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Pitx2 is an upstream activator of extraocular myogenesis and survival

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ABSTRACT

The transcription factors required to initiate myogenesis in branchial arch- and somite-derived muscles are known, but the comparable upstream factors required during extraocular muscle development have not been identified. We show *Pax7* is dispensable for extraocular muscle formation, whereas *Pitx2* is cell-autonomously required to prevent apoptosis of the extraocular muscle primordia. The survival requirement for *Pitx2* is stage-dependent and ends following stable activation of genes for the muscle regulatory factors (e.g. *Myf5*, *MyoD*), which is reduced in the absence of *Pitx2*. Further, PITX2 binds and activates transcription of the *Myf5* and *MyoD* promoters, indicating these genes are direct targets. Collectively, these data demonstrate that PITX2 is required at several steps in the development of extraocular muscles, acting first as an anti-apoptotic factor in pre-myogenic mesoderm, and subsequently to activate the myogenic program in these cells. Thus, *Pitx2* is the first demonstrated upstream activator of myogenesis in the extraocular muscles.

Introduction

Throughout development, networks of transcription factors activate the step-wise progression of cell fate decision-making, enabling phases of competence, specification, and differentiation. Often, these networks are re-used spatially and temporally during development with different variations such as substituting paralogs, swapping repressors for activators, or utilizing a different transcription factor at the top of a transcriptional cascade. In skeletal muscle development, the common cassette of muscle regulatory factors (MRFs), the transcription factors *Myf5* (GeneID: 17877), *Mrf4* (17878), *MyoD* (17927), and *Myogenin* (17928), induces specification and differentiation in all muscle progenitors, but upstream activator(s) of MRFs differ between the somitic, branchial arch, and extraocular muscles. Therefore, extraocular muscles provide an excellent opportunity to study a common transcriptional network that has evolved to generate specialized functions.

Extraocular muscles (EOMs) have evolved properties that enhance binocular vision, such as extreme speed, contractile precision, and fatigue resistance, which make them unique among the skeletal muscles (reviewed in Spencer and Porter, 2006). EOMs also have unique gene expression profiles relative to other skeletal muscles, 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 (Porter et al., 2006). These unique properties make the EOMs resistant to many forms of muscular dystrophy (Porter et al., 2003).

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 versus craniofacial myogenesis have been well documented; the trunk muscles develop from somites, whereas the craniofacial muscles develop from unsegmented prechordal and paraxial mesoderm (reviewed in Noden and Francis-West, 2006). The response of myogenic cells to extracellular signals also differs between the trunk and head (Tzahor et al., 2003). *Pax3* (18505), which activates MRF expression in the somites, is not expressed in the developing craniofacial muscles (Bajard et al., 2006; Horst et al., 2006). However, the development of the extraocular muscles also differs from craniofacial muscles formed in the branchial arches. *Tbx1* (21380), *Musculin* (*MyoR*, 17681), and *Tcf21* (*Capsulin*, 21412) are upstream activators of MRFs in the branchial arches, but individually they are not required for EOM formation (Kelly et al., 2004; Lu et al., 2002). Analogous transcriptional activators of the MRF myogenic cascade have not been identified in the extraocular muscles.

In contrast, the MRF transcriptional cassette is required for myogenesis in all muscle lineages, and a recent analysis by Sambasivan et al. identified the specific functions of the MRFs in

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EOM development. They showed *Myf5* and *Mrf4* have redundant functions that are absolutely required for EOM formation. Mice lacking either *Myf5* or *Mrf4* have largely normal EOMs, whereas mice with null mutations in both genes lack differentiated EOMs. The authors attribute this loss to the failure to activate *MyoD*, which rescues muscle specification in the absence of *Myf5*/*Mrf4* in the somites, and the subsequent apoptosis of the EOM primordia beginning at e11.0, suggesting that the MRFs function as survival factors. A small number of MYOD/MYOGENIN positive cells remain in the *Myf5*/*Mrf4* double mutant animals, suggesting other pathways can activate *MyoD*, although this is apparently insufficient to generate differentiated muscle (Sambasivan et al., 2009). These data clarify the roles of the MRFs of the EOMs, but leave the identity of the upstream activators unresolved.

Two transcription factors, *Pax7* (18509) and *Pitx2* (18741), have been proposed to be upstream activators of the MRF cascade in the EOM primordia (Diehl et al., 2006; Mootoosamy and Dietrich, 2002; Shih et al., 2007a). *Pax7* is expressed in the developing cranial muscles and it can activate MRF expression in development and regeneration (Kuang et al., 2006; Mootoosamy and Dietrich, 2002; Relaix et al., 2006; 2005). However, the potential functions of *Pax7* in EOM myogenesis have never been examined in detail, although recent experiments suggest it may not be required for EOM development (Relaix et al., 2004; Sambasivan et al., 2009). In contrast, the homeodomain transcription factor *Pitx2* is the only single gene shown to be required for extraocular muscle development. Mice lacking *Pitx2* function have no extraocular muscles and their formation is dependent on *Pitx2* gene dose (Diehl et al., 2006; Gage et al., 1999; Kitamura et al., 1999). Hypomorphic *Pitx2* mutants with approximately 20% of normal gene dose also lack extraocular muscles. Heterozygous *Pitx2* embryos have no oblique muscles, smaller rectus muscles, and expression of the MRFs *Myf5*, *MyoD*, and *Myogenin* is reduced to 10–20% of wildtype levels (Diehl et al., 2006). In adult EOMs, *Pitx2* continues to be expressed in satellite cells. Post-natal knockdown of *Pitx2* in the extraocular muscles showed a dramatic loss of MRF expression levels, suggesting *Pitx2* may be important for satellite cell function (Zhou et al., 2009). Overexpression of a *Pitx2-engrailed* dominant repressor construct reduces MRF expression in chick somites, suggesting regulation of the MRFs by PITX2 may be direct (Abu-Elmagd et al., 2010). The expression of *Pitx2* is unaffected in the *Myf5*;*Mrf4* double mutants, providing further evidence that *Pitx2* functions upstream of the MRFs (Sambasivan et al., 2009).

In addition to the mesodermal cells that are fated to form myofibers, the developing EOMs also receive contributions from the ocular neural crest, which produce tendons and connective fascia (Gage et al., 2005). Recent work has highlighted the complex interactions between neural crest and mesodermal cells in craniofacial development (Evans and Noden, 2006; Grenier et al., 2009; Rinon et al., 2007). Although *Pitx2* is expressed in both populations, its function is not required in the neural crest for initiation of extraocular myogenesis (Evans and Gage, 2005). This suggests *Pitx2* is required in the mesoderm lineage for EOM formation, where it may activate MRF expression.

Here we show that *Pax7* does not function in EOM specification, based on the timing of its expression relative to the MRFs and the presence of EOMs in *Pax7* mutant mice. We use lineage-specific knockout mice to demonstrate that EOM development requires *Pitx2* expression in the pre-myogenic mesoderm, and we show that EOM primordia lacking *Pitx2* undergo apoptosis prior to MRF activation. Delaying the ablation of *Pitx2* permits EOM precursor survival, however *Pitx2* continues to be required to prevent apoptosis at later stages as well. We found MRF levels are reduced in the absence of *Pitx2* and PITX2 can bind and activate the promoters of two key MRFs, *Myf5* and *MyoD*. These results indicate PITX2 plays key roles in both the survival and specification of extraocular muscles, in part through activation of the MRFs.

Materials and methods

Mouse husbandry

All experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Experimental Animals and all procedures involving mice were approved by the University of Michigan Committee on Use and Care of Animals. Mice were mated to generate timed pregnancies. The relevant crosses include: *T-Cre; Pitx2*$^{+/null}\times$ *Pitx2*$^{flox/+}$; *R26R/R26R, UBC-CreER*^{T2}; *Pitx2*$^{+/null}\times$ *Pitx2*$^{flox/flox}$; *R26R/R26R*, *Pitx2*$^{+/null}\times$ *Pitx2*$^{+/null}$, *Pax7*$^{LacZ/+}\times$ *Pax7*$^{LacZ/+}$. If indicated, a single intraperitoneal injection of tamoxifen (Sigma) suspended in corn oil at a dose of 100 μg/g body weight was administered to the pregnant dam at noon on the day noted. The resulting embryos were genotyped for *Cre* or *Pitx2* using PCR-based methods as per the Jackson Laboratories (Suh et al., 2002), and processed for histology as previously described (Evans and Gage, 2005).

