2002; Robb et al., 1998). In fact, no upstream activators of the MRF myogenic cascade in the extraocular muscles have been identified, although *Pitx2* and *Pax7* have been proposed to play this role (Diehl et al., 2006; Mootoosamy and Dietrich, 2002).

The homeodomain transcription factor *Pitx2* is the only single gene shown to be required for extraocular muscle formation (Diehl et al., 2006; Gage et al., 1999; Kitamura et al., 1999). In the many organs in which *Pitx2* is required for development, it has been

implicated in controlling cell fate specification as well as cell proliferation and survival (Charles et al., 2005; Kioussi et al., 2002; Liu et al., 2003; Marcil et al., 2003; Quentien et al., 2002b; Rodriguez-Leon et al., 2008; Shih et al., 2007a; Suh et al., 2002). Mice lacking *Pitx2* function have no extraocular muscles and their formation is dependant on *Pitx2* gene dose (Diehl et al., 2006; Gage et al., 1999; Kitamura et al., 1999). Mouse embryos with approximately 20% of normal *Pitx2* dose also lack extraocular muscles (Figure 1.4). Mice heterozygous for a null allele of *Pitx2* have no oblique muscles and smaller rectus muscles and expression of the MRFs *Myf5*, *MyoD*, and *Myogenin* is reduced to 10-20% of their wildtype levels (Figure 1.5) (Diehl et al., 2006). A recent report of post-natal knockdown of *Pitx2* in the extraocular muscles also showed a dramatic loss of MRF expression levels (Zhou et al., 2009). Although *Pitx2* is required for muscle precursor survival in the branchiomeric muscles, no changes in cell death or proliferation were reported in *Pitx2*$^{-/-}$ extraocular muscle primordia, although only later timepoints were examined (Diehl et al., 2006; Dong et al., 2006; Shih et al., 2007a).

Pitx2 has other critical functions in eye development; it is expressed in both the mesodermal and neural crest lineages of the periocular mesenchyme (Evans and Gage, 2005; Gage et al., 2005). In addition to the loss of extraocular muscles, *Pitx2*$^{-/-}$ mice have thickened corneas with agenesis of the anterior segment, scleral agenesis, and hypoplastic hyaloid blood vessels. They also have non-cell autonomous defects in the development of the optic stalk and RPE (Evans and Gage, 2005; Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). Mice with reduced *Pitx2* levels also have eyelid closure defects and the optic cup is rotated ventrally (Adam Diehl, personal communication). Many of these structures receive contributions from both the neural crest and mesodermal lineages. Recent work has highlighted the complex interactions between neural crest and mesodermal cells in craniofacial development. The neural crest is important for patterning the developing mesoderm and inducing branchiomeric myogenesis, and may play a role in EOM development as well (Evans and Noden, 2006; Grenier et al., 2009; Noden and Trainor, 2005; Rinon et al., 2007). Similarly, the developing muscles signal to the neural crest to induce tendon formation (Grenier et al., 2009; Grifone et al., 2008; Murchison et al., 2007; Pryce et al., 2007). We recently described a neural crest-specific

knockout of *Pitx2* and found that these mice recapitulated many features of the global knockout. However, these mice have fused eyelids at e18.5, and the extraocular muscles are present and differentiated by e14.5. It was not possible to evaluate tendon formation in these mice because the globe of the eye, to which the muscles normally attach, is shifted in position relative to the EOMs (Evans and Gage, 2005).

To determine the mesoderm specific functions of *Pitx2* in eye development, we created mesoderm-specific knockout mice using the Cre-lox system. *T-Cre* was used to convert the *Pitx2^flox* allele to the *Pitx2^null* allele in all mesoderm at gastrulation (Perantoni et al., 2005). The resulting *Pitx2* mesoderm knockout mice (*Pitx2-mko*) lacked extraocular muscles, have eyelids that failed to close and have retinal coloboma at a reduced penetrance. To further investigate the EOM phenotype, we examined the fates of the former EOM precursors and discovered that these cells undergo apoptosis at e10.5 and that this process depends on *Pitx2* gene dose. In attempt to circumvent the requirement of *Pitx2* for cell survival, we used an inducible *CreER^TM* to delete *Pitx2* at various timepoints in development, but found that *Pitx2* continues to be required for cell survival.

Materials & Methods

Mouse Strains: The *Pitx2^flox* and *Pitx2^null* alleles were created from different recombination products from CMV-Cre transfected mouse R1 ES cells carrying a *Pitx2* allele containing a neomycin resistance cassette and three LoxP sites. The *Pitx2^flox* allele loses the PGK-*neo^R* cassette, but carries LoxP sites on either side of the 5th exons that encodes the homeodomain required for DNA binding, while the *Pitx2^null* allele loses the cassette and the 5th exon (Gage et al., 1999; Suh et al., 2002). The TgN(Cga-cre)S3SAC mice, referred to here as *αGSU-Cre*, were a gift from Sally Camper. This transgene contains nuclear-localized Cre cDNA with a β-actin polyadenylation sequence under the control of a 4.6 kb (-5000 to +46) promoter for the pituitary glycoprotein α subunit (Cushman et al., 2000). The Tg(T-cre)1Lwd mice, referred to here as *T-Cre*, were a gift from Mark Lewandoski. This transgene includes 650 bp promoter of *T*, including 500 bp

upstream of the transcriptional start site, the bacterial recombinase Cre with a nuclear localization sequence and a β-actin polyadenylation sequence (Perantoni et al., 2005). The Tg(UBC-cre/ESR1)1Ejb mice, referred to here as *UBC-CreER^{T2}*, were obtained from the Jackson Laboratories. This transgene contains Cre recombinase fused to a modified form (G400V/M543A/L544A) of the human estrogen receptor which binds tamoxifen specifically and is inactive in the absence of tamoxifen. This fusion protein is under the control of a 2 KB fragment of the human *UBIQUITIN C* promoter. The transgenic line was created through lentitransgenesis by lentiviral injection of one cell zygotes and is present in a single copy (Gruber et al., 2007; Ruzankina et al., 2007). The Tg(CAG-cre/Esr1)5Amc mice, referred to here as *Cagg-CreER^{TM}*, carry a transgene which also contains a Cre recombinase/modified estrogen receptor fusion protein under the control of a chicken β-actin promoter/enhancer coupled with the cytomegalovirus immediate-early enhancer (Hayashi and McMahon, 2002). These mice were obtained from the Jackson Laboratories. Gt(Rosa)26Sor mice, referred to here as *R26R*, carry a ubiquitously expressed Cre-reporter construct, which contains a floxed stop codon upstream of a LacZ expression cassette (Soriano, 1999). More detailed descriptions of all mouse lines used in these experiments can be found in the original references noted here.

Mouse Husbandry: Mice were mated to generate timed pregnancies. The relevant crosses include:

αGSU-Cre; Pitx2^{+/-} X Pitx2^{flox/+}; R26R/R26R,
T-Cre; Pitx2^{+/-} X Pitx2^{flox/+}; R26R/R26R,
Cagg-CreER^{TM}; Pitx2^{+/-} X Pitx2^{flox/flox}; R26R/R26R,
UBC-CreER^{T2}; Pitx2^{+/-} X Pitx2^{flox/flox}; R26R/R26R,
Pitx2^{+/-} X Pitx2^{+/-}

The morning after mating was designated as embryonic day 0.5. If indicated, a single intraperitoneal injection of tamoxifen (Sigma, T5648-1G) suspended in corn oil at a dose of 100 μg per gram body weight was administered to the pregnant dam at noon on the day noted. If noted, intraperitoneal injections of pifithrin-α (Alexis Biochemicals, ALX-270-287) suspended in PBS were administered daily beginning at e8.5 at a dose of 0.2 μg per gram of body weight. Embryos were collected by C-section after euthanizing the pregnant dam. The resulting embryos were genotyped for *Cre* or *Pitx2* using PCR-based

methods (Suh et al., 2002). The Cre genotyping includes IL-2 positive control primers (CTAGGCCACAGAATTGAAAGATCT and GTAGGTGGAAATTCTAGCATCATCC) and Cre-specific primers (GCGGTCTGGCAGTAAAAACTATC and GTGAAACAGCATTGCTGTCACTT) run for 42 cycles with an annealing temperature of 51˚C and an extension time of 1 minute, as per the Jackson Laboratories. All procedures involving mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

Histology: All embryos were fixed in 4% paraformaldehyde in phosphate buffered saline, washed, dehydrated, embedded in paraffin, and sectioned at 7 μm. Mounted sections for morphological analysis were dewaxed, rehydrated and stained with hematoxylin and eosin (H & E).

Immunofluorescence: Paraffin sections were stained as previously described (Evans and Gage, 2005). Primary antibodies against PITX2 (gift from T. Hjalt), β-galactosidase (Eppendorf 5-prime and a gift from T. Glaser), FOXC1 (ab5079, Abcam), FOXC2 (ab5060, Abcam), Collagen IV (2150-1470, Biogenesis), developmental myosin heavy chain (vp-M664, Vector), Ki67 (clone TEC3, Dako Cytomation), phospho-histone H3 (06-570, Upstate Biotechnology), MYOD (ab788, Abcam), and Myogenin (clone F5D, Santa Cruz). An appropriate fluorescently labeled secondary antibody (Jackson Immuno) or tyramide signal amplification kit (Molecular Probes) was used for detection.

In situ hybridization: Antisense riboprobes for *Pitx2* were generated and labeled with digoxigenin according to standard procedures (Martin et al., 2002). Paraffin sections were processed for *in situ* hybridization as previously described (Cushman et al., 2001). Probes were hybridized at 57˚C.

TUNEL staining: Terminal dUTP nick end labeling (TUNEL) was performed using an *In situ* Cell Death Detection kit (Roche) per manufacturer protocol. Briefly, paraffin

sections were dewaxed, rehydrated, and enzymatically treated with Proteinase K (PCR grade, Roche) at a final concentration of 10 μg/mL in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer for 15 min at 37°C. Slides were rinsed twice in PBS for 5 minutes and then incubated with the manufacturer specified mix of enzyme and labeling solution for 1 hour at 37°C in the dark. Slides were rinsed three times with PBS and either coversliped with ProLong Gold antifade reagent with DAPI (Molecular Probes) or followed with an antibody staining, beginning with the incubation of blocking solution, and proceeding as usual.

Imaging and Analysis: All imaging of sections was performed on a Nikon Eclipse 800 fluorescent microscope using ACT-1 software. Whole mount imaging was performed using a Leica MZ12.5 dissecting microscope with FireCam software. Images were analyzed using Photoshop 7.0 for Macintosh.

Statistics: To evaluate the possiblity that the percentage of cells undergoing cell death was different between wildtype and *Pitx2* heterozygote extraocular muscle primordia, the following analysis was undertaken. Three to four non-adjacent sections of three wildtype and four heterozygous e10.5 embryos were stained for TUNEL, PITX2 immunostaining, and DAPI to label nuclei. The number of TUNEL/PITX2/DAPI triple-positive cells in the location of the EOM precursors was divided by the number of PITX2/DAPI double-positive cells to generate the percentage of EOM precursors undergoing cell death. 9 total wildtype observations were compared to 15 total *Pitx2* heterozygote observations using a two-tailed student's t-test assuming equal variances.

Results

Mesoderm specific Pitx2 knockout mice

It was previously shown that mice with neural crest-specific knockout of *Pitx2* recapitulate most aspects of the *Pitx2* global knockout, except the extraocular muscles are unaffected (Evans and Gage, 2005). We hypothesized that the primary function of *Pitx2*

in the mesoderm lineage during eye development is to direct extraocular muscle formation. To test this hypothesis, mesoderm-specific *Pitx2* knockout mice were created using mice from one of two different Cre recombinase transgenic lines. *αGSU-Cre* is expressed in the cranial mesoderm as shown in Gage et al., 2005, but its expression is not fully penetrant (Cre recombinase activity is not seen in all cells), and we found that it was not expressed in the cranial mesoderm prior to the expression of *Pitx2* (Figure 3.1A, B)(Gage et al., 2005). Based on Cre-recombinase activity, mesoderm cells are initially found ventral to the optic vesicle (Figure 3.1A, B). By e11.5, the mesoderm condenses into a wedge shape, which breaks into individual muscle primordia beginning at e12.5 (Figure 3.1H, I). Other mesoderm cells are found in the hyaloid vasculature and adjacent to the optic fissure (Figure 3.1C). Mesoderm cells are also found in the developing eyelid mesenchyme beginning at e12.5, but the presence of unlabeled cells suggests that neural crest cells may contribute as well (Figure 3.1D, E).

