

# Canonical Wnt/ $\beta$ -Catenin Signaling Is Required for Maintenance But Not Activation of *Pitx2* Expression in Neural Crest During Eye Development

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*Pitx2* is a paired-like homeodomain gene that acts as a key regulator of eye development. Despite its significance, upstream regulation of *Pitx2* expression during eye development remains incompletely understood. We use neural crest-specific ablation of *Ctnnb1* to demonstrate that canonical Wnt signaling is not required for initial activation of *Pitx2* in neural crest. However, canonical Wnt signaling is subsequently required to maintain *Pitx2* expression in the neural crest. Eye development in *Ctnnb1*-null mice appears grossly normal early but significant phenotypes emerge following loss of *Pitx2* expression. LEF-1 and  $\beta$ -catenin bind *Pitx2* promoter sequences in ocular neural crest, indicating a likely direct effect of canonical Wnt signaling on *Pitx2* expression. Combining our data with previous reports, we propose a model wherein a sequential code of retinoic acid followed by canonical Wnt signaling are required for activation and maintenance of *Pitx2* expression, respectively. Other key transcription factors in the neural crest, including *Foxc1*, do not require intact canonical Wnt signaling. *Developmental Dynamics* 239:3215–3225, 2010. © 2010 Wiley-Liss, Inc.

**Key words:** homeodomain transcription factor; Wnt signaling; *Pitx2*; eye development; anterior segment; neural crest

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## INTRODUCTION

It is a well-established paradigm that the net expression patterns of important developmental transcriptional regulators result from the integration of multiple inductive signaling inputs, but the underlying molecular details are poorly understood in most systems. The mammalian eye provides an excellent system for further detailed analysis of this model because extensive inductive signaling contributed by multiple major signaling pathways is passed back and forth between the

four embryonic primordial contributing to the mature organ. For example, inductive signaling from the neuroectoderm-derived optic vesicle is required for initiation of the lens differentiation program in the overlying surface ectoderm and the newly induced lens placode in turn signals back to the optic vesicle, specifying the position where the presumptive retina will emerge within the neuroectoderm (Chow and Lang, 2001).

Development of the ocular anterior segment, including the cornea, limbus, conjunctiva, eyelids, and ciliary body,

is particularly complex. It requires the interaction of neuroectoderm, surface ectoderm, and neural crest and mesoderm mesenchyme. The mature cornea, limbus, conjunctiva, eyelids, and ciliary body are chimeric, arising from an integration of neuroectoderm (ciliary body) or surface ectoderm (cornea, limbus, conjunctiva, and eyelids) and mesenchyme. Development of the primordia to these structures commences immediately after transition of the optic vesicle to the optic cup and separation of the lens vesicle from the surface ectoderm, when mesenchyme cells

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consisting primarily of neural crest begin to migrate between the anterior optic cup/lens vesicle and the overlying surface ectoderm (Cvekl and Tamm, 2004; Gould et al., 2004). Retinoic acid (RA) signaling from the optic cup, lens, and surface ectoderm crest plays a critical role in orchestrating the morphogenesis of the anterior segment at this stage of development by activating expression of key transcription factor genes in the neural crest (Matt et al., 2005, 2008; Molotkov et al., 2006).

A critical downstream target of RA signaling in the ocular neural crest at this stage is the homeobox gene, *Pitx2* (Matt et al., 2005, 2008; Molotkov et al., 2006). Global or neural crest-specific loss of *Pitx2* in mice results in severe dysmorphogenesis of the eye, including loss of any recognizable anterior segment structures except the lens (Gage et al., 1999; Evans and Gage, 2005). These defects closely phenocopy those found in mice in which RA signaling is disrupted, in part because *Pitx2* expression is lost (Matt et al., 2005, 2008; Molotkov et al., 2006). Heterozygous mutations in human *PITX2* are one genetic cause of Axenfeld-Rieger Syndrome, which features anterior segment defects and an associated high risk for early-onset glaucoma (Semina et al., 1997; Alward, 2000). An important downstream target of *PITX2* is *Dkk2*, which encodes an extracellular antagonist of canonical Wnt/ $\beta$ -catenin signaling. *DKK2* is required to moderate Wnt/ $\beta$ -catenin signaling activity in both the neural crest and overlying surface ectoderm of the anterior segment (Gage et al., 2008). Collectively, these observations implicate *PITX2* as an important integration node between these two major signaling pathways during eye development (Gage et al., 2008; Gage and Zacharias, 2009; Kumar and Duester, 2010). In addition, *PITX2*,  $\beta$ -catenin, and *LEF-1* interact synergistically to regulate the *Lef1* promoter, further strengthening the connection between *PITX2* and canonical Wnt signaling (Vadlamudi et al., 2005).