Immunostaining and in situ hybridization

Paraffin sections were immunostained as previously described (Evans and Gage, 2005). Primary antibodies against PITX2 (a gift from T. Hjalt), PITX1 (a gift from J. Drouin), b-galactosidase (Eppendorf 5′), developmental myosin heavy chain (Vector), Ki67 (Dako Cytomation), MYOD (Abcam), Myogenin (Santa Cruz), MYF5 (Santa Cruz), and PAX7 (developed by A. Kawakami and obtained from NICHD/Developmental Studies Hybridoma Bank) were used. Digoxigenin-labeled riboprobes against *Pitx2* were generated and used to stain paraffin sections as previously described (Cushman et al., 2001; Martin et al., 2002).

Cell death analysis

Three to four non-adjacent sections from three wildtype and four heterozygous e10.5 embryos were stained for Terminal dUTP nick end labeling (TUNEL), PITX2 immunostaining, and DAPI using an *In situ* Cell Death Detection kit (Roche) per manufacturer protocol, followed by a standard immunostaining protocol with the hydrogen peroxide blocking step omitted. The number of TUNEL/PITX2/DAPI-labeled EOM primordia was divided by the number of PITX2/DAPI-labeled cells for the percentage of EOM primordia undergoing cell death. Nine total wildtype observations were statistically compared to 15 total *Pitx2* heterozygote observations using a Student's t-test.

Quantitative RT-PCR

Quantitation of MRF expression levels was performed using TaqMan Gene Expression Assays and Master Mix (Applied Biosystems) as previously described (Gage et al., 2008). cDNA was generated as previously described from the heads of e11.5 mouse embryos of the noted genotypes, which were microdissected to remove the branchial arches and portions of the brain, leaving the eyes and the surrounding tissues. Relative fold changes compared to controls and standard error were calculated from 3–4 samples of each genotype using the $2^{-\Delta\Delta Ct}$ method and normalized to *Hprt*.

Chromatin immunoprecipitation (ChIP)

C2C12 and mEOM (Porter et al., 2006) cells were grown to 80% confluency and subjected to ChIP assays as previously described (Gummow et al., 2006). For immunoprecipitation, two polyclonal antibodies specific for PITX2 were used (Santa Cruz, goat C-16 and 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 S1.

Luciferase assays

The PITX2 wildtype and T30P mutant expression constructs were a gift from Michael Walter and have been previously described (Kozlowski and Walter, 2000). A 317 bp *MyoD* minimal promoter fragment contains 124 bp upstream of the transcriptional start site (TSS) and the 5′UTR cloned into the pFL-basic luciferase reporter vector, and a similar plasmid containing 410 bp of the Myf5 minimal promoter includes 202 bp upstream of the TSS and the 5′UTR. Luciferase assays using these reporter vectors were carried out as previously described (Gage et al., 2008).

Results

Expression of transcription factors during myogenesis

To evaluate the proposed factors upstream of the MRF myogenic cascade, we examined the presence of PITX2 and PAX7 protein during primary myogenesis in wildtype EOMs. PITX2 is expressed in the unspecified pre-myogenic mesoderm fated to form EOMs (referred to here as EOM primordia) as early as e8.5 (Shih et al., 2007b), whereas expression of MYF5, the earliest MRF, is present in a small number of cells at e10.5 and expands by e11.5 (Figs. S1E, F). PAX7 and the other MRFs, MYOD, and MYOGENIN, are not observed until e11.5 (Fig. S1). The expression of PITX2 is more widespread than the MRFs, throughout the EOM primordia at both e11.5 and e12.5, suggesting not all EOM precursors have activated MRF expression by e12.5 (Figs. S1U–X). Since the MRFs are functional markers of muscle specification, this indicates EOM cells are specified during a period of several days, beginning at e10.5 and extending beyond e12.5. Persistent expression of PITX2 and the MRFs in a large proportion of EOM cells remains as late as e16.5 (Fig. S1). Overall, these data indicate *Pitx2* is temporally upstream of the MRFs, which is consistent with a potential role in activating them.

In contrast, the presence of MRF expression prior to the activation of *Pax7* indicates *Pax7* is unlikely to be an upstream regulator of the MRFs. We confirmed this by examining $Pax7^{LacZ/LacZ}$ knockout mice (MGI:3047633), which have normal expression of the MRFs and PITX2 in the extraocular muscle precursors at e12.0 (Fig. S2). *Pax7* mutant EOMs differentiate normally by e14.5 as indicated by the presence of myosin heavy chain (Figs. S2I, J), demonstrating *Pax7* is not required for the embryonic specification and differentiation of the extraocular muscles and it is not the upstream activator of the MRFs during early development. We therefore focused our efforts on determining the functions of *Pitx2* during EOM development and evaluating its potential to activate the MRFs.

Mesoderm-specific Pitx2 knockout mice lack EOMs

It was previously shown that mice with neural crest-specific knockout of *Pitx2* recapitulate most aspects of the *Pitx2* global knockout ocular phenotype, except that initiation of myogenesis in the extraocular muscles is unaffected (Evans and Gage, 2005). We hypothesized that a primary function of *Pitx2* in the mesodermal lineage during eye development is to direct extraocular muscle formation. To test this, mesoderm-specific *Pitx2* knockout (*Pitx2-mko*) mice were created using the T-Cre transgene (MGI:3605847), which is broadly expressed in mesoderm at gastrulation, with a conditional $Pitx2^{flox}$ allele (MGI:1857844) (Perantoni et al., 2005). Based on Cre-mediated activation of a *LacZ* reporter, mesoderm cells are found ventral to the optic vesicle at e9.5 (Fig. S3). By e11.5, the mesoderm condenses into a wedge shape, which separates into individual developing EOMs beginning at e12.5 (Fig. S3).

Since $Pitx2^{+/null}$ embryos have a reduced EOM phenotype, both *T-Cre+;* $Pitx2^{+/null}$ and *T-Cre+;* $Pitx2^{+/+}$ embryos were used as controls for the *T-Cre;* $Pitx2^{flox/null}$ mutants. *Pitx2-mko* mutant embryos have extremely reduced or completely absent extraocular muscles (Fig. 1). The *Pitx2-mko* embryos also have open eyelids at e16.5, occasional retinal lamination defects, and failure of body wall closure similar to the global *Pitx2* knockout (Zacharias et al., in preparation; Gage et al., 1999). The absence of EOMs in the *Pitx2-mko* mice suggests that loss of *Pitx2* causes a cell-autonomous defect in the mesoderm-derived myoblasts that form the EOMs.

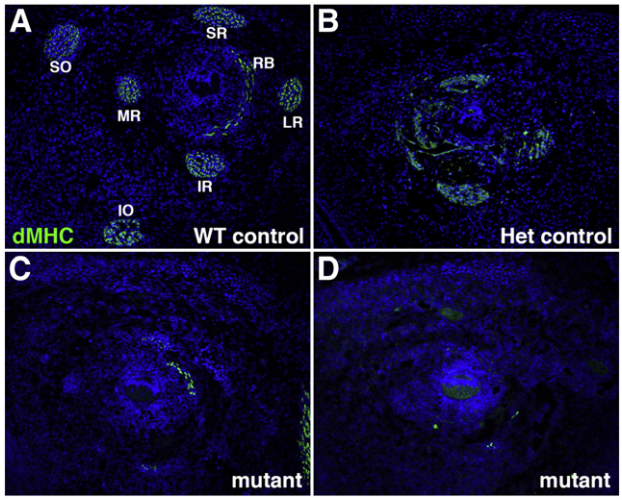


Fig. 1. Mesoderm-specific knockout of *Pitx2* results in the absence of extraocular muscles. Sagittal sections behind the globe of the eye allow for visualization of all seven extraocular muscles at later developmental timepoints, such as e14.5 (A). Immunohistochemistry for developmental myosin heavy chain (dMHC) shows *T-Cre+;* $Pitx2^{flox/null}$ mutant embryos have little (C) to no (D) differentiated extraocular muscle at e14.5, as compared to either *T-Cre+;* $Pitx2^{+/+}$ (A) or *T-Cre+;* $Pitx2^{+/null}$ (B) controls. SO, superior oblique; SR, superior rectus; MR, medial rectus; RB, retractor bulbus; LR, lateral rectus; IR, inferior rectus; IO, inferior oblique.