αGSU-Cre+; *Pitx2$^{flox/-}$* mice were observed to have small or absent extraocular muscles, open eyelids at e16.5, and retinal coloboma (Figure 3.2A-D, data not shown), but all of these defects were seen with reduced penetrance, probably because the knockout of *Pitx2* was late and not fully complete. The underlying defect in eyelid closure was not identified, but it was not caused by changes in FOXC1 and FOXC2 expression, two transcription factors required for eyelid closure (Figure 3.2E-H) (Kidson et al., 1999; Kume et al., 1998; Smith et al., 2000). Although the mutant embryos often had a pale, blanched appearance, the ocular blood vessels, which are mesoderm derived and express *Pitx2*, were not dramatically affected (Figure 3.2B, M-P). The pale appearance may be connected with the requirement for *Pitx2* in the hematopoetic stem cell niche, which includes mesoderm-derived cells (Chagraoui et al., 2003; Kieusseian et al., 2006; Zhang et al., 2006). Since *Pitx2$^{+/null}$* embryos have an EOM phenotype, *Cre+; Pitx2$^{+/+}$* embryos were used as controls in all of the mesoderm specific knockout experiments.

To attempt to circumvent the problems caused by the late expression of *αGSU-Cre* relative to *Pitx2*, we used a *T-Cre* transgene that drives Cre expression using a 500

bp promoter of *T*, the mammalian homologue of *Brachyury*. *T-Cre* is expressed in all mesoderm at gastrulation, and Cre-recombinase activity is observed in the cranial mesoderm at e9.0 (Figure 3.1F, G) (Perantoni et al., 2005). The transgene is also ectopically expressed in some neural tissues and the lens, but *Pitx2* is not expressed in these locations, so it is not a concern (Figure 3.1H, I). We verified that *T-Cre* is active in the extraocular primordia using the *R26R* Cre-reporter allele (Figure 3.1H, I). We found that *T-Cre* activity, as indicated by the presence of β-galactosidase, was also not present in all cranial mesoderm cells that express *Pitx2*, but it was expressed in a greater percentage than *αGSU-Cre*. *T-Cre; Pitx2$^{flox/-}$* mesoderm specific knockout mice display the same ocular phenotypes as the *αGSU-Cre* knockouts, but with greater penetrance (Figure 3.2I-L). The extraocular muscles in particular were more severely affected; some mice had completely absent EOMs (Figure 3.2Q-T). This suggests that *Pitx2* may have critical functions at very early timepoints in EOM development. The *T-Cre Pitx2-mko* mice also displayed defects in body wall closure similar to those seen in global *Pitx2* knockout mice, although they did survive up to two days longer than the global knockouts, until e16.5 in one case (data not shown) (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Perantoni et al., 2005).

Pitx2 is required for EOM survival in a dose dependant manner

In order to determine what fates were adopted by the EOM precursors in the absence of functional *Pitx2*, we examined *Pitx2* mRNA expression. The *Pitx2^{null}* allele produces a stable mRNA transcript that can be identified by a *Pitx2* probe targeting the 3' UTR. However, any protein product produced by the mutant mRNA is either non-functional or degraded, because the homozygous null mice phenocopy other *Pitx2* knockout mice (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Perantoni et al., 2005). Although robust *Pitx2* mRNA expression is seen in the neural crest cells that surround the optic cup in *Pitx2$^{null/null}$* embryos at e10.5, little to no expression is seen in the location where the mesodermal EOM precursors are normally present (Figure 3.3A-C). The severe reduction in the number of EOM precursors could be caused by defects in proliferation or an increase in cell death.

To examine the possibility that the EOM precursors were lost to cell death, TUNEL staining was performed. In *Pitx2^{null/null}* embryos, a massive increase in cell death was observed in the region where *Pitx2*-expressing EOM precursors are normally found (Figure 3.3E, G). Cell death was also increased in *Pitx2^{+/null}* EOM precursors (Figure 3.3F), and the number of PITX2-positive cells that also labeled with TUNEL was significantly greater than wildtype littermates (p<0.007) (Figure 3.3D). No difference in apoptosis was observed at e9.5 or e11.5 (data not shown).

Because *Pitx2* has been implicated in cell proliferation and disruptions in cell cycle progression often lead to apoptosis (Charles et al., 2005; Hipfner and Cohen, 2004; Kioussi et al., 2002; Kleinschmidt et al., 2009; Martinez-Fernandez et al., 2006), cell proliferation was examined in the EOM precursors at both e9.5, prior to the observed apoptosis, and at e10.5. Ki67, which marks all proliferating cells, and phospho-histone H3, which marks all cells in metaphase, were used to label proliferating cells (Paulson and Taylor, 1982). The number of cells proliferating in the wildtype EOM primordia was generally low, but no differences were seen between mutant and wildtype embryos (Figure 3.3H-O).

Pitx2 is required at multiple timepoints for EOM precursor survival

Other data generated by our lab suggests that *Pitx2* has a role in EOM specification as well as survival (see Chapter 4)(Diehl et al., 2006). However, *Pitx2^{null/null}* EOM precursor cells undergo apoptosis before they express any markers of muscle specification (see Chapter 4). To determine if *Pitx2* has functions in later EOM development, we endeavored to create a temporal knockout of *Pitx2* using a ubiquitously expressed CreER^{TM} as diagrammed in Figure 3.4. We initially selected the *Cagg-CreER^{TM}* transgene, which has been used successfully by many groups (Hayashi and McMahon, 2002). We bred *Cagg-CreER^{TM}+; Pitx2^{+/null}* males to *Pitx2^{flox/flox}* females and injected the pregnant dams with the appropriate dose of tamoxifen at e10.5. Of 91 resulting pups, only 3 were *Cre+;Pitx2^{flox/null}* and none were *Pitx2^{flox/+}* without the transgene. All embryos were harvested at e14.5 or earlier, which is within the window of

viability for $Pitx2^{null/null}$ embryos. Based on these numbers, we conclude that $Pitx2$ and the $Cagg$-$CreER^{TM}$ transgene are in linkage, approximately 3 cM apart.

To effectively induce a temporal knockout of $Pitx2$, we switched to using the UBC-$CreER^{T2}$ transgene, which is driven by the ubiquitin promoter (Gruber et al., 2007; Ruzankina et al., 2007). The results seen were identical to those observed in the few mutants that were obtained with the $Cagg$-$CreER^{TM}$ transgene. Although wild type $Pitx2$ mRNA has been shown to be relatively unstable unless positively regulated, it was not known how long it would take for the existing PITX2 protein to decay. Others have shown effective induction of Cre recombinase activity as soon as 6 hours post-injection, and we observed almost complete loss of PITX2 protein expression 24 hours post-injection (Figure 3.5A, C). The vast majority of cells expressed no PITX2 protein, but a few cells had very weak labeling with the PITX2 antibody, suggesting that PITX2 expression was still decaying. A very small number of cells had robust PITX2 expression, suggesting that Cre was either not expressed or not active. For all temporal knockout experiments UBC-$CreER^{T2}$+; $Pitx2^{flox/null}$ mice were used as mutants, and because $Pitx2^{+/null}$ mice have a reduced EOM phenotype, $Pitx2^{flox/null}$ littermates without the transgene were used as controls.

Apoptotic EOM precursors are seen in $Pitx2^{null/null}$ embryos at e10.5, indicating that the requirement for $Pitx2$ for cell survival begins earlier, possibly at e9.5. To test this hypothesis, we deleted $Pitx2$ expression at e9.5 by injecting timed pregnant dams with tamoxifen. In contrast to the T-Cre; $Pitx2$ knockout mice, the expression of $Pitx2$ can be initiated normally in the EOM precursors at e8.5 (Shih et al., 2007b). EOM development was assessed at e10.5 and e11.5. No markers of myogenic development were seen and $Pitx2$ mRNA expression was absent from the mesodermal wedge at e11.5 (Figure 3.5E-H). Increased TUNEL staining was seen in the mesodermal cells at e10.5 (Figure 3.5B, D), indicating that apoptosis occurred rapidly after the deletion of $Pitx2$. No change in apoptosis was observed at e11.5 (data not shown) and areas of reduced cellularity were often found in the normal location of the EOM precursors, indicating that the dead cells had already been cleared. This indicates that $Pitx2$ expression in the cranial mesoderm

prior to e9.5 is not sufficient to prevent cell death, and that *Pitx2* is required for EOM precursor survival prior to the visualization of apoptosis by TUNEL staining at e10.5.

To determine if survival of the EOM precursors could be rescued by *Pitx2* expression at e9.5, and to evaluate the role of *Pitx2* in activating muscle specification at e11.5, we delayed the ablation of *Pitx2* until e10.5. Timed pregnant dams were injected with tamoxifen at e10.5 and embryos were examined at both e11.5 and e14.5 for presence of EOM precursors and markers of myogenesis. In eight of eight eyes from four mutants examined at e11.5, the EOM precursors could be identified by *Pitx2* mRNA expression, and some expressed MYOD and MYOG (Figure 3.6A-L). The size of the mesodermal wedge of EOM precursors in mutants was generally smaller than that of the controls, and there were some regions that did not express any MRFs (Figure 3.6E, H, K, arrowheads). TUNEL staining in the EOM wedge was comparable between the control and mutant embryos (Figure 3.6M-O).

By e14.5, embryos with loss of *Pitx2* function at e10.5 had little to no extraocular muscle, as indicated by the almost complete absence of MYOD and MYOG expression (Figure 3.7G-L). The small amount of muscle present was associated with cells that retained PITX2 expression (Figure 3.7C), and the muscle is differentiated based on myosin heavy chain expression (Figure 3.7O). There is almost no expression of *Pitx2* mRNA, indicating that most of the EOM precursors are gone (Figure 3.7D-F). Because the mutant eyes were sometimes sunken (Figure 3.8I), more distal sections through the eye were also examined, but no evidence of extraocular muscle precursors was found (data not shown). The precursor cells have probably undergone apoptosis, but no increase in TUNEL staining was seen at e14.5 (Figure 3.8D-F), presumably because the dead cells have already been cleared. A large decrease in the number of cells surrounding the optic nerve was observed, which further indicates that cell death has taken place (Figure 3.8A-C). The reduced number of cells caused the optic nerves of mutant mice to be shifted closer to the oculo-sphenoid bone of the skull. This indicates that EOM precursors require *Pitx2* for survival, even after e10.5.

EOM cell death is not p53 mediated

In an attempt to prevent the cell death that occurs in the absence of *Pitx2*, we treated timed-pregnant *Pitx2^{null}* females with pifithrin-α, a chemical inhibitor of p53, a critical mediator of apoptosis (Culmsee et al., 2001; Komarov et al., 1999). Although p53 is not implicated in normal developmental apoptosis, it may be important for abnormal developmental apoptosis, like we see in the *Pitx2* mutant mice. p53 is required for the cell death that is seen in *Tcof1* mutant mice, a model for Treacher Collins Syndrome (Jones et al., 2008). Although mice were treated with pifithrin-α beginning at e8.5, well before the onset of apoptosis in the EOM primordia of *Pitx2* mutant embryos, it was unable to prevent apoptosis in the mutant EOM precursors, because reduced EOM primordia were seen at e11.5 (Figure 3.9I-K). Other sites of developmental apoptosis, such as the optic cup and trigeminal ganglion, were also not affected by pifithrin-α (Figure 3.9A-H), implying that p53 function is not required for either normal or *Pitx2*-deficient developmental apoptosis.

Discussion

We used mesoderm-specific and temporal *Pitx2* knockout mice to identify mesoderm-specific functions of *Pitx2* in the development of the extraocular muscles, eyelids, and optic fissure. We have identified *Pitx2* as a survival factor in mesoderm-derived extraocular muscle precursors.