Pitx2 is required for EOM survival in a dose-dependent manner

To determine the fates adopted by EOM precursors in the absence of functional *Pitx2*, we examined expression of the mutant *Pitx2* mRNA produced by the $Pitx2^{null}$ allele. This mutant transcript is stable because it can be identified by a *Pitx2* probe targeting the 3′UTR. However, any protein produced by the mutant mRNA is either non-functional or degraded, because the homozygous $Pitx2^{null/null}$ mice phenocopy other *Pitx2* knockout mice (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). In the wildtype and heterozygous embryos, expression of *Pitx2* mRNA is observed in both the neural crest cells that surround the optic cup and the mesodermal cells that lie adjacent to the optic stalk (Figs. 2A, B). Although robust *Pitx2* mRNA expression is seen in the neural crest at e10.5 in $Pitx2^{null/null}$ global knockout (*Pitx2-gko*) embryos, little to no expression is seen in the location where mesoderm cells fated to form EOMs are normally present (Fig. 2C, arrow). Therefore, the EOM primordia are absent in *Pitx2-gko* mice.

To examine the possibility the EOM primordia were lost to cell death, TUNEL staining was performed. In $Pitx2^{null/null}$ embryos, widespread cell death is observed in the region where *Pitx2*-expressing EOM primordia are normally found (Fig. 2F). Cell death was also increased in $Pitx2^{+/null}$ EOM primordia (Fig. 2E), and the number of PITX2-positive cells co-labeled with TUNEL was significantly greater than wildtype littermates ($p<0.007$) (Fig. 2G). No difference in apoptosis was observed at e9.5 or e11.5 (data not shown), indicating that in EOM primordia lacking *Pitx2*, coordinated apoptosis occurs during a narrowly defined period, just prior to e10.5.

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), cell proliferation was examined in the EOM primordia at e9.5, prior to the

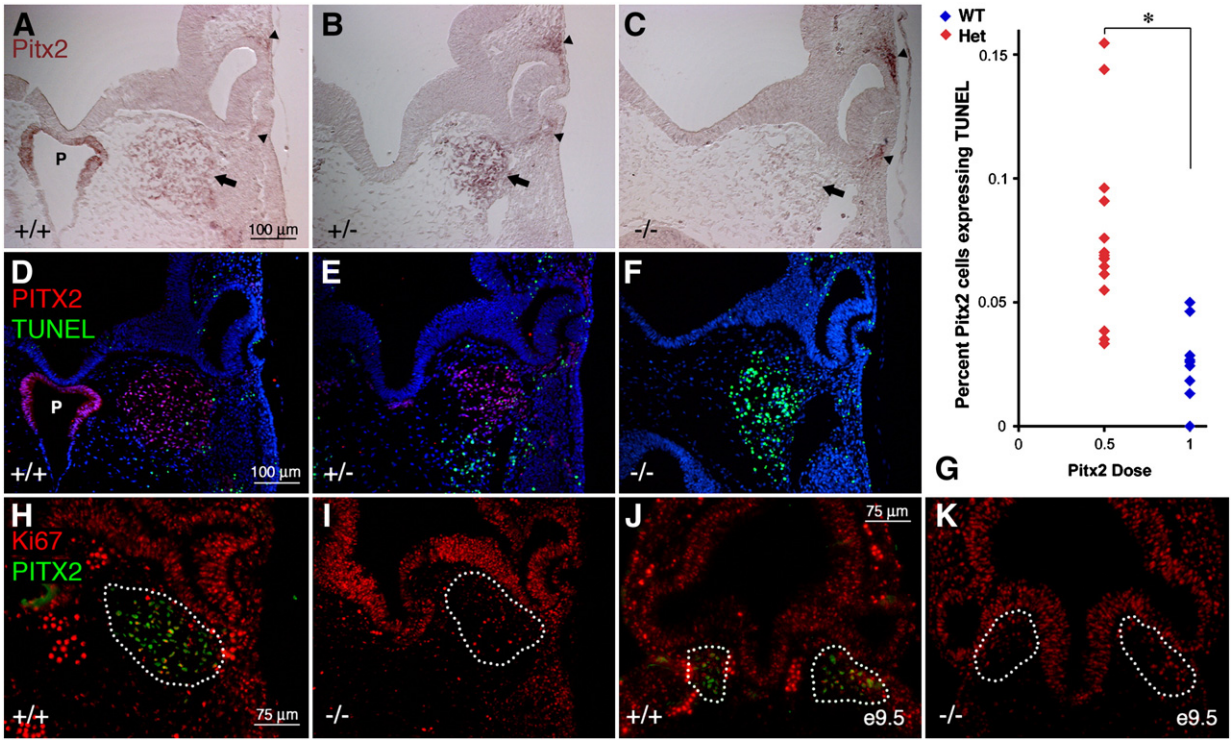


Fig. 2. Loss of *Pitx2* causes apoptosis in EOM primordia but does not affect cell proliferation. In both control (A) and heterozygote (B) e10.5 eyes, *Pitx2* mRNA is expressed in the neural crest cells which surround the optic cup (arrowheads) and the mesoderm cells which lie just adjacent to the optic stalk (arrow). In $Pitx2^{null/null}$ mutant eyes (C), expression surrounding the optic cup remains (arrowheads), but expression in the mesoderm is strikingly absent (arrow), indicating the absence of the EOM primordia. TUNEL staining shows an increase in the percentage of PITX2-positive cells also labeled with TUNEL in the *Pitx2* heterozygote (E) EOM primordia as compared to the control (D), which is statistically significant (G). In $Pitx2^{null/null}$ mutant eyes (F), there is a massive increase in TUNEL labeled cells in the region where the EOM primordia are normally found, demonstrating these cells are lost to apoptosis. Very few PITX2-positive EOM primordia cells (dotted line) also labeled with the proliferation marker Ki67 in wildtype embryos (H, J), and an approximately equal numbers of cells labeled with Ki67 in Pitx2 mutant EOM primordia (dotted lines) at both e10.5 (I) and e9.5 (K).

observed apoptosis, and e10.5 by staining for Ki67. There are few cells proliferating in the wildtype EOM primordia, but no differences were noted between mutant and wildtype embryos (Figs. 2H–K). Thus, defects in proliferation are unlikely to be the mechanism underlying the dramatic increase in apoptosis seen in the EOM primordia of *Pitx2-gko* embryos.

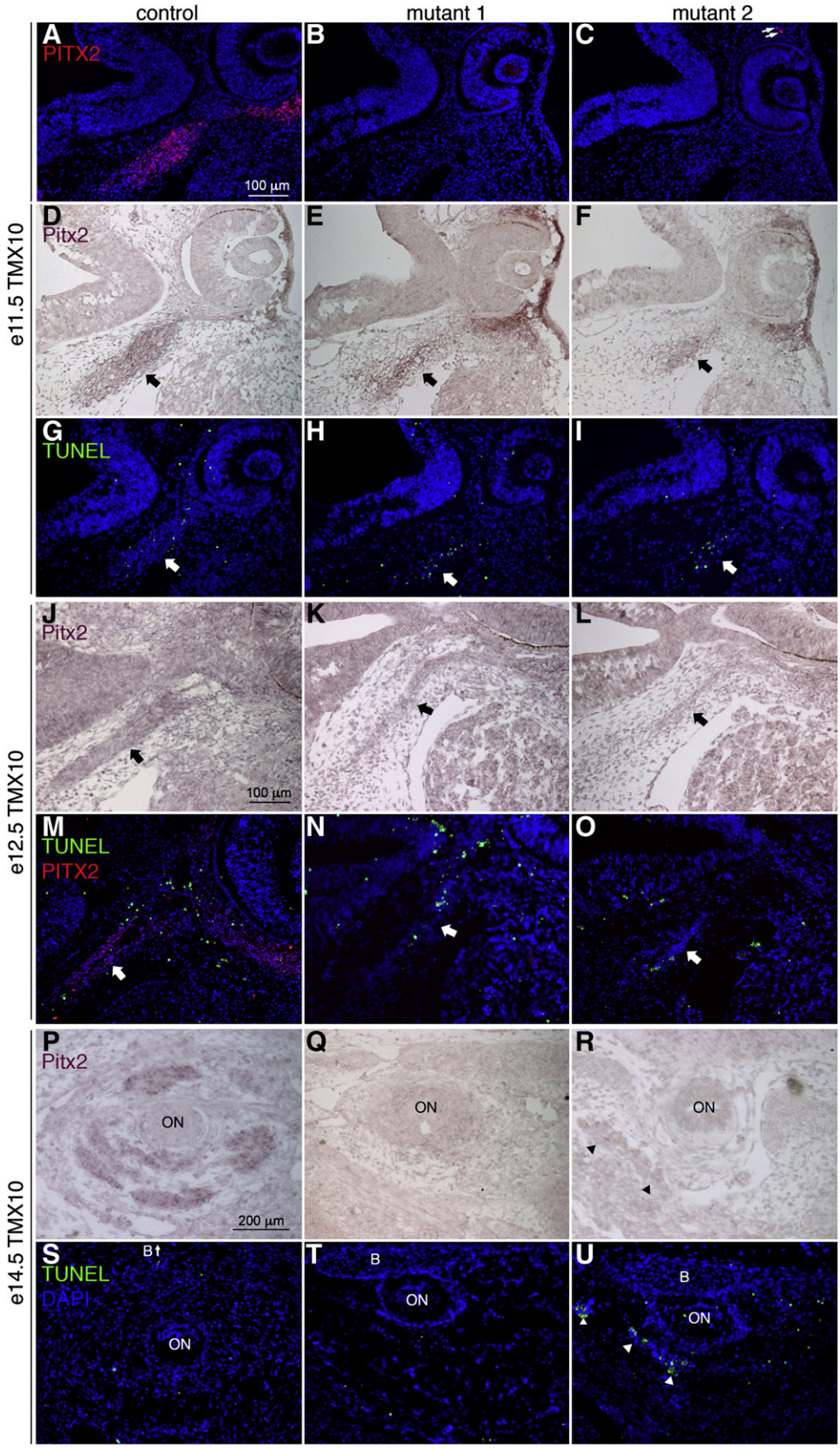
Pitx2 is required for EOM precursor specification and survival

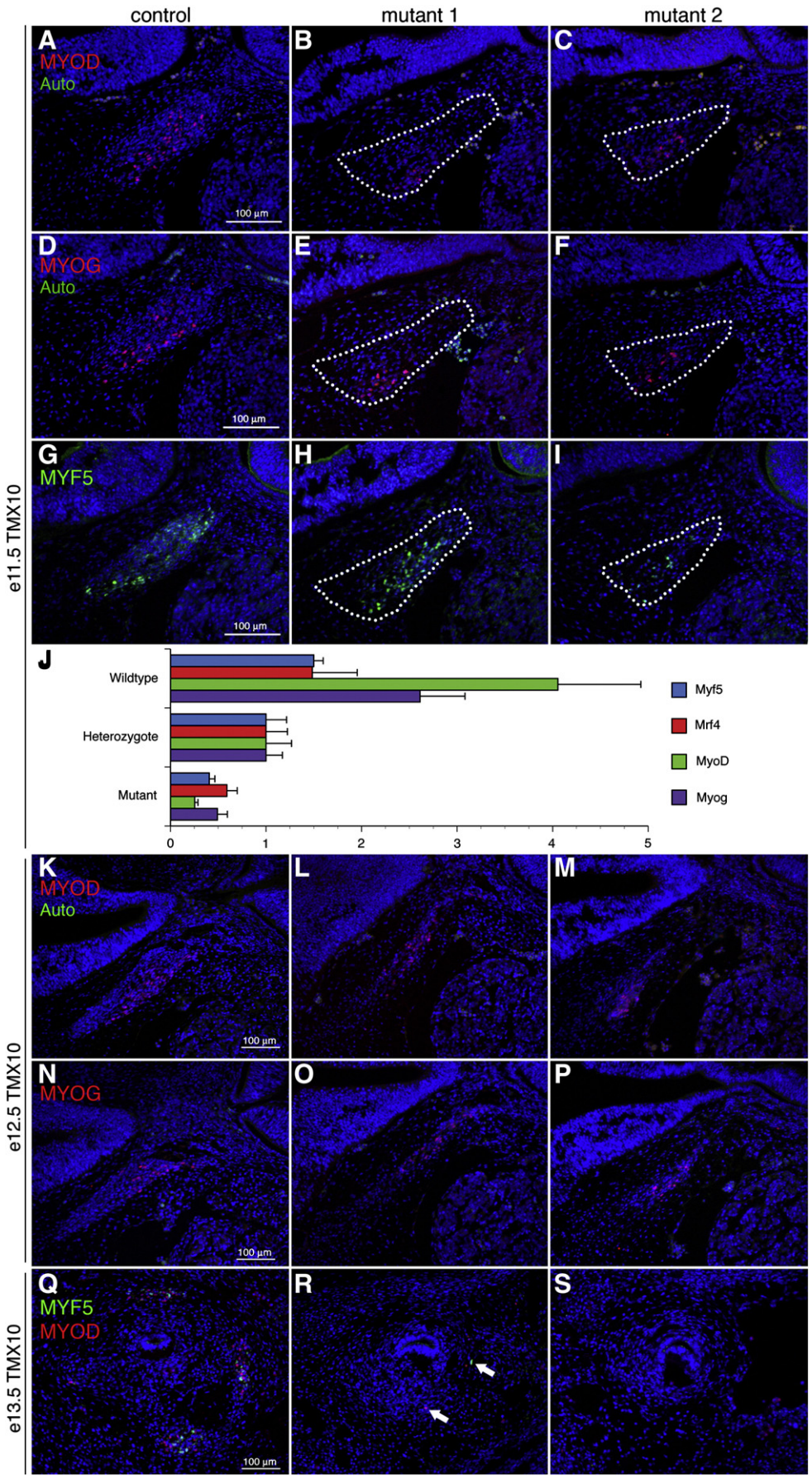
Previous data suggested *Pitx2* has a role in EOM specification as well as survival of the EOM primordia (Diehl et al., 2006). However, $Pitx2^{null/null}$ EOM primordia undergo apoptosis before they express any MRF markers of muscle specification (Fig. S1). To determine if *Pitx2* has functions in later EOM development, we generated mutants in which the loss of *Pitx2* function is delayed until after the initial stage when it is required for cell survival. This was accomplished by creating a temporal knockout of *Pitx2* using a ubiquitously expressed $CreER^{T2}$ (see Fig. S4, MGI:3707333)(Ruzankina et al., 2007). We bred *UBC-*$CreER^{T2}$+; $Pitx2^{+/null}$ males to $Pitx2^{flox/flox}$ females and injected the pregnant dams with the appropriate dose of tamoxifen at e10.5 (abbreviated TMX10, etc.). A nearly complete loss of PITX2 protein expression was observed 24 h post-injection (Figs. 3B, C). Tamoxifen treatment at other timepoints resulted in equally efficient ablation of PITX2 (data not shown). For all temporal knockout experiments *UBC-*$CreER^{T2}$+; $Pitx2^{flox/null}$ mutants were compared to $Pitx2^{flox/null}$ heterozygote littermates, because $Pitx2^{+/null}$ mice have an EOM phenotype.