Pitx2 is required in the mesoderm for eyelid and optic fissure closure

We have identified requirements for *Pitx2* in the processes of eyelid closure, failure of which results in open eyelids at birth, and optic fissure closure, failure of which results in coloboma. The mesoderm specific requirement of *Pitx2* in eyelid closure is somewhat surprising. *Pitx2* is expressed in both the mesoderm of the eyelid mesenchyme and cells that do not label as mesoderm, which are presumably neural crest (Figure 3.1E). In the anterior segment of the eye, *Pitx2* is required to activate expression of *Dkk2* in the

neural crest cells. Mice lacking *Dkk2* have anterior segment defects as well as a failure of eyelid closure. *Dkk2* mutant eyelids lose FOXC1 and FOXC2 expression in the conjunctival epithelium of the eyelid, but this expression is unaffected in *Pitx2-mko* mice. Although the expression of *Dkk2* in the eyelids has not been fate mapped, this indicates that *Pitx2* expression in the mesoderm of the eyelids does not mediate closure by activating *Dkk2* (Gage et al., 2008). The eyelids also have the filopodia-like periderm extensions that are associated with closure, so the mechanism of failure is unclear (Fujii et al., 1995; Tao et al., 2005). It may be due to reduced proliferation of the eyelid mesenchyme, which prevents the eyelids from extending enough to close. This suggests that *Pitx2* may have functions in both the neural crest and mesoderm lineages that are required for eyelid closure.

The requirement for *Pitx2* in closure of the retinal fissure further extends our knowledge of the non-cell autonomous functions of *Pitx2*. *Pitx2* global and neural-crest specific knockout mice have severe optic nerve defects that resemble coloboma, but retinal coloboma has not been previously reported (Evans and Gage, 2005; Gage et al., 1999). Since, *Pitx2* is not expressed in the retina, this indicates that it has non-cell autonomous functions in the mesoderm as well as in the neural crest during eye development. Cells expressing *Pitx2* surround the optic cup and there is a small patch of mesodermal cells adjacent to the optic fissure at e12.5. The signals that induce closure of the optic fissure could come from these cells, or possibly from the hyaloid vasculature, which is also adjacent to the optic fissure. *Pitx2* function in the mesodermal lineage is not required for the formation of the hyaloid vasculature of the eye. The number of vessels may be slightly reduced, but any differences are difficult to quantify with the methods used here. This indicates that *Pitx2* influences ocular blood vessel formation primarily through the neural crest-derived pericytes (Evans and Gage, 2005).

Pitx2 is required for survival of extraocular muscle precursors

Previously, it has been shown that *Pitx2* is required for the formation of the extraocular muscles and that EOM size and number depend on *Pitx2* dose (Diehl et al.,

2006; Gage et al., 1999; Kitamura et al., 1999). However, no underlying mechanism had been identified, and a requirement for *Pitx2* function in the neural crest for extraocular muscle development had been ruled out (Evans and Gage, 2005). We have demonstrated that *Pitx2* is required in the mesoderm lineage for extraocular muscle formation and that *Pitx2* expression in the mesodermal EOM precursor cells is required for their survival in a dose dependant manner. This provides a mechanism to explain why EOM size and number are correlated with *Pitx2* dose, and further emphasizes the critical role for *Pitx2* in EOM development. The requirement for *Pitx2* in EOM precursor survival parallels the role of *Pax3/Pax7,* which are redundantly required for muscle precursor survival in the somites (Relaix et al., 2005).

The requirement for *Pitx2* in EOM precursor survival begins at approximately e10.0. In *Pitx2^{null/null}* embryos, cell death is seen in the EOM precursors at e10.5, but not earlier. TUNEL staining marks sheared DNA, the final stage of apoptosis, indicating that the process of cell death initiated earlier. Similarly, embryos that lose *Pitx2* function shortly after e9.5 have apoptosis in the EOM precursor cells at e10.5. This indicates that although *Pitx2* is expressed in the cranial mesoderm prior to e8.5, it is not required for survival until approximately e10.0. *Pitx2* is also required in the mesoderm of the first branchial arch for the survival of those muscle precursors at an earlier stage (Dong et al., 2006; Shih et al., 2007a). TUNEL staining is seen at e9.5 in the branchial arches of *Pitx2* mutant mice, prior to the expression of other myogenic markers (Shih et al., 2007a). The difference in timing may be due to the fact that myogenesis is delayed in the EOMs relative to the branchial arches (Kelly et al., 2004; Noden and Francis-West, 2006). The requirement for *Pitx2* in the survival of myogenic precursors may represent an important developmental step in myogenesis. The pathways that activate apoptosis in both normal and aberrant development are not well understood (reviewed in (Mirkes, 2008)). The mechanism by which loss of *Pitx2* function causes cell death in the EOM precursors remains to be identified. There are many pathways that lead to cell death, but the pathway activated in *Pitx2* mutant EOMs does not appear to be mediated by p53.

More work needs to be done to pinpoint the time window in which *Pitx2* is required for EOM precursor survival. We have identified the initial requirement at e9.5 and determined that it is still required after e10.5. Muscle-specific post-natal knockout of *Pitx2* did not result in any changes in extraocular muscle mass or fiber number at three weeks post-natal, although cell death was not specifically assessed (Zhou et al., 2009). This indicates that *Pitx2* is not required post-natally for the survival of the extraocular muscles. The latest timepoint at which *Pitx2* is still required for EOM precursor survival can be determined experimentally using the temporal knockout system by injecting tamoxifen at various timepoints between e11.5 and e18.5. The fact that *Pitx2* continues to be required for survival in extraocular muscle development indicates it is not merely required for a single developmental checkpoint, but plays an active role in cell survival, which is notable for a homeodomain transcription factor in a non-neural cell type.

Pitx2 is required for cell survival in the development of other tissues, besides the extraocular muscles. It is required for the survival of branchial arch muscle precursors and Rathke's pouch, the precursor to the pituitary. In these tissues, *Pitx2* is implicated in regulating other transcription factors that are required for survival, *Musculin* and *Lhx3*, respectively (Charles et al., 2005; Dong et al., 2006; Lu et al., 2002; Shih et al., 2007a). There are no transcription factor candidates to play this role in extraocular muscle development; all known factors are either expressed later in EOM development than the observed cell death, or have no proven requirement in EOM formation. While an as yet unknown survival factor may exist, this suggests that *Pitx2* may play a more direct role in regulating cell survival.

Pitx2 has been implicated in regulating proliferation and the cell cycle by affecting c-Jun, CyclinD1 and CyclinD2 expression and mRNA stability, and *Pitx2* has been demonstrated to affect proliferation in the C2C12 limb muscle progenitor cell line (Briata et al., 2003; Kioussi et al., 2002; Martinez-Fernandez et al., 2006). Cell cycle progression and apoptosis are intimately linked—cells that fail to progress in the cell cycle are often induced to undergo apoptosis (Hipfner and Cohen, 2004; Kleinschmidt et al., 2009). Although we were not able to detect changes in markers of proliferation in

Pitx2^{null/null} EOM precursors, delays in cell cycle progression in the absence of *Pitx2* may be the underlying cause of apoptosis. However, the function of *Pitx2* in cell survival is cell-type specific. Tooth buds and the neural crest portion of the ocular mesenchyme require *Pitx2* for their normal development, but not survival (Evans and Gage, 2005; Kitamura et al., 1999; Liu et al., 2003). This further highlights the differences in *Pitx2* function between the two ocular lineages and indicates that these cell types may have different survival requirement or different co-factors that affect *Pitx2* function.

In many tissues, *Pitx2* is required for cell fate specification as well as control of proliferation and survival (Charles et al., 2005; Dong et al., 2006; Shih et al., 2007a). The continued requirement for *Pitx2* in the survival of extraocular muscle precursors has made it difficult to determine if *Pitx2* has other functions in EOM development. Although the *Pitx* genes are expressed too late to play a role in activating myogenesis in the somites, the early expression of *Pitx2* in the EOM and branchial arch muscle precursors makes it a strong candidate to activate myogenesis there (L'Honore et al., 2007; Shih et al., 2007b). Our data do not contradict this hypothesis. The fact that some of the EOM precursors fail to express MRFs suggest that *Pitx2* may be required for MRF expression. While some of the EOM precursors that lack PITX2 protein express MYOD and MYOG, it is not clear how long PITX2 has been absent in these cells. Prior to its decay, PITX2 may have already activated the expression of the MRFs or other events that lead to their expression. It may be necessary to treat satellite cells derived from *UBC-Cre+; Pitx2^{flox/null}* adult EOMs with tamoxifen to determine if *Pitx2* is required for MRF activation, although we cannot be sure that *Pitx2* is not required for satellite cell survival.

There are other additional functions *Pitx2* could have in extraocular muscle formation. The mechanisms that control the compaction of the EOM precursors into a wedge and then split them into 7 different muscles are not known. The *Pitx2* temporal knockout mice that were induced at e10.5 have a typical mesodermal wedge at e11.5, suggesting that *Pitx2* may not affect cell adhesion at this stage. However, it is possible that PITX2 protein may not have been absent long enough to affect cell adhesion. *Pitx2* could also be important for the formation of separate muscle primordia or specifying

differences in the fibers of the global and orbital layers. It may be possible to assess these potential functions with later temporal knockouts of *Pitx2*, or it may be that the only way to determine the function of *Pitx2* in these processes is to identify and inhibit the pathway that causes apoptosis in the absence of *Pitx2*.

Acknowledgements

Figure 3.1: Mesoderm-specific Cre expression patterns. *R26R*-labeled *αGSU-Cre* (**A-E**) and *T-Cre* (**F-I**) control embryos. *αGSU-Cre* recombinase activity is absent from the mesoderm at e9.0, but PITX2 is already present (**A**). Recombinase activity driven *αGSU-Cre* is first observed in the mesoderm at e10.0 (**B**). Mesodermal cells are found adjacent to the optic fissure (**C**, arrowhead) and in the hyaloid vasculature (**C**, arrow), as well as in the eyelid mesenchyme (**D**) at e12.5. At e16.5, some of the PITX2-positive cells in the eyelid are mesoderm labeled (**E**, arrow), while others are not (**E**, arrowhead). The absence of Cre-recombinase activity in large areas of the eyelid mesenchyme indicates a neural crest contribution. *T-Cre* expression is seen in the cranial mesoderm as at e9.0 (**F**) and e10.5 (**G**) (red arrows, eye is marked in red). A high percentage of the mesodermal wedge (arrow) show *T-Cre* recombinase activity at e11.5 (**H**) and in the developing extraocular muscles (**I**, arrows) at e12.5, indicating the transgene is effective. PITX2 expression in the neural crest cells is seen surrounding the optic cup (**H**, **I**, arrowheads). Ectopic expression of *T-Cre* transgene is also seen in the neural tube (N), retina (R), and lens (L), but these are not areas of PITX2 expression. Cornea (C). Images **F** and **G** are from Perantoni et al., 2005.
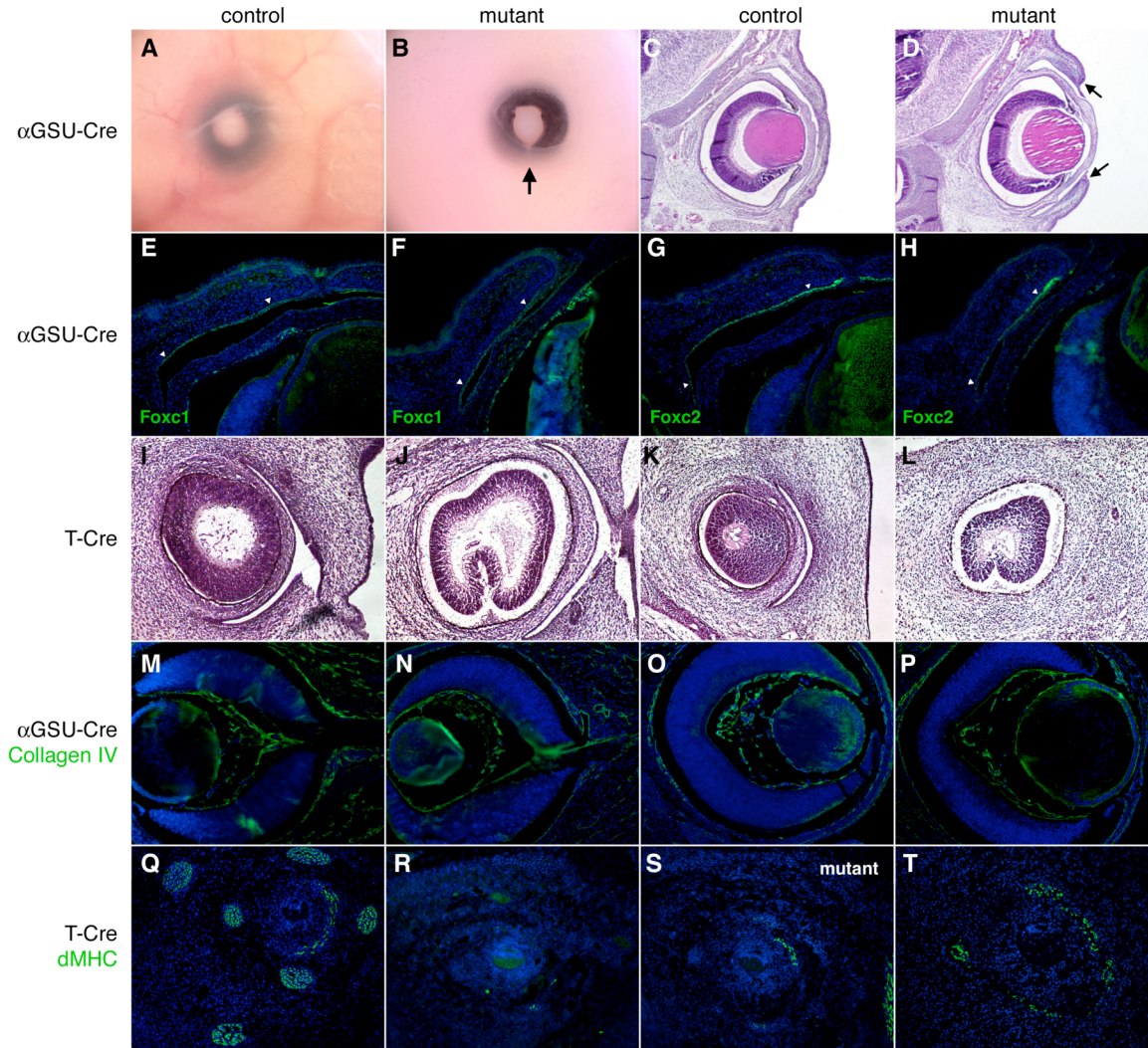
Figure 3.2: *Pitx2* **mesoderm-specific knockout phenotype.** Control (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, **Q**) and mutant (**B**, **D**, **F**, **H**, **J**, **L**, **N**, **P**, **R**, **S**, **T**) mesoderm-specific *Pitx2* knockout embryos. *αGSU-Cre*; *Pitx2-mko* mice have open eyelids, despite the presence of periderm (**A-D**, arrows), and retinal coloboma (**B**, arrow). The open eyelid defect is not due to changes in FOXC1 (**E**, **F**, arrowheads) or FOXC2 (**G**, **H**, arrowheads) expression. *T-Cre; Pitx2-mko* embryos also have retinal coloboma (**I-L**), seen in sagittal sections. *Pitx2-mko* mice do not have obvious defects in hyaloid blood vessel formation, labeled here with Collagen IV (**M-P**). The mice have absent (**R**) or severely reduced (**S**, **T**) extraocular muscles, as compared to the control (**Q**). Embryos in **A-H** are e16.5, **I-L** are e15.5, **M-P** are e14.5, **Q-S** are e14.5 and **T** is e15.5.
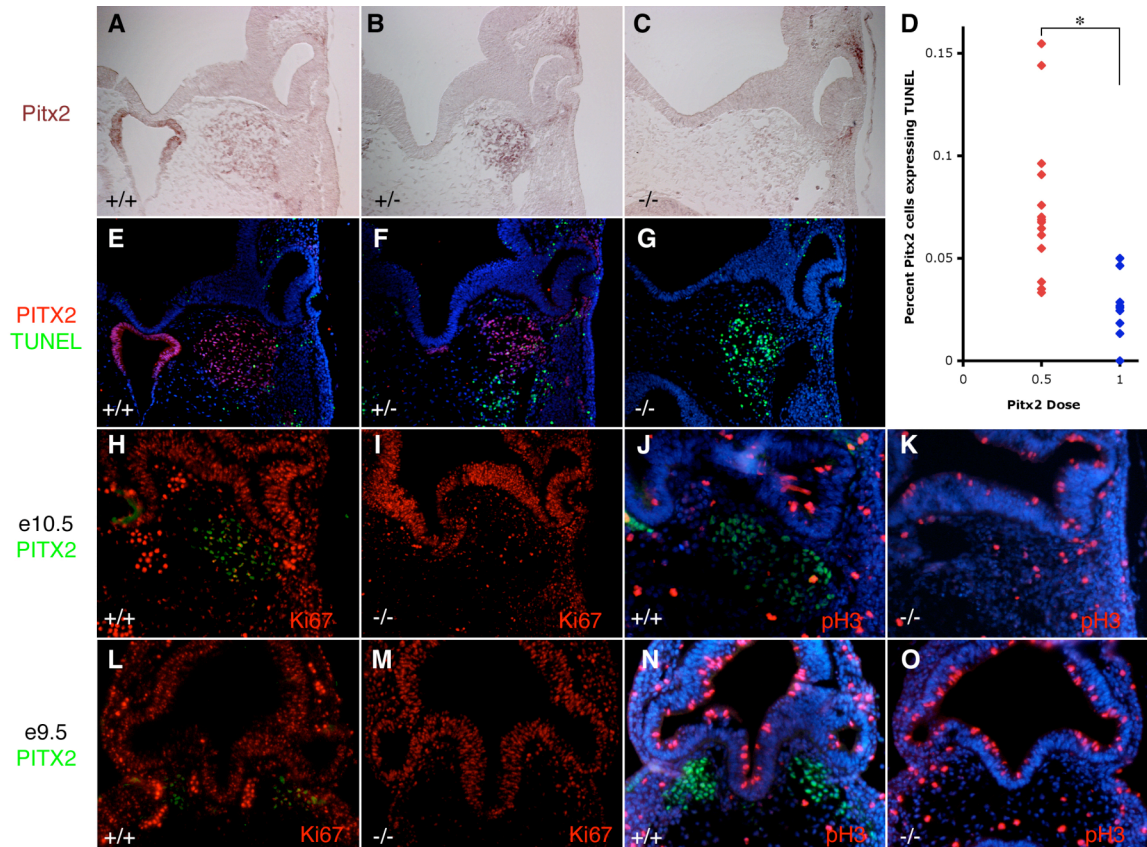
Figure 3.3: Cell death and proliferation in *Pitx2^null* extraocular muscle primordia.
Wildtype (**A**, **E**, **H**, **J**, **L**, **N**), Heterozygote (**B**, **F**) and mutant (**C**, **G**, **I**, **K**, **M**, **O**) *Pitx2^null*
embryos. *Pitx2* mRNA is lost in the mesoderm of *Pitx2^-/-* embryo at e10.5(**C**) but not the
neural crest surrounding the optic cup, as compared to the wildtype (**A**) and heterozygote
(**B**). A massive increase in TUNEL staining is seen in the mutant (**G**) where the