To determine if survival of the EOM precursors could be rescued by early *Pitx2* expression and to evaluate the role of *Pitx2* in activating muscle specification at e11.5 and beyond, timed pregnant dams were injected with tamoxifen at e10.5 (TMX10). Embryos were harvested at e11.5, e12.5, e13.5 and e14.5 and analyzed for the presence of EOM primordia and expression of myogenic markers. To determine if expression of *Pitx2* prior to e10.5 could rescue the EOM primordia from apoptosis, we examined Pitx2 mRNA to identify the primordia and TUNEL labeling to identify apoptotic cells. In eight of eight eyes from four mutants examined at e11.5, the EOM primordia could be identified by *Pitx2* mRNA expression, indicating early expression of *Pitx2* extended cell survival (Figs. 3E, F arrows). Unlike the *Pitx2-gko*, apoptosis in the EOM primordia was not strikingly affected, but the percentage of cells labeled with TUNEL is reproducibly increased in the mutant embryos as compared to the controls (Figs. 3G–I). By e12.5, the EOM primordia of TMX10 mutant embryos were noticeably reduced in size compared to the heterozygous controls (Figs. 3J–L). Apoptosis was also increased in mutant primordia

Fig. 3. Delaying *Pitx2* deletion until e10.5 can temporarily rescue EOM primordia survival. *UBC-Cre+; Pitx2*$^{flox/null}$ mutant embryos treated with tamoxifen at e10.5 were examined for PITX2 protein expression 24 h later at e11.5. While control embryos (A) had normal PITX2 expression, mutant embryos either had no PITX2 expression (B), or a few remaining cells that expressed PITX2 at high levels (C, arrows), indicating the Cre-mediated excision of the $Pitx2^{flox}$ allele had failed. *Pitx2* mRNA expression (D–F) indicates the EOM primordia cells (arrow) are still present in e11.5 *UBC-*$CreER^{T2}$*+; Pitx2*$^{flox/null}$ embryos treated with tamoxifen at e10.5 (TMX10 mutants). However, the TMX10 mutant EOM primordia display increased TUNEL staining (H, I) at e11.5 as compared to controls (G). At e12.5, *Pitx2 in situ* hybridization shows that the TMX10 mutant EOM primordia (arrows) are smaller than controls (J–L) and have increased TUNEL staining (M–O). By e14.5, TMX10 mutants have no EOM primordia cells, based on the expression of *Pitx2* mRNA (P–R). The e14.5 TMX10 mutants do not show increased TUNEL staining, but instead show a reduced number of cells based on DAPI staining (S–U), causing the optic nerve (ON) to be shifted closer to the oculo-sphenoid bone of the skull (B). Red blood cells also infiltrate the empty spaces (R, U, arrowheads).

(Figs. 3M–O). By e13.5, most TMX10 mutant eyes (5/7) lacked EOM condensations, but a few (2/7) had small EOM condensations expressing *Pitx2* mRNA (data not shown). Apoptosis was increased in the mesenchyme of e13.5 TMX10 mutant eyes compared to the heterozygote controls (data not shown). By e14.5, there is no expression of *Pitx2* mRNA, indicating that the EOM precursors are




control
mutant 1
mutant 2
A
MYOD
Auto
B
C
100 µm
D
MYOG
Auto
E
F
100 µm
G
MYF5
H
I
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e11.5 TMX10
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Wildtype
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0
1
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N
MYOG
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e12.5 TMX10
Q
MYF5
MYOD
R
S
100 µm
e13.5 TMX10

Table 1
Summary of phenotypes in TMX10 mutant embryos.

	e11.5	e12.5	e13.5	e14.5
EOM primordia present	8/8	8/8 reduced	2/7	0/8
MRF+ cell present	8/8	8/8	2/7	0/8
Regions lack MYF5	2/8	1/8	1/2	–
Regions lack MYOD/MYOG	8/8	1/8	1/2	–
TUNEL increased	8/8	8/8	7/7	7/8 reduced cellularity

completely absent (Figs. 3P–R). No increase in TUNEL staining was seen in e14.5 TMX10 mutants (Figs. 3S–U), presumably because the dead cells were already cleared. There is a noticeable decrease in the number of cells surrounding the optic nerve, suggesting prior cell death in this region (Figs. 3S–U). Together, these data demonstrate that even after e10.5, EOM precursors require *Pitx2* for survival.

Deletion of *Pitx2* at e10.5 provided temporary rescue of the apoptosis seen in *Pitx2-gko* EOM primordia, which enabled us to examine the role of *Pitx2* in MRF activation. At e11.5, 24 h after *Pitx2* ablation, some cells still expressed MYF5, MYOD and MYOGENIN, but the number of EOM precursors expressing no MRFs was significantly increased (Figs. 4A–I, S5). MYF5 expression by immunohistochemistry was not spatially restricted, but the percentage of EOM primordia cells (as defined by the expression of *Pitx2* mRNA) labeled with MYF5 protein decreased significantly from 46% on average to just 15% (Fig. S5). All EOM primordia had regions lacking MYOD and MYOGENIN protein expression, with two of eight showing complete loss of expression. In primordia that retained expression, the percentage of cells expressing MYOD and MYOGENIN decreased from 10% and 7% to 3% and 4% respectively, representing statistically significant decreases (Fig. S5). The changes in percent expression indicate that the decrease in MRF expression cannot be explained merely by reduction in primordia size. To further quantitate the effect of the loss of *Pitx2* on MRF expression, we used qRT-PCR to measure transcript levels in e11.5 TMX10 dissected embryos. We found *Myf5*, *Mrf4*, *MyoD* and *Myogenin* were significantly reduced in mutants relative to both wildtype and heterozygous embryos, with *MyoD* being the most severely affected (Fig. 4J). At e12.5, the MRF proteins MYF5, MYOD, and MYOGENIN were expressed throughout the smaller TMX10 mutant EOM primordia, although the overall number of MRF-positive cells was decreased relative to the control (Figs. 4K–P, data not shown). In the two e13.5 TMX10 embryos that had EOM primordia (2/7), both had a few cells that expressed MRFs, although the number was substantially reduced relative to controls (Figs. 4Q, R). The e13.5 and e14.5 TMX embryos that lacked EOM primordia also had no expression of MRFs or markers of muscle differentiation, indicating the complete absence of extraocular muscle (Fig. 4S, Table 1, data not shown).

To identify the window during which *Pitx2* is required for the survival of EOM primordia, we delayed the tamoxifen injections until e11.5, e12.5 or e14.5. Treatment with tamoxifen at e11.5 or e12.5 resulted in partial restoration of EOM development in mutants, as evidenced by the presence of EOM primordia with substantially more widespread MRF expression than was observed in TMX10 mutants (compare Figs. 5A–I vs. Fig. 4). However, apoptosis in TMX11 and TMX12 mutant EOM primordia remains higher than in control EOMs (Figs. 5D–I). Differentiated muscles are present at e16.5 in TMX12 mutants, based on the expression of desmin (Figs. 5J–L), indicating *Pitx2* function may not be required for EOM differentiation. However, the requirement for *Pitx2* to ensure cell survival is not complete by e12.5 as evidenced by the increased apoptosis (Figs. 5G–I) and reduced EOM size in TMX12 mutants as compared to control littermates (Figs. 5J–L). Tamoxifen treatment at e14.5 results in normal EOM development, as judged by MRF expression, absence of apoptosis, and overall muscle size (data not shown). This indicates the developmental period during which *Pitx2* is required for EOM precursor survival ends between approximately e12.5–14.5.

Pitx2 can bind and activate MRF promoters

The initial reduction of the MRFs at e11.5 in response to the loss of PITX2, together with data from previous reports, lead us to hypothesize that PITX2 may directly activate the expression of the MRFs (Abu-Elmagd et al., 2010; Diehl et al., 2006; Zhou et al., 2009). We focused on the ability of PITX2 to activate *Myf5* and *MyoD*, because *MyoD* was most strongly reduced and *Myf5* is more dramatically affected by the loss of *Pitx2* than *Mrf4* (Fig. 4J), and it is expressed at sixteen fold higher levels than *Mrf4* (data not shown), suggesting *Myf5* may be the dominant early MRF in the EOMs. We did not examine the ability of *Pitx2* to activate the expression of *Myogenin*, because it is downstream of both *MyoD* and *Myf5* (Cheng et al., 1995; Yee and Rigby, 1993). We focused on three elements previously shown to drive *LacZ* expression in the correct time and place during EOM development, the *Myf5* promoter, the *MyoD* promoter, and the *MyoD* 258 bp enhancer (Goldhamer et al., 1995; 1992; Patapoutian et al., 1993). These regions were examined, and predicted *bicoid*-like PITX2 binding sites were identified in each (Fig. 6A). Chromatin immunoprecipitation (ChIP) was used to determine if PITX2 physically associates with these regions in two muscle precursor lines: C2C12 cells, which are limb derived, and a mouse EOM (mEOM) primary cell line (Porter et al., 2006). Both cell lines express PITX2 endogenously (data not shown). ChIP with an antibody against PITX2 shows enrichment for the *Myf5* proximal promoter as compared to control antibodies, but no enrichment is observed for the negative control region (Fig. 6B). ChIP also indicates that PITX2 binds the *MyoD* proximal promoter, but not the 258 bp *MyoD* enhancer or negative control (Fig. 6B). Similar results were obtained in both limb muscle and EOM precursors. These results confirm that PITX2 physically interacts with the promoters of MRF genes.

To examine if PITX2 binding to the MRF promoters is functionally significant, we tested the ability of the *Myf5* and *MyoD* proximal promoters to drive luciferase expression in response to PITX2 in C2C12 cells. Both the *Myf5* and *MyoD* proximal promoters respond to increasing doses of PITX2-expression vector, but not to transcriptionally-deficient mutations of PITX2 (Fig. 6C and data not shown) (Kozlowski and Walter, 2000). These data indicate PITX2 can activate the *Myf5* and *MyoD* promoters *in vitro*.

Discussion

The transcription factors upstream of trunk and branchial arch myogenesis have been defined, but the factors upstream of

Fig. 4. Loss of *Pitx2* at e10.5 reduces MRF expression in the EOM primordia. The loss of *Pitx2* in TMX10 mutants results in a significantly reduced number of cells in the EOM primordia (dotted line, based on *Pitx2* mRNA expression in Fig. 4) labeled with MYOD (B, C) and MYOG (E, F) as compared to controls (A, D). MYF5 expression is not as dramatically affected in TMX10 mutant EOMs (G–I). Quantitative RT-PCR on wildtype, heterozygote control and mutant e11.5 TMX10 embryos demonstrates the MRF mRNA levels are significantly reduced (J). The relative expression levels +/− SEM are normalized to the heterozygote controls to show the differences between mutant, heterozygote and wildtype. Levels of *Myf5*, *Myod*, and *Myog* are reduced at a greater level of statistical significance than *Mrf4* (J). At e12.5, the TMX10 mutants have expression of MYOD (K–M), MYOG (N–P), and MYF5 (data not shown) throughout their smaller EOM primordia. At e13.5, most TMX10 mutants have no EOM primordia cells and lack MRF expression (S), but a few have some expression of MYF5 and MYOD (R), although the number of labeled cells is dramatically reduced as compared to control EOMs (Q, arrows). Autofluorescent red blood cells appear as yellow or green in some panels (A–F, K–M).

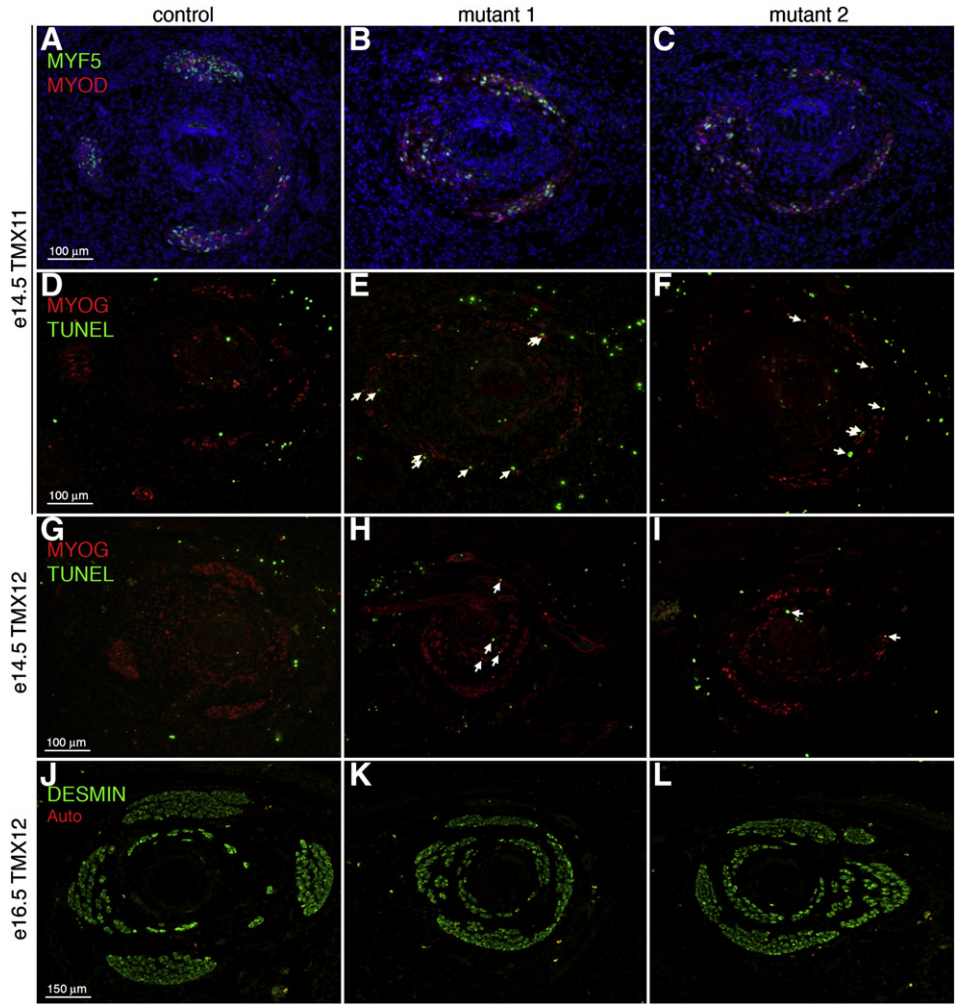

Fig. 5. Further delaying *Pitx2* deletion restores MRF expression but results in reduced EOM size. EOMs of e14.5 TMX11 mutants retain expression of MYF5 and MYOD (A–C), as well as MYOG (D–F). However, an increased number of mutant EOM precursor cells, their location marked by the expression of MYOG, label with TUNEL (arrows) in both e14.5 TMX11 (D–F) and TMX12 (G–I) embryos, indicating an increase in apoptosis in the absence of *Pitx2*. Desmin expression is unaffected in e16.5 TMX12 mutant EOMs (K, L), but they are smaller than the control EOMs (J). Autofluorescent red blood cells appear yellow or orange in panels J–L.

myogenesis in the extraocular muscles were unknown until now. Here we show *Pax7* is dispensable for extraocular muscle formation, whereas *Pitx2* plays multiple roles in primary extraocular myogenesis, controlling both EOM primordia survival, by an as yet unknown mechanism, as well as muscle specification by directly activating expression of MRFs.