mesoderm is normally found in the wildtype (**E**). The heterozygous *Pitx2^+/-* mice also
have an increase in apoptosis in the PITX2-labeled mesoderm (**F**), which is statistically
significant, (**D**, *p<0.007). Proliferation is unchanged in the EOM primordia of e10.5
Pitx2^-/- embryos by Ki67 (**H**, **I**) and phospho-histone H3 (**J**, **K**) staining. Proliferation is
similarly unaffected at e9.5 (**L-O**).
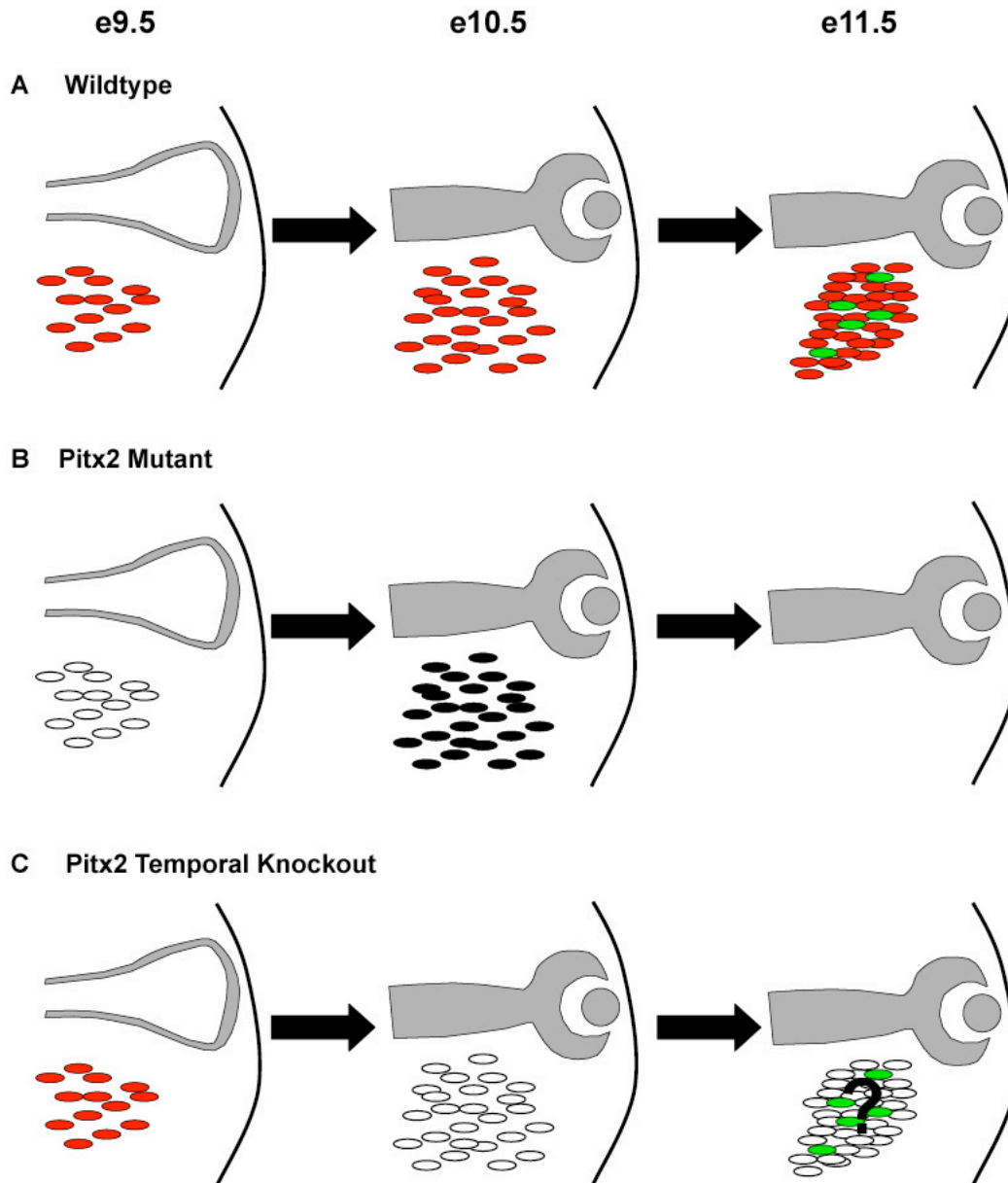
Figure 3.4: Strategy for temporal knockout of *Pitx2*. In wildtype embryos, PITX2-positive extraocular muscle precursors (red) increase in number between e9.5 and e10.5 (**A**). These precursors condense into a wedge shape and activate the expression of MRF proteins (green) by e11.5 (**A**). In *Pitx2^{null/null}* mutants, apoptotic cells (black) are seen at e10.5, indicating that the process of cell death was initiated earlier due to the absence of PITX2 (**B**). The loss of the EOM precursors makes it impossible to evaluate the role of PITX2 in MRF activation *in vivo*. Using a ubiquitously expressed *CreER^{T2}*, *Pitx2* was deleted after the initial timepoint when it is required for EOM precursor survival, so the role of *Pitx2* in MRF activation and continued precursor survival could be evaluated (**C**).
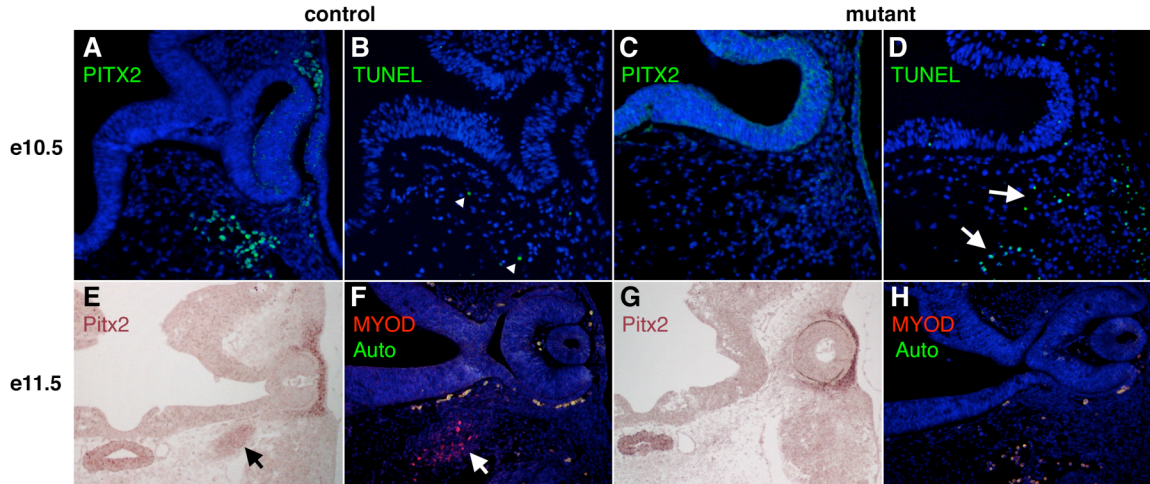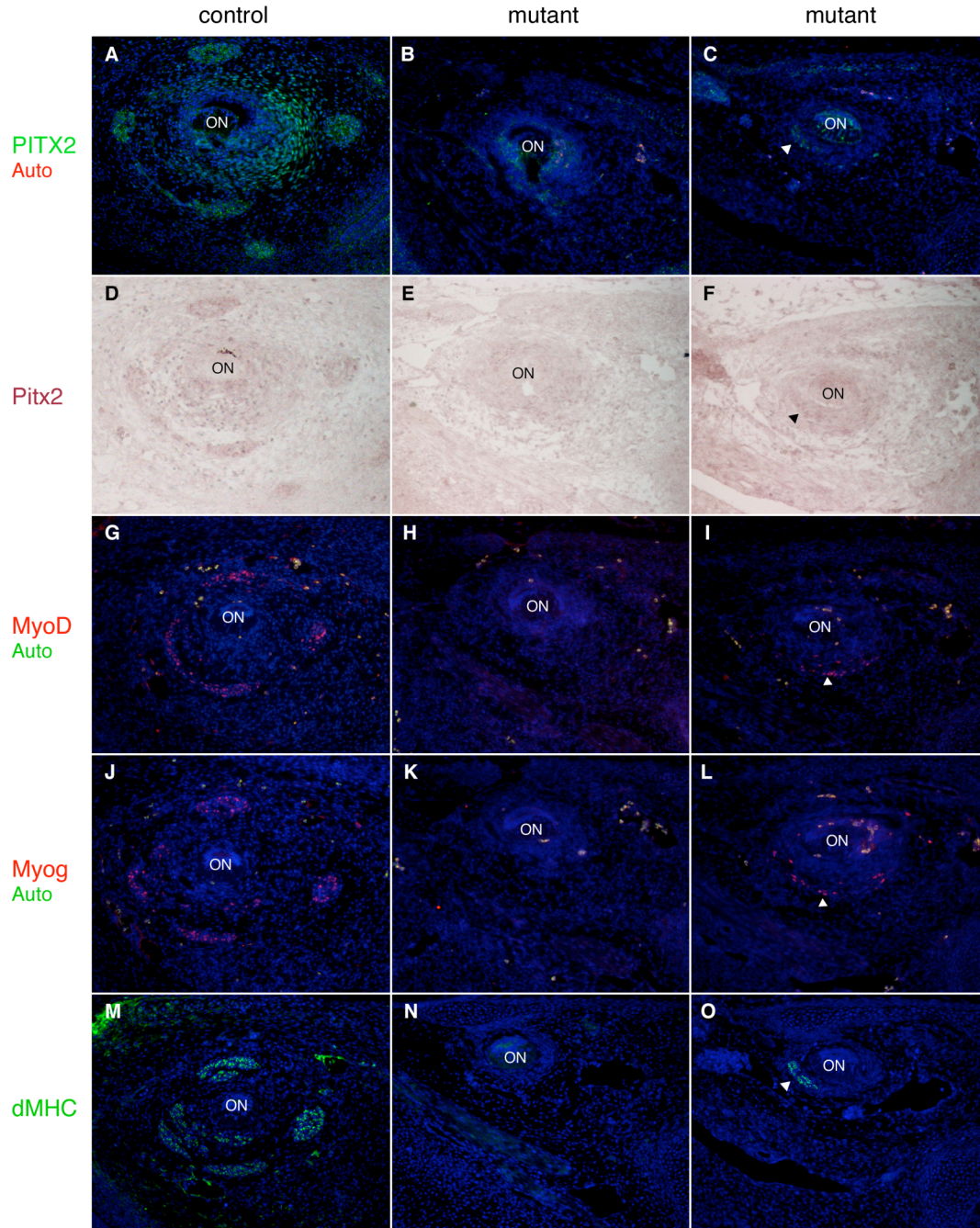
Figure 3.5: Temporal knockout of *Pitx2* at e9.5. Control (**A**, **C**, **E**, **G**) and mutant (**B**, **D**, **F**, **H**) *Ubiquitin-CreER^TM* embryos. Tamoxifen-induced knockout of *Pitx2* at e9.5, results in efficient knockout of PITX2 protein (**A**, **C**), and an increase in cell death at e10.5 as indicated by TUNEL staining (arrows, **B**, **D**). By e11.5, the EOM primordia are absent in the mutants as indicated by *Pitx2* mRNA expression (**G**) and MYOD expression (**H**), as compared to the control embryo (arrows, **E**, **F**). Autofluorescent red blood cells are shown in green for **F** and **H**.
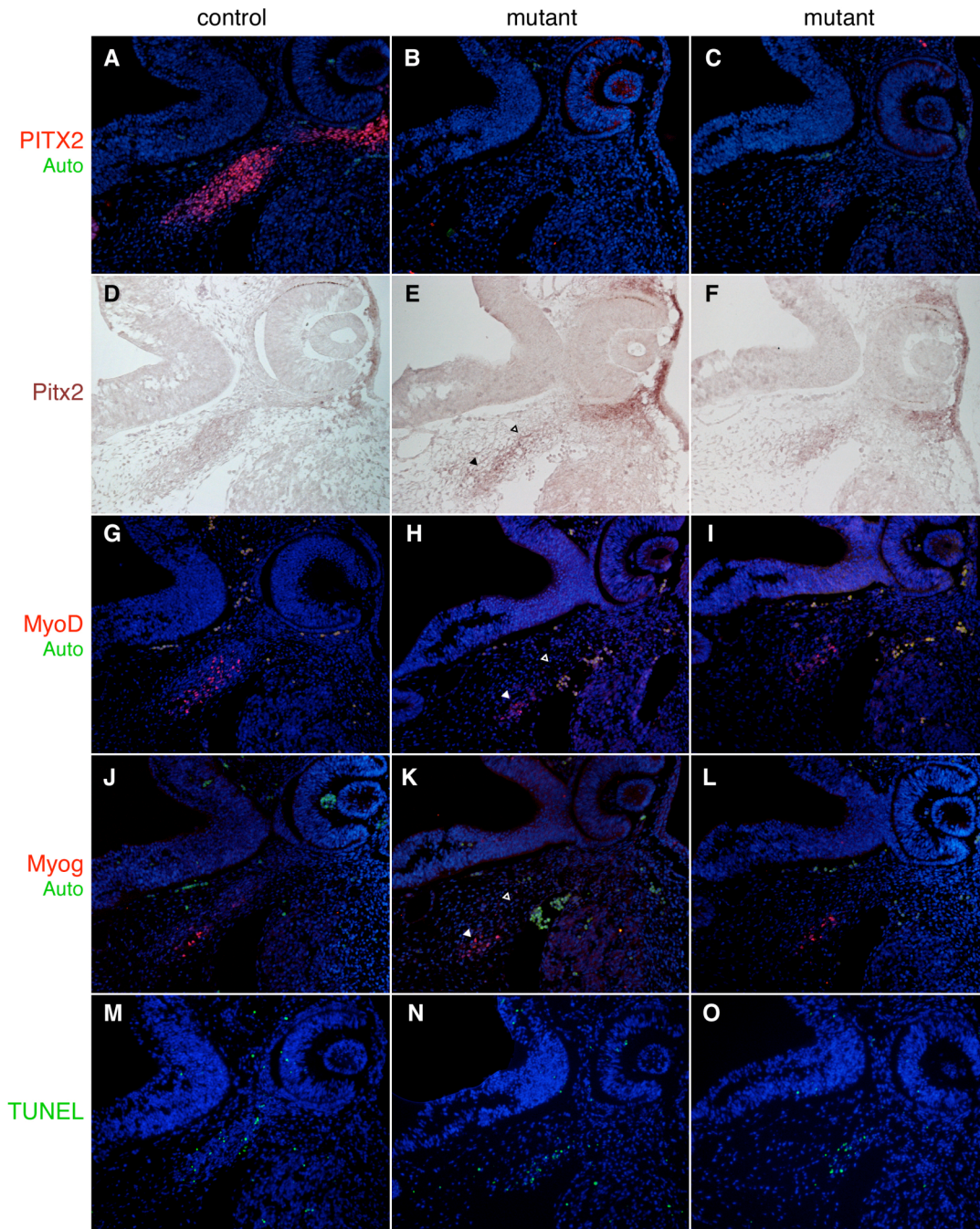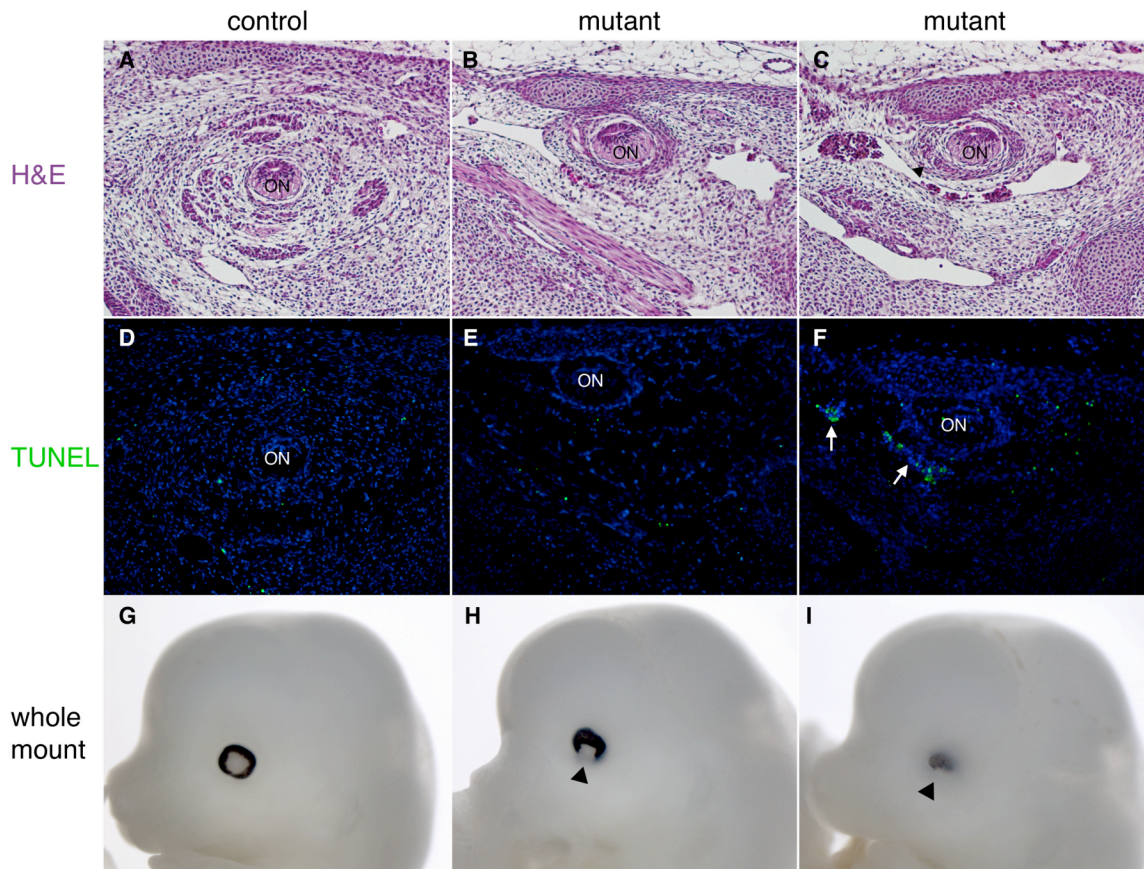
Figure 3.6: Temporal knockout of *Pitx2* results in reduced MRF expression.
Representative control (**A**, **D**, **G**, **J**, **M**) and Mutant (**B**, **C**, **E**, **F**, **H**, **I**, **K**, **L**, **N**, **O**)
Ubiquitin-CreER^{TM} e11.5 embryos. Tamoxifen-induced knockout of *Pitx2* at e10.5
results in efficient knockdown of PITX2 protein (**A-C**). Extraocular muscle primordia are
still present at e11.5 by *Pitx2* mRNA expression (**E**, **F**), but are generally smaller than
controls (**D**). MYOD (**G-I**) and MYOG (**J-L**) are lost in some EOM precursors (**H**, **K**,
arrowheads), although patches of expression remain (**H**, **I**, **K**, **L**), possibly because
insufficient time has elapsed to see the full effects of PITX2 loss. TUNEL staining labels
a comparable number of cells in controls (**M**) and mutants (**N**, **O**). Autofluorescent red
blood cells are shown in green (**A-C**, **G-L**).