Pitx2 is required for EOM survival

We show *Pitx2* is cell-autonomously required in EOM primordia cells for their survival prior to e10.5. *Pitx2* is required in a dose-dependent manner to prevent apoptosis in the mesoderm-derived muscle precursors, which explains why EOM size correlates with *Pitx2* dose, as previously reported (Diehl et al., 2006). The absence of *Pitx2* or removal of *Pitx2* function at e9.5 results in the apoptosis of the EOM primordia by e10.5, prior to activation of MRFs (Fig. 2 and data not shown). This is earlier than the apoptosis reported in EOM precursors lacking *Myf5* and *Mrf4*. In these embryos, apoptosis is first observed at e11.0, and a significant number of $Myf5^{nlacZ}$ positive EOM precursors remain at e12.5, indicating that the death of the EOM primordia occurs gradually (Sambasivan et al., 2009). Thus, there is an earlier, more absolute requirement for *Pitx2* in EOM survival than there is for the MRFs.

Stable expression of the MRFs and continued survival are dependent on Pitx2

Use of an inducible *Cre-Lox* system to delay deletion of *Pitx2* until e10.5 revealed that *Pitx2* is required for full activation of the MRFs. Our data suggest the MRF-negative cells observed at e11.5 are preferentially lost as a result of failing to activate or maintain the myogenic program. However, activation of the MRFs is apparently insufficient to prevent apoptosis in the absence of *Pitx2*. The progressive reduction in the size of the EOM precursor population in TMX10 mutants indicates that cells are progressively lost to apoptosis, until all of the EOM precursors are gone. Thus, even EOM precursors that express MRFs are unable to survive in the absence of *Pitx2*. Interestingly, *Pitx2* is similarly unable to prevent apoptosis in *Myf5;Mrf4* double mutant EOM primordia (Sambasivan et al., 2009). Therefore, expression of *Pitx2* and the MRFs must both be required for EOM primordia survival.

In contrast to the effects of deletion of *Pitx2* at e10.5, post-natal deletion of *Pitx2* does not appear to affect survival of the extraocular muscles, suggesting its requirement is transient (Zhou et al., 2009). Here we used temporal deletion to demonstrate that *Pitx2* function beyond e10.5 enables EOM development to reach a point after which the survival requirement for *Pitx2* is greatly diminished. Further, they suggest *Pitx2* is not required for MRF expression once it has been

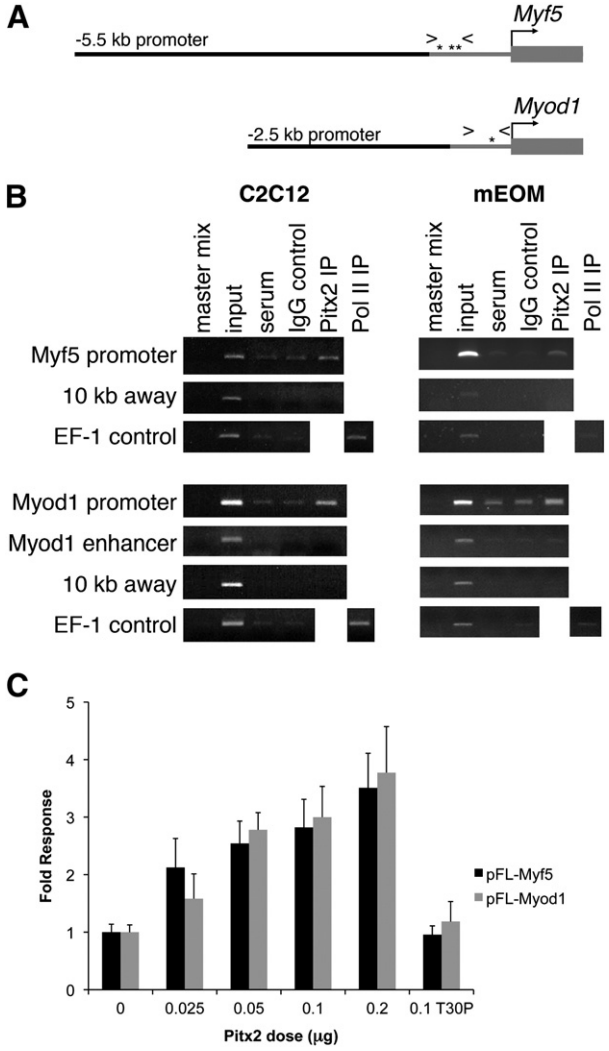

Fig. 6. PITX2 binds and activates the *Myf5* and *Myod1* promoters. Diagrams indicating the previously described *Myf5* and *Myod1* promoters (A, not to scale) show the transcriptional start site (arrows), the predicted PITX2 binding sites (*), and the location of the primers used for ChIP (> <). The regions in gray correspond to the minimal promoters used to drive luciferase expression. Chromatin immunoprecipitation (B), shows PITX2 binds to the *Myf5* and *Myod1* promoters more strongly than control antibodies (serum, IgG control) in both C2C12 limb muscle precursor cells and mEOM primary cells. The *Myod1* 258 bp enhancer and negative control regions 10 kb away from the promoters are not bound. The binding of a Pol II antibody to EF-1 is shown as a positive control. The minimal *Myf5* and *Myod1* promoters respond positively to increasing doses of PITX2 expression vector in luciferase reporter experiments (C). Neither promoter responds to the transcriptionally dead T30P mutation in PITX2, or to other transcriptionally compromised mutations (data not shown).

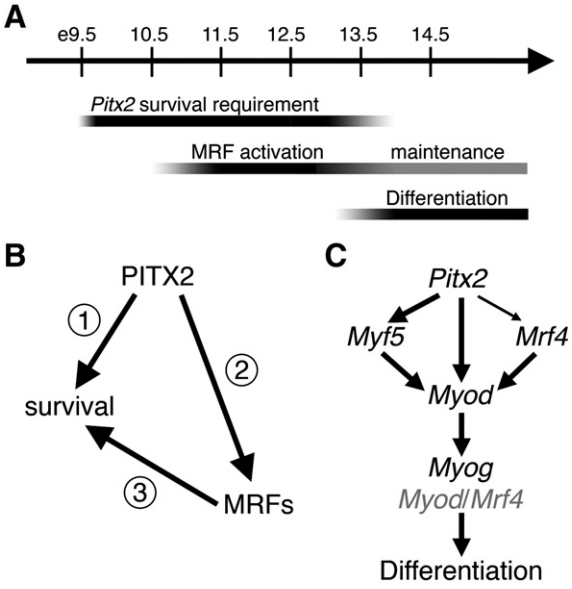

Fig. 7. Model for *Pitx2* functions in EOM development. A timeline (A) shows how the *Pitx2* requirement for survival in EOM primordia both precedes and overlaps with the activation of MRF expression. Differentiation markers appear during the same time frame that the requirement of *Pitx2* for EOM survival ends. A three-step model for *Pitx2* function in EOM development (B) shows that PITX2 is initially required for EOM primordia survival (1), and is later required for MRF activation (2) and subsequently the MRFs are also required for EOM survival (3). A model for the functions of PITX2 in MRF activation in the EOMs (C, adapted from the model proposed by Sambasivan et al.) shows that *Pitx2* directly activates the expression of *Myf5* and possibly *Mrf4*, which regulate *Myod*, a gene that is also directly activated by *Pitx2*. *Myod* then activates *Myog*, which induces differentiation, a process in which *Myod* and *Mrf4* may also play a role, based on the development of other skeletal muscles.

stably initiated, and may not be required for terminal differentiation of EOMs. However, *Pitx2* continues to be required for cell survival in at least a subset of the EOM primordia. We hypothesize that this subset represents unspecified EOM primordia cells that have not yet activated MRF expression, which we observed at both e11.5 and e12.5 (Fig. S1). Without *Pitx2*, these unspecified cells are unable to activate MRF expression, and without either of these critical anti-apoptotic factors, they undergo apoptosis. Data from late temporal knockouts indicates that the requirement for *Pitx2* ends by e14.5. Therefore, the period in which *Pitx2* is required for EOM precursor survival and specification begins at e9.5 and lasts until e12.5–e14.5, an endpoint which overlaps with the end of MRF activation and the initiation of EOM differentiation (Fig. 7A). An investigation of the role for *Pitx2* in secondary fiber formation in the time window between our studies and the post-natal studies of Zhou et al. remains to be done.

Based on our results, we propose a three-step model for the functions of *Pitx2* and the MRFs in EOM specification and survival (Fig. 7B). First, *Pitx2* is required for the survival of EOM primordia, prior to the activation of the myogenic program. Second, *Pitx2* is required to specify the EOM primordia as muscle by activating MRF expression. Finally, MRFs are required, but not sufficient, to ensure EOM precursor survival. Thus, *Pitx2* has dual functions in EOM primordia survival. It is required for a survival checkpoint at e9.5 and *Pitx2* continues to be required to prevent apoptosis until the EOM primordia are fully specified by robustly activating MRFs. Once all EOM precursors are specified and expressing MRFs at e14.5 and beyond, *Pitx2* is no longer required for their survival. The finding that MRF expression is insufficient to prevent apoptosis in TMX10 mutant EOM precursors suggests that *Pitx2* has other survival functions in addition to MRF activation between e10.5 and e14.5. The identification of these functions, as well as the mechanism by which *Pitx2* prevents cell death at the e9.5 checkpoint, is an important future direction.

Pitx2 directly activates expression of MRFs

Despite the constraints of the temporal knockout, we observe a substantial reduction in the number of cells expressing MYF5, MYOD, and MYOGENIN, and significant decreases in the expression levels of all four MRF mRNAs in e11.5 TMX10 mutant EOMs. Consistent with this, deletion of *Pitx2* in the EOMs post-natally, when it is expressed primarily in satellite cells, results in dramatic decreases in the expression levels of *Myf5*, *MyoD* and *Myogenin* (Zhou et al., 2009). These genetic data indicate *Pitx2* is an upstream activator of MRF expression in the EOMs.

The ability of PITX2 to bind and directly activate the promoters of *Myf5* and *MyoD*, two key MRFs, demonstrates that these genes are direct targets of PITX2. It is intriguing that PITX2 can utilize the promoters of *Myf5* and *MyoD* to activate their expression. A series of complex enhancers regulate the expression of *MyoD* and in particular *Myf5* in most locations of expression, including the branchial arches. However, an EOM specific enhancer has never been described, and the promoters are sufficient to drive expression in the developing EOMs for both *Myf5* and *MyoD* (Carvajal et al., 2001, 2008; Goldhamer et al., 1992; Patapoutian et al., 1993). The exact mechanism(s) of activation of the promoters by PITX2 remain to be determined. We hypothesize that PITX2 functions as a typical site-specific transcription factor, but PITX2 could also act as a chromatin-remodeling factor and enhance transcription through epigenetic mechanisms, similar to *Pax7* (McKinnell et al., 2008). This remains an important area for further study.

Our results suggest a model for MRF activation in primary EOM myogenesis (Fig. 7C). Sambasivan et al. recently showed *Myf5* and *Mrf4* activate *MyoD*, which then activates *Myogenin* (Sambasivan et al., 2009). We now provide genetic and biochemical evidence that PITX2 directly activates expression of *Myf5* and genetic evidence that it acts upstream of *Mrf4* as well. Our results also affirm *Myogenin* acts largely downstream of *MyoD*, as MYOGENIN is lost in mutant EOM precursors where MYOD is also lost at e11.5. However, we showed PITX2 directly activates expression of *MyoD* (Fig. 7C). Sambasivan et al. showed a small but significant population of cells expressing MYOD in *Myf5;Mrf4* double mutant EOM primordia, indicating that there is an independent pathway for activating *MyoD* expression (Sambasivan et al., 2009). We propose that *Pitx2* is part of this *Myf5;Mrf4* independent pathway, suggesting that *Pitx2* alone is sufficient to activate *MyoD* in a subset of EOM precursors. This is consistent with the ability of PITX2 to activate the human *MYOD* promoter in CHO cells that lack MRF expression (Fig. S6). The loss of MYOD expression in some EOM precursor cells that express MYF5 protein (Figs. 4A–I), and the greater reduction in *MyoD* mRNA expression than *Myf5* or *Mrf4* (Fig. 4J), suggest that *Pitx2* is also necessary to activate *MyoD* expression, even if *Myf5* is present. We also found that PITX2 is able to activate the human *MYOD* promoter in C2C12 muscle precursor cells at levels three fold greater than in CHO cells (Fig. S6). This suggests that PITX2 may have a muscle-specific co-factor that enhances activation of the *MyoD* promoter. This factor could be *Myf5*, *Mrf4*, or one of their downstream target genes such as *Mef2c* (17260) (Dodou et al., 2003). Thus we propose that in wildtype EOM precursors, PITX2 works with MYF5 and MRF4 to activate *MyoD*. The ability of *Pitx2* to activate MRF expression distinguishes it as the primary activator of EOM cell fate specification, in addition to its critical role in EOM precursor survival.

Despite the important roles we have demonstrated here for *Pitx2* in extraocular muscle specification, other factors are likely to be critical for extraocular myogenesis. These include a constantly changing milieu of extracellular signals, which may emanate from the neural tube, the neural crest or the eye itself (Mootoosamy and Dietrich, 2002; Rinon et al., 2007; Tzahor et al., 2003). The input of extracellular signals likely underlies why PITX2 is present in EOM primordia for several days before myogenesis is initiated. Additionally, other transcription factors not yet identified may also be required, or some of the myogenic transcription factors required in the branchial arches, such as *Tbx1* and *Musculin*, may play supporting roles in extraocular myogenesis.

Pitx2 in the development of other muscle populations

Similar to its functions in extraocular muscle development, *Pitx2* is required for both precursor survival and cell fate specification in the muscles of the first branchial arch (Dong et al., 2006; Shih et al., 2007a). Likewise, in the pituitary gland, *Pitx2* is first required for the survival of the precursor, Rathke's pouch, and subsequently for the activation of lineage-specific transcription factor genes (Charles et al., 2005; Suh et al., 2002). Although *Pitx2* does not play an anti-apoptotic role in all tissues where it is expressed, other genes that activate the MRFs display this multifunctionality. *Pax3* and *Pax7* are required in the somites for normal proliferation, survival and MRF activation (Collins et al., 2009; Relaix et al., 2006; 2005).

Given its essential role in EOM and branchial arch myogenesis, it is intriguing that *Pitx2* is not required for myogenesis in the trunk and limb, where it is also expressed. This may be due to redundancy in the expression of the homologous genes *Pitx1* (18740) and *Pitx3* (18742) (L'Honore et al., 2007; Lanctot et al., 1999). Double knockouts would be necessary to uncover these functions, but mounting evidence suggests that *Pitx* genes do play a role in trunk and limb myogenesis (Abu-Elmagd et al., 2010; L'Honore et al., 2007). While *Pitx2* is expressed too late to initiate MRF expression in the somites, our results with the C2C12 limb muscle precursor cells indicate that *Pitx2* may maintain MRF expression in the somites. The functions of *Pitx* genes in the specification of the craniofacial and somite-derived muscles may be conserved, but the temporal aspect of these functions has changed.

Conclusions

Here we have shown that *Pitx2* functions in the activation of the muscle regulatory factors *Myf5*, *Mrf4*, *MyoD*, and *Myogenin*. Like other activators of myogenesis, *Pitx2* plays a multi-functional role in extraocular muscle development by regulating cell survival and cell fate specification. The role of *Pitx2* in activating MRF transcription may extend to myogenesis throughout the developing embryo, but currently only in the extraocular muscles does *Pitx2* operate alone at the top of the myogenic cascade.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.10.028.

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