Figure 3.7: Temporal knockout of *Pitx2* eventually results in the loss of extraocular muscle precursors. Representative control (**A**, **D**, **G**, **J**, **M**) and mutant (**B**, **C**, **E**, **F**, **H**, **I**, **K**, **L**, **N**, **O**) *Ubiquitin-CreER^TM* e14.5 embryos. Tamoxifen-induced temporal knockout of *Pitx2* at e10.5 results in efficient loss of PITX2 protein by e14.5 (**B**) as compared to controls (**A**), except in a small patch of cells (**C**, arrowhead). However, all *Pitx2* mRNA expression is lost in the extraocular muscles (**D**, **E**), except for a small patch of cells (**F**, arrowhead). In one eye, this patch of cells is associated with MYOD, MYOG and developmental myosin heavy chain (dMHC) expression (**I**, **L**, **O**, arrowheads), while the expression of these genes is lost in the other mutant eye (**H**, **K**) as compared to the control (**G**, **J**).

Figure 3.8: Temporal knockout of *Pitx2* eventually results in the loss of extraocular muscle and disrupted eye development. Representative control (**A**, **D**, **G**) and mutant (**B**, **C**, **E**, **F**, **H**, **I**) *Ubiquitin-CreER^{TM}* e14.5 embryos, which were treated with tamoxifen at e10.5. Hematoxylin and Eosin staining reveals that the mutant optic nerves (**B**, **C**, labeled ON) are shifted closer to the adjacent oculo-sphenoid bone (labeled B) of the cranial vault and there are fewer cells in the regions where the EOM are normally present (**A**). Very few TUNEL positive cells are found in the control or mutant embryos by e14.5 (**D-F**, arrows in **F** indicate autofluorescent red blood cells). Whole mount views of the embryos show that the mutant eyes are slightly ventrally rotated and have retinal coloboma (**H**, arrowhead) or a sunken appearance (**I**, arrowhead).
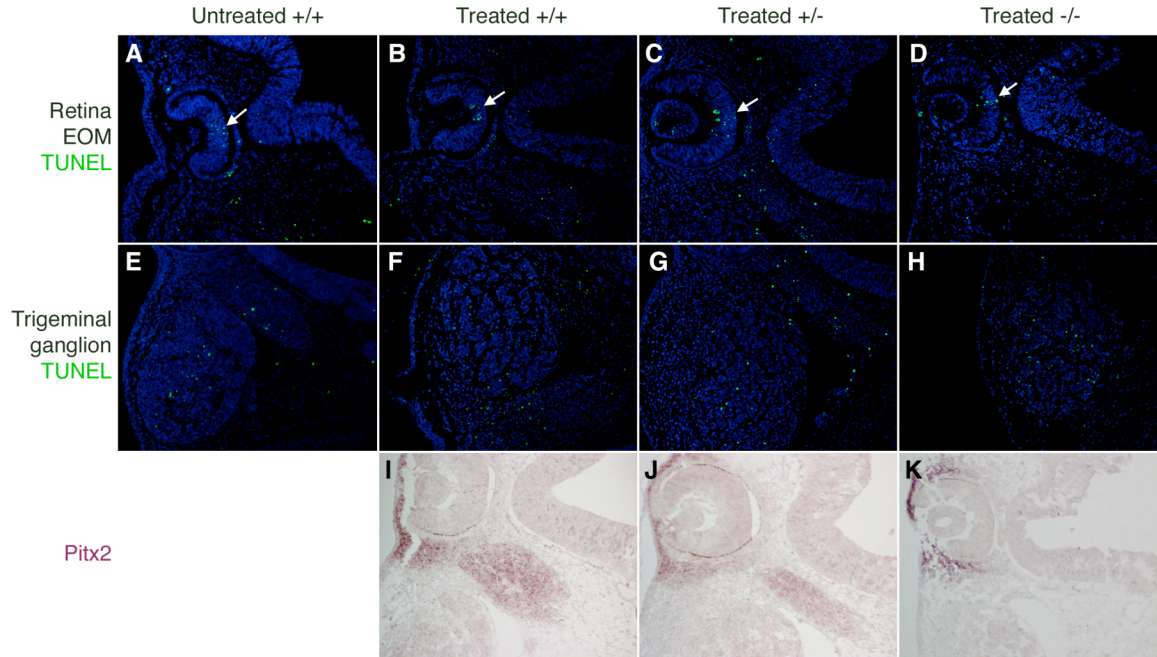
Figure 3.9: Treatment with a p53-inhibitor does not prevent EOM precursor death in the absence of *Pitx2*. Untreated wildtype (**A**, **E**) and pifithrin-α treated wildtype (**B**, **F**, **I**), heterozygote (**C**, **G**, **J**) and *Pitx2$^{-/-}$* (**D**, **H**, **K**) e11.5 embryos. Treatment with p53 inhibitor pifithrin-α does not prevent naturally occurring cell death in the retina (**A-D**, arrows) or trigeminal ganglion (**E-H**) at e11.5 as indicated by TUNEL staining. Cell death in the EOM primordia of *Pitx2* mutants is inferred to be unchanged at e10.5, because the EOM primordia are reduced in the heterozygote (**J**) and completely absent in the null (**K**) at e11.5, based on *Pitx2* mRNA expression.

Chapter 4: *Pitx2* regulates expression of the muscle regulatory factors in extraocular muscle development

Introduction

Extraocular muscles (EOMs) have many properties that make them unique among the skeletal muscles (reviewed in (Porter, 2002; Spencer and Porter, 2006). In contrast to other skeletal muscles, the function of the EOMs, to move the eye, requires a constant load, which has enabled their specialization and unique physiology. The EOMs have evolved to be extremely fast, precise and fatigue-resistant in order to meet the demands of binocular vision. They have unique muscle fiber types, including multiply innervated fibers that can undergo graded, non-propagating contractions. EOMs also have unique gene expression profiles, which include the presence of embryonic and cardiac muscle proteins as well as higher levels of enzymes that lead to improved calcium homeostasis and reduced oxidative stress relative to other skeletal muscles (Khanna et al., 2003; Porter et al., 2006). Some of these unique properties make the EOMs resistant to many forms of muscular dystrophy, while the need for extreme precision can cause visual deficits if the EOMs are not functioning perfectly, as seen in strabismus or "cross eyes"(Gronlund et al., 2006; Porter et al., 2003b).

Not surprisingly, the development of these unique muscles has many aspects that distinguish it from the development of other skeletal muscles (reviewed in Noden and Francis-West, 2006). The EOMs form from the most anterior portion of the mesoderm and are not derived from somites like trunk muscles or the branchial arches (BAs) like the other cranial muscles. The initiation of myogenesis in the EOMs is delayed relative to muscles derived from the somites and branchial arches, as indicated by the expression of the muscle regulatory factors (MRFs), *Myf5*, *MyoD* and *Myogenin* (*Mrf4* is not expressed in the head) (Kelly et al., 2004). The MRFs are so named because they initiate a genetic cascade that can convert differentiated cells to muscle and they are required for muscle

development (Braun et al., 1989a; Braun et al., 1989b; Edmondson and Olson, 1989; Thayer et al., 1989; Weintraub et al., 1989). *Myf5* and *MyoD* can each induce myogenic specification, while their downstream target *Myogenin* acts primarily to induce differentiation (Arber et al., 1994; Rudnicki et al., 1993). The MRFs activate the myogenic program in all skeletal muscle lineages, but the upstream transcription factors that regulate their expression are different between muscle populations. *Pax3* and *Pax7*, and to a lesser extent *Six1* and *Six4*, activate MRF expression in the somites (Bajard et al., 2006; Grifone et al., 2005; Relaix et al., 2005). *Tbx1*, *Musculin* (*MyoR*), and *Tcf21* (*Capsulin*) activate MRF expression in the branchial arches (Dastjerdi et al., 2007; Kelly et al., 2004; Lu et al., 2002). However, no upstream activator of MRF expression in the EOMs has been identified. Many of the genes demonstrated to activate MRF expression in other locations are absent in the EOMs (*Pax3*, *Tcf21*) or not required for their normal development (*Tbx1*, *Musculin*) (Grenier et al., 2009; Kelly et al., 2004; Lu et al., 2002). *Six1*/*Six4* double mutant mice have small EOMs, indicating that they may play a role, but are not required for MRF expression (Grifone et al., 2005). Other genes important for myogenesis, such as *Eya1*, *Eya2*, and *Dach2*, are expressed in the EOMs, but a role in EOM development has not been reported (Davis et al., 2008; Davis et al., 2001; Grifone et al., 2007; Xu et al., 1997).

Two genes, *Pax7* and *Pitx2*, have been proposed to be the upstream activator of MRF expression in EOM development (Diehl et al., 2006; Mootoosamy and Dietrich, 2002). *Pax7* is a homeodomain transcription factor in the *paired* family. During skeletal muscle development, it acts primarily to specify a population of muscle precursor cells as satellite cells, the muscle stem cells (Seale et al., 2004; Seale et al., 2000). In the absence of *Pax3*, *Pax7* acts as an activator of MRF expression during myogenesis (Relaix et al., 2004, 2005). Unlike *Pax3*, *Pax7* is expressed in the cranial mesoderm, so it is an excellent candidate to activate MRF expression there (Horst et al., 2006; Mootoosamy and Dietrich, 2002). However, *Pax7* expression lags behind MRF expression in the branchial arches, and *Pax7* mutant mice are reported to have normal head muscle formation (Horst et al., 2006; Relaix et al., 2004). The functional role of *Pax7* in EOM development remains to be determined.

Pitx2 is another homeodomain transcription factor proposed to regulate MRF expression in the EOMs. *Pitx2* is required in a dose-dependent manner for extraocular and branchiomeric muscle precursor survival (Diehl et al., 2006; Dong et al., 2006; Shih et al., 2007a). *Pitx2* is also expressed in somite-derived muscles, but it is not required for their development, possibly because of redundancy with paralogs *Pitx1* and *Pitx3* (Marcil et al., 2003). Although it has not been possible to prove an *in vivo* requirement for *Pitx2* in MRF expression during development because of the survival requirement, there are indications that it is important. In *Pitx2^{+/null}* heterozygous mice, expression of *Myf5*, *MyoD*, and *Myogenin* is reduced to 21%, 14%, and 13%, respectively, of wildtype levels, even though the reduction in the number of muscle precursors is not as severe (Figure 1.5). Terminal muscle differentiation is also reduced in these mice, as indicated by the presence of myosin heavy chain (Diehl et al., 2006). Recently, a post-natal muscle specific knockout of *Pitx2* was reported, and these mice have drastically reduced expression of *Myf5*, *MyoD*, and *Myogenin* 21 days after *Pitx2* deletion. Other muscle specific proteins were affected on a much longer time scale (3 months), leading the authors to suggest that *Pitx2* might directly regulate the MRFs. They also report that *Pitx2* expression is found in satellite cells, muscle stem cells that lie quiescent until they are activated to express the MRFs, proliferate and differentiate (Zhou et al., 2009).

Here, we examine the potential for *Pitx2* and *Pax7* to directly regulate the MRFs in the extraocular muscles. We found that *Pitx2* is expressed prior to *Pax7* and the MRFs and that mice lacking *Pax7* function have normal pre-natal EOM development. We show that *Pitx2* binds the promoters of *Myf5*, *Myod1*, and *Myogenin* and can activate the *Myod1* promoter, demonstrating that *Pitx2*, not *Pax7*, directly activates MRF expression in the extraocular muscles.

Materials and Methods

Mice: Mice carrying the *Pax7^{tm2Pgr}* allele, referred to here as *Pax7^{LacZ}*, were a gift from Michael Rudnicki. These mice have a *LacZ-neo* cassette inserted in frame into the first

exon of the paired box of *Pax7*. This disrupts the expression of the protein and generates β-galactosidase staining which recapitulates the *Pax7* expression pattern (Mansouri et al., 1996; Seale et al., 2000). Pax7$^{LacZ/+}$ or wildtype mice were mated to generate timed pregnancies. The morning after mating was designated as embryonic day 0.5. Embryos were collected by C-section after euthanasia of the mother and genotyped using PCR-based methods. The Pax7 genotyping protocol uses one forward (GGGCTTGCTGCCTCCGATAGC), and two reverse primers (GTGGGGTCTTCATCAACGGTC and TCGTGCTTTACGGTATCGCCGCTCCCG) and a PCR program which requires 65°C annealing temperature and a two minute extension time for 35 cycles. All procedures involving mice were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

Immunofluorescence: All embryos were fixed in 4% paraformaldehyde in phosphate buffered saline, washed, dehydrated, embedded in paraffin, and sectioned at 7 μm. Sections were dewaxed, rehydrated and stained as previously described (Evans and Gage, 2005). Primary antibodies against PITX2 (gift from T. Hjalt), PAX7 (developed by A. Kawakami and obtained from NICHD/Developmental Studies Hybridoma Bank), MYOD (Ab 788, Abcam), Myogenin (clone F5D, Santa Cruz), and MYF5 (SC-302, Santa Cruz) were used.

Immuocytochemistry: Cells were plated on coverslips in normal media, grown overnight, and fixed with cold methanol for 15 minutes. Cells were washed three times in PBS for five minutes and incubated with antibody block for one hour, then incubated with the primary antibody overnight. Cells were washed three times in PBS for 5 minutes, incubated in an appropriate fluorescently labeled secondary antibody (Jackson Immuno) diluted 1:500 in antibody block, washed again three times in PBS for 5 minutes and mounted to slides with ProLong Gold antifade reagent (Molecular Probes). Primary antibodies against PITX2 (gift from T. Hjalt) and MYOD (Ab 778, Abcam) were used.

Cell culture: CHO (Chinese Hamster Ovary) and C2C12 limb muscle precursor cells were obtained from ATCC (Puck et al., 1958; Yaffe and Saxel, 1977). They were grown in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with antibiotics and L-glutamine plus 10% fetal bovine serum (GIBCO), and passaged every two to three days. The C2C12 cells were never allowed to reach greater than 80% confluency, in order to prevent spontaneous differentiation. mEOM primary mouse extraocular muscle cells were obtained from Henry Kaminski (Porter et al., 2006). They were grown on plates coated with 0.5% gelatin (Sigma, #G1393), in F-10C media (GIBCO) supplemented with 15% horse serum (GIBCO), 2 ng/mL FGF (Sigma, #F0291), 1.2 mM $CaCl_2$, and antibiotics. These cells regularly took 5-7 days to reach 80% confluency and were supplemented with fresh media every other day.

Chromatin Immuno Precipitation (ChIP): C2C12 and mEOM cells were grown to 80% confluency and subjected to ChIP assays as previously described (Gummow et al., 2006). Briefly, cells were fixed to cross-link DNA and proteins, then nuclear extracts were prepared. The resulting chromatin was sheared with a sonicator and verified to be an average of 500 bp in length. The chromatin fragments were immunoprecipitated, heated to reverse the cross-linking and purified. For immunoprecipitation, two polyclonal antibodies specific for PITX2 were used (Santa Cruz, goat C-16 & rabbit H-80), as well as control antibodies as previously described (Gage et al., 2008). Purified DNA fragments were analyzed by PCR using the primers described in Table 1. The PCR program used for all primers has a 59.5°C annealing temperature with a 45 second extension time.

Vectors, Cloning and Mutagenesis: The PITX2 expression constructs in the pCI-HA tag vector were a gift from Kathy Kozlowski and Michael Walter and have been previously described (Kozlowski and Walter, 2000). The names of mutations T30P, K50E, and R53P indicate the position of the mutation within the homeodomain, not the full protein. A -2.5 kb human *MYOD1* promoter in the pGL3-basic luciferase reporter vector was a gift from David Goldhamer. The -2.5 kb promoter was previously described (Goldhamer et al., 1992). An analogous fragment of the mouse *Myod1* promoter (-2.7 kb promoter)

and a shortened version (-1.6 kb promoter) were generated by high-fidelity PCR of a mouse BAC containing the *Myod1* gene (RP23 149N5, ResGen), with primers tagged with restriction sites to facilitate cloning into the XhoI and SpeI sites of the pFL-basic luciferase reporter vector (Table 2). The pFL-basic vector has the same backbone as the pGL3-basic vector, but a different multiple cloning site. A 317 bp *Myod1* minimal promoter, which contains 124 bp upstream of the transcriptional start site and the 5'UTR cloned into in the pFL-basic vector, was a gift from Jeff Ishibashi and Michael Rudnicki. Deletion and mutagenesis of the *Myod1* minimal promoter were carried out using the QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer protocol. Mutagenesis primers were designed using the manufacturer's website: http://www.stratagene.com/sdmdesigner/default.aspx. For the mutagenesis of PITX2 sites, non-complementary transversion was used to convert every other base pair to the pyridine/purine it does not complement (i.e. A to C, G to T), a common method of binding site mutagenesis (Scott Barolo, personal communication). For deletions, a standard primer design program was used to create primers that could be used to amplify the sequence to be deleted. The reverse complement of the second primer was attached to the first and vice-versa to create two long primers that form a bridge, forcing the polymerase to omit the sequence between them. The sequence for all primers used for the mutations and deletions of the *Myod1* minimal promoter are listed in Table 3. All mutations and deletions were verified with sequencing, and an average of 4/5 clones were correct.

Luciferase Assays: Transfections were carried out in CHO or C2C12 cells using FuGENE6 (Roche) according to standard techniques. Cells were transfected with various *Myod1* promoter-luciferase reporter plasmids and either an expression vector encoding human wildtype or mutant PITX2A protein or empty pCI-HA vector (a gift from Michael Walter). For all conditions, the constitutively expressing renilla vector pPolIII-RL was included as a control for transfection efficiency (Nybakken et al., 2005). Cells were lysed 48 h after transfection, and luciferase and renilla levels assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Normalized luciferase values were compared to the promoter-luciferase reporter transfected with the

empty HA vector to determine the extent of activation over background levels. Results are presented as relative activity and are expressed as mean±S.D. of three experiments, each performed in triplicate. Additionally, for the mutagenesis/deletion analysis of the *Myod1* minimal promoter, three individual clones of each mutation/deletion were analyzed to control for accidental mutagenesis of the backbone vector sequence. All reporters showed activation greater than the empty reporter vector (data not shown).

Results

Pax7 is dispensable for EOM formation

Pax7 has been shown to activate MRF expression in other muscles, and proposed to activate MRF expression in the developing extraocular muscles (Mootoosamy and Dietrich, 2002; Relaix et al., 2004, 2005). Therefore, we examined extraocular muscle development in *Pax7*$^{LacZ/LacZ}$ mutant embryos (Mansouri et al., 1996). The extraocular muscles of these embryos were histologically normal (data not shown) so MRF expression was examined. *Pax7*$^{LacZ/LacZ}$ mutant embryos were found to have normal expression of MYF5, MYOD, MYOG and PITX2 at e12.0 (Figure 4.1A-H). By e14.5, all seven EOMs were present and differentiated normally, as indicated by the presence of developmental myosin heavy chain (Figure 1I, J). This shows that *Pax7* is not required for MRF activation in EOM development. The role of *Pax7* in EOM satellite cell formation and post-natal development remains to be examined.

Pitx2 is expressed prior to the muscle regulatory factors

The exclusion of *Pax7* as an activator of MRF expression in the extraocular muscles leaves *Pitx2* as the only proposed candidate. However, the hierarchy of gene expression was not known in mouse extraocular muscle precursors. To determine the timing of activation of myogenic genes in the extraocular muscle, their expression was examined at different stages in wildtype mice. PITX2 is expressed in the EOM primordia as early as e8.5, while expression of MYF5 is seen in a small patch of cells at e10.5 and

the other myogenic genes, PAX7, MYOD, and MYOG are not seen until e11.5 (Figure 4.2). This indicates that *Pitx2* is temporally upstream of the MRFs. PITX1, homologue of *Pitx2*, is also found in a subset of extraocular muscle precursors beginning at e11.5, but it is not required for EOM development (Adam Diehl, personal communication). At e12.5, MYOD and MYOG are co-expressed with PITX2 in many cells, while others express only MYOD or MYOG (Figure 4.2 F, I), indicating that some cells may downregulate *Pitx2* expression once the MRFs have been activated. Experiments to prove that all extraocular muscle cells express *Pitx2* during their development remain to be done (the proper reagents only recently became available), but the absolute requirement for *Pitx2* in extraocular muscle formation suggests that this is the case (Donna Martin, personal communication).

Pitx2 binds MRF promoters

We hypothesized that *Pitx2* is directly activating the muscle regulatory factors. The previously characterized promoters of *Myf5*, *MyoD*, and *Myogenin*, which were shown to drive expression in the EOMs, were examined, and predicted PITX2 binding sites were identified in each of them (Figure 4.3) (Cheng et al., 1995; Goldhamer et al., 1995; Goldhamer et al., 1992; Patapoutian et al., 1993). Chromatin immunoprecipitation was used to determine if PITX2 was binding these promoters in two cell types. Both the C2C12 muscle precursor cell line, which is derived from mouse lower limb satellite cells, and a mouse EOM (mEOM) primary cell line, which is derived from neonatal (p4-6) extraocular muscle, were examined (Porter et al., 2006; Yaffe and Saxel, 1977). It was first verified that both cell lines express PITX2 protein endogenously (Figure 4.4). In the ChIP experiments, sequences that were enriched in the PITX2 IP over a control IgG IP, as shown by increased PCR product, were considered to be bound by PITX2. PITX2 binds specific regions with predicted PITX2 binding sites in the *Myf5*, *MyoD*, and *Myogenin* promoters (Figure 4.5). In the *Myod1* promoter, PITX2 binds the more proximal B and C regions, but not the distal A region or the 258 bp *Myod1* enhancer. In the *Myf5* promoter, PITX2 binds the B and D regions, but not the A and C regions. The more proximal regions of the *Myog* promoter are also bound by PITX2. Generally, the

same sites were bound in both the C2C12 and mEOM cells, although a few sites in the *Myog* promoter were bound in C2C12 cells but not in the mEOM cells (Figure 4.5E, F). This is likely an artifact caused by the close spacing of the *Myog* ChIP regions (Figure 4.3); regions B, C, and D are only separated by a few hundred base pairs, and the resolution of ChIP is considered to be about 1 kb. It should be noted that chromatin immunoprecipitation cannot discriminate between direct protein binding to DNA or indirect binding via a co-factor. These results confirm that PITX2 interacts with the promoters of the MRF genes.

Pitx2 activates the MYOD1 promoter

To determine whether PITX2 binding to the MRF promoters might be functionally significant, we tested the human -2.5 Kb *MYOD1* promoter, described by Goldhamer et al. that was used to drive *LacZ* expression in the developing EOMs (Figure 4.3A) (Goldhamer et al., 1995; Goldhamer et al., 1992). We selected MyoD1 as a representative MRF to test here because it is involved in both the specification and differentiation of muscle (Blais et al., 2005; Cao et al., 2006; Weintraub et al., 1989). Luciferase promoter assays found that the *MYOD1* promoter responds to increasing doses of PITX2-expression vector, but not to mutant forms of PITX2 that have been shown to be transcriptionally deficient (Figure 4.6). Furthermore, the *MYOD1* promoter responds at greater levels over baseline in the C2C12 muscle precursor cell line than in the CHO Chinese hamster ovary cell line (Figure 4.6). These data indicate that PITX2 can activate the *MYOD1* promoter and that the muscle cell line may have factors that enhance this activation.

A minimal Myod1 promoter fragment contains the PITX2-responsive element

To localize which part of the *Myod1* promoter is responding to PITX2, we carried out serial deletions of the promoter (Figure 4.7A). Because the ChIP experiments that identified PITX2 binding to the *Myod1* promoter were carried out using mouse cells, the mouse sequence was used for all subsequent experiments. The mouse -2.7 kb *Myod1*

promoter responded to PITX2 dose in a similar manner as the human promoter in C2C12 cells (Figure 4.7B). A 1.1 kb region containing the A ChIP region was deleted from the distal end of the *Myod1* promoter to create the -1.6 kb promoter (Figure 4.7A). The -1.6 kb promoter responds to PITX2 dose at levels equal to the full length promoter, as expected since PITX2 does not bind the A region. A further deletion of 1.5 kb containing the B ChIP region yields the -0.12 kb promoter, a 317 bp fragment of the *Myod1* promoter that contains 124 bp upstream of the transcriptional start site and the 5'UTR (Figure 4.7C). Although PITX2 binds the B ChIP region of the *Myod1* promoter, its deletion does not affect the ability of the -0.12 kb promoter to respond to PITX2 dose. This identifies a minimal 317 bp *Myod1* promoter that responds to PITX2 and contains the ChIP C region, which binds PITX2. It remains to be determined if the B ChIP region is sufficient for PITX2 promoter activation in the absence of the PITX2-responsive site(s) in the minimal promoter.

Identification of the PITX2 responsive site in the minimal Myod1 promoter

The *Myod1* minimal promoter contains two predicted PITX2 binding sites; one is conserved between mouse and human, the other is not (Figure 4.7C, 4.9A). To assess the functionality of these sites, we mutagenized each site by non-complementary transversion of every other base pair (Table 3). Mutagenesis of the conserved predicted PITX2 binding site in ChIP site C did not affect the ability of the minimal *Myod1* promoter to respond to PITX2 (Figure 4.8). Similarly, a non-conserved predicted PITX2 binding site in the 5' UTR was not required for PITX2 responsiveness (data not shown).

With all predicted PITX2 binding sites ruled out, we sought to localize the PITX2 responsive region(s) to either the promoter region upstream of the *Myod1* start site or the *Myod1* 5' UTR (Figure 4.9A). Deletion of the promoter region upstream of the *Myod1* predicted start site resulted in an almost complete loss of PITX2 responsiveness, while deletion of the 5'UTR had no effect (Figure 4.9B). The 83 bp promoter region was divided into two overlapping halves (Deletions A and B), which were each deleted. Both

halves are required for PITX2 responsiveness, which indicates that there are either multiple responsive sites, or a single site in the overlapping region (Figure 4.9C).

To further narrow the location of the PITX2 responsive region(s), scanning adenine mutagenesis of 7-9 bp regions in the *Myod1* promoter region was carried out. Six of seven mutations analyzed responded to PITX2 dose statistically the same as the wildtype construct (Figure 4.9D). The mutation of one 8 bp region in the area of overlap between Deletions A and B resulted in significantly reduced PITX2 response from wildtype levels, indicating that it is required for PITX2 responsiveness (Figure 4.9D). The center of this region is 80 bp from the transcriptional start site. The sequence of this region (with 4 base pairs on either side) is (CCCG)CCCCCAGC(CTCC) and the reverse complement is (GGAG)GCTGGGGG(CGGG). This region does not resemble any known PITX2 binding sites or contain the TAAT sequence associated with stereotypical homeodomain binding sites (Berger et al., 2008). It does contain SP1, KLF15, and MAZ binding sites, all of which are involved in transcription in both muscle and non-muscle cell types (Genomatix, William Zacharias, personal communication) (Aiba et al., 2008; Almeida-Vega et al., 2009; Cullingford et al., 2008; de Leon et al., 2005; de Wolf et al., 2006; Figliola et al., 2008; Fisch et al., 2007; Gray et al., 2002; Himeda et al., 2008; Otteson et al., 2005; Wu et al., 2007). Several of the adenine mutations have yet to be analyzed.

Discussion

Here we have excluded *Pax7* as a regulator of myogenic development in the extraocular muscles and showed that the *Pitx2* expression pattern is consistent with a role in MRF activation in extraocular muscle. We have demonstrated that PITX2 binds MRF promoters and activates the *Myod1* promoter through a novel site. The ability of PITX2 to activate the promoters of *Myf5* and *Myog* remains to be determined.

Pax7 is not required for MRF activation

We have shown that *Pax7* is not required for the activation of MRF expression in the extraocular muscles. We also showed that *Pax7* is not required for the differentiation of the extraocular muscles, although its potential role in later developmental functions was not examined. *Pax7* may have functions in late EOM development, because *Pax7* is required for normal post-natal muscle size and fiber growth in somitic muscles. Another function of *Pax7* is to specify satellite cells during somitic muscle development (Seale et al., 2000). The EOMs and several other craniofacial muscles have high numbers of satellite cells per fiber as compared to other muscles, but the factors that lead to this increased number are not known (Karpati et al., 1988). The role of *Pax7* in satellite cell formation in the EOMs and other craniofacial muscles has not been investigated (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000); the differences between craniofacial and trunk myogenesis make this an important area for future study. With the elimination of *Pax7* as a potential activator of the MRFs in the developing extraocular muscles, this promotes *Pitx2* as the likely candidate, since other known activators of MRF expression are either not expressed or not required for EOM development.

Pitx2 activates MRF expression

Pitx2 is the remaining proposed candidate to activate MRF transcription in the extraocular muscle precursors. We have identified several lines of evidence that indicate *Pitx2* has an essential role in activating MRF transcription. We have shown that PITX2 binds the promoters of *Myf5*, *Myod1*, and *Myogenin* in a specific manner in both limb and extraocular muscle precursor cells, which is consistent with the hypothesis that they are direct targets of *Pitx2*. The function of PITX2 in binding these promoters may be to activate transcription, as we showed with the *Myod1* promoter. Wild-type PITX2 can transactivate the promoter *in vitro*, while transcriptionally dead mutations in PITX2 have no effect on the *Myod1* promoter. The promoter responds to PITX2 more robustly in a muscle precursor cell line than in an unrelated cell-line, indicating that PITX2 has one or

more muscle specific co-factor(s) that enhances its ability to activate the *Myod1* promoter. The human *MYOD1* promoter responds with greater fold activation than the mouse *Myod1* promoter to the same doses of PITX2 in C2C12 cells. While it is possible that differences in the cloning vector used can account for the change, it is not known how *in vitro* differences in promoter activation actually affect cellular processes like cell fate specification. It may be that the *MYOD1* promoter does respond more strongly *in vivo*, but that both are sufficient to activate the myogenic program, especially since it is self-perpetuating.

We have localized the PITX2-responsive regions to an 8 bp sequence in an 83 bp region upstream of the *Myod1* transcriptional start site. The exact site(s) and their mechanisms of function remain to be identified, and these will need to be verified as PITX2-binding regions with electromobility shift-assays (EMSA). None of the regions contain identifiable PITX2 binding sites. This suggests that PITX2 may be binding a novel site or interacting with a co-factor that modifies its binding site. It may even be that PITX2 does not bind DNA directly, but is acting as part of a transactivation complex. The identified 8 bp region does include binding sites for the transcription factors, SP1, KFL15, and MAZ. This raises the possiblity that these factors may be part of such a complex. These factors can be functionally evaluated for their presence in CHO and C2C12 cell lines, their ability to enhance PITX2-dependant transcription of *Myod1*, and their ability to bind PITX2. Few proteins have been identified that directly interact with PITX2, and no muscle specific factors have been identified (Amendt et al., 1999; Berry et al., 2006; Huang et al., 2009; Vadlamudi et al., 2005). Immunoprecipitation of PITX2 followed by mass-spectrometry analysis or a yeast two-hybrid using a muscle specific library could be used to identify such a co-factor.

Although we have shown that PITX2 can activate MRF expression, there is no direct *in vivo* evidence that *Pitx2* is required for MRF activation in developing EOM primordia because of the prior requirement for PITX2 in EOM precursor survival (see Chapter 3). However, all current evidence is consistent with a *Pitx2* requirement for MRF activation. *Pitx2*$^{+/null}$ heterozygous mice lose some EOM cells because of cell death, but

have an even greater reduction of MRF expression levels in the remaining EOMs (Diehl et al., 2006). Post-natal knockout of *Pitx2* in EOMs results in a severe loss of MRF expression (Zhou et al., 2009). Mice that lose *Pitx2* expression at e10.5 have patches of EOM precursors with no MRF expression. All of these phenotypes are consistent with *Pitx2*-dependent MRF activation.

Functions of Pitx genes in muscle development

Similar to its functions in extraocular muscle development, *Pitx2* is required for both organ precursor survival and cell fate specification in the pituitary and first branchial arch (Charles et al., 2005; Dong et al., 2006; Shih et al., 2007a). *Pitx2* has been shown to activate *Tbx1* expression in the first branchial arch, and is likely to activate MRF expression directly as it does in the EOMs (Shih et al., 2007a). *Pitx2* has also been shown to increase proliferation in myogenic cells in culture, but this function has not been verified *in vivo (Kioussi et al., 2002; Martinez-Fernandez et al., 2006)*. Other genes that activate the MRFs display this multifunctionality. *Pax3* and *Pax7* in the somites are required for normal proliferation, survival and MRF activation (Collins et al., 2009; Relaix et al., 2006; Relaix et al., 2005). Mice lacking both *Musculin* and *Tcf21* lose MRF expression in the first branchial arch, which is followed by apoptosis (Lu et al., 2002). Our results show that *Pitx2* plays multiple roles in extraocular muscle development, similar to other upstream activators of myogenesis.

While *Pitx2* plays critical upstream roles in myogenesis in the head, it is first expressed after the initiation of myogenesis in the somites (L'Honore et al., 2007; Shih et al., 2007b). Although it cannot be the initial activator of MRF expression in the somites, our results with the C2C12 limb muscle precursor cells indicate that *Pitx2* may help maintain MRF expression. In developing trunk and limb muscles, PITX2 expression overlaps with MYOD and MYOG expression, as well as PAX3 and PAX7, which is consistent with this role (L'Honore et al., 2007; Shih et al., 2007b). However, *Pitx2*$^{null/null}$ embryos have no defects in somite-derived muscle development, which may be due to functional redundancy with the other *Pitx* genes. *Pitx1* and *Pitx3* are expressed in the

trunk and limb muscles and may have similar functions to *Pitx2* and thus be able to compensate in *Pitx2*$^{null/null}$ embryos (L'Honore et al., 2007; Lanctot et al., 1997; Shang et al., 1997). In EOM development, *Pitx1* is expressed too late to prevent EOM precursor apoptosis. The *Pitx* gene family may play critical roles in MRF activation and maintenance during muscle development throughout the embryo, but double mutant mice have been nearly impossible to generate (Jacques Drouin, personal communication) (Marcil et al., 2003). It may be necessary to use the Cre-Lox system to test this hypothesis.

Summary

Here we have shown that while *Pax7* is dispensable for early extraocular muscle development, *Pitx2* functions in the activation of the muscle regulatory factors *Myf5*, *MyoD1*, and *Myogenin*. Like other activators of myogenesis, *Pitx2* plays a multi-functional role in extraocular muscle development by regulating cell survival, cell proliferation and cell fate specification. We have identified a non-canonical PITX2 responsive site in the *MyoD1* minimal promoter and other evidence that suggests PITX2 has muscle-specific binding partner(s) that enhance its activation of MRF promoters. The role of *Pitx2* in activating MRF transcription may extend to myogenesis throughout the developing embryo, but only in the extraocular muscles does *Pitx2* operate alone at the top of the myogenic cascade.

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