*Pitx2* itself has been implicated as a transcriptional target of canonical Wnt/ $\beta$ -catenin signaling in the developing pituitary gland through sequences located 5' (Kioussi et al., 2002) and 3' (Ai et al., 2007) of the gene. Canonical Wnt signaling may also play a

role in post-transcriptional stabilization of the *Pitx2* mRNA (Briata et al., 2003). Treatment of embryos with LiCl, an agonist of canonical Wnt signaling, stimulates expression of *Pitx2* within the periocular mesenchyme (Kioussi et al., 2002). In addition, *PITX2* protein levels remain persistently high in the ocular neural crest of *Dkk2*-deficient mice, providing evidence for an auto-regulatory feedback loop (Gage et al., 2008). Although the precise mechanism underlying this expression change is not known, these data also suggested that a combinatorial code including both RA and canonical Wnt/ $\beta$ -catenin signaling may be required to activate *Pitx2* expression in the neural crest.

In this report, we present evidence that canonical Wnt/ $\beta$ -catenin signaling is not required for initial activation of *Pitx2*, but it is required for maintenance of its continued expression, likely via direct binding of *Lef-1* and  $\beta$ -catenin to the *Pitx2* promoter. Thus, the two signaling pathways do contribute to a code required for normal *Pitx2* expression but they act sequentially, rather than in concert. Transient expression of *Pitx2* is sufficient to activate a genetic program that is required to anchor the anterior optic cup/lens to the overlying surface ectoderm. Finally, the dependence of *Pitx2* expression upon canonical Wnt/ $\beta$ -catenin signaling appears to be unique among a panel of known regulators of early anterior segment development.

## RESULTS

### Neural Crest-Specific Loss of $\beta$ -Catenin Is Efficient and Does Not Notably Alter Early Eye Development

$\beta$ -catenin is expressed throughout the tissues of the developing eye, including the neural crest, through the period during which *Pitx2* expression is activated (Fig. 1A). This expression pattern was consistent with the possibility that intact responsiveness to canonical Wnt/ $\beta$ -catenin signaling by the neural crest might be required for activation of *Pitx2* in these cells, as well as more generally for normal eye development during this stage.

To test these hypotheses, we generated mice lacking  $\beta$ -catenin in neural

crest by mating mice carrying the conditionally null *Ctnnb1<sup>tm2Kem</sup>* allele (Brault et al., 2001) with mice transmitting the neural crest-specific *Wnt1Cre1* transgene (Danielian et al., 1998). Since  $\beta$ -catenin is the essential downstream effector of the pathway, this genetic approach very efficiently and comprehensively prevents neural crest in these mice from responding to canonical Wnt signaling (Brault et al., 2001). However, the effects of ablating  $\beta$ -catenin on eye development were not previously described (Brault et al., 2001)  $\beta$ -catenin is undetectable in neural crest of *Ctnnb1-ncko* (*Wnt1Cre;Ctnnb1<sup>tm2Kem/tm2Kem</sup>*) embryos at e10.5 (Fig. 1E). Eyes of the mutant embryos are grossly normal at this time point (Fig. 1B vs. F), indicating that intact Wnt signaling in neural crest is not required for their migration into the eye field and may not be required for early stages of eye morphogenesis. In contrast, neural crest in mutants with constitutively activated  $\beta$ -catenin does not efficiently populate the developing eye (data not shown) (Lee et al., 2004). *Ctnnb1-ncko* eyes begin to exhibit a thick, neuroblastic optic stalk that is different from wild type controls and a modest misorientation of the optic cup towards the ventral side by e11.5 (Fig. 1C vs. G). These features are more accentuated by e12.5, when the optic stalk remains abnormally thick and contiguous with the ventral diencephalon, which is displaced peripherally, and the optic cup is rotated ventrally (Fig. 1D vs. H). Collectively, these data suggest that the overt requirement for Wnt/ $\beta$ -catenin responsiveness in neural crest begins at approximately e11.5 during mouse eye development.

### Canonical Wnt/ $\beta$ -Catenin Signaling Is Required for Maintenance of *Pitx2* Expression in Neural Crest

We next assessed whether intact canonical Wnt/ $\beta$ -catenin signaling is required for activation or maintenance of *Pitx2* expression in neural crest by immunostaining *Ctnnb1-ncko* embryos for both  $\beta$ -catenin and *PITX2*. Activation of the *R26R* Cre reporter locus (Soriano, 1999) was used to fate map neural crest in the mutant eyes and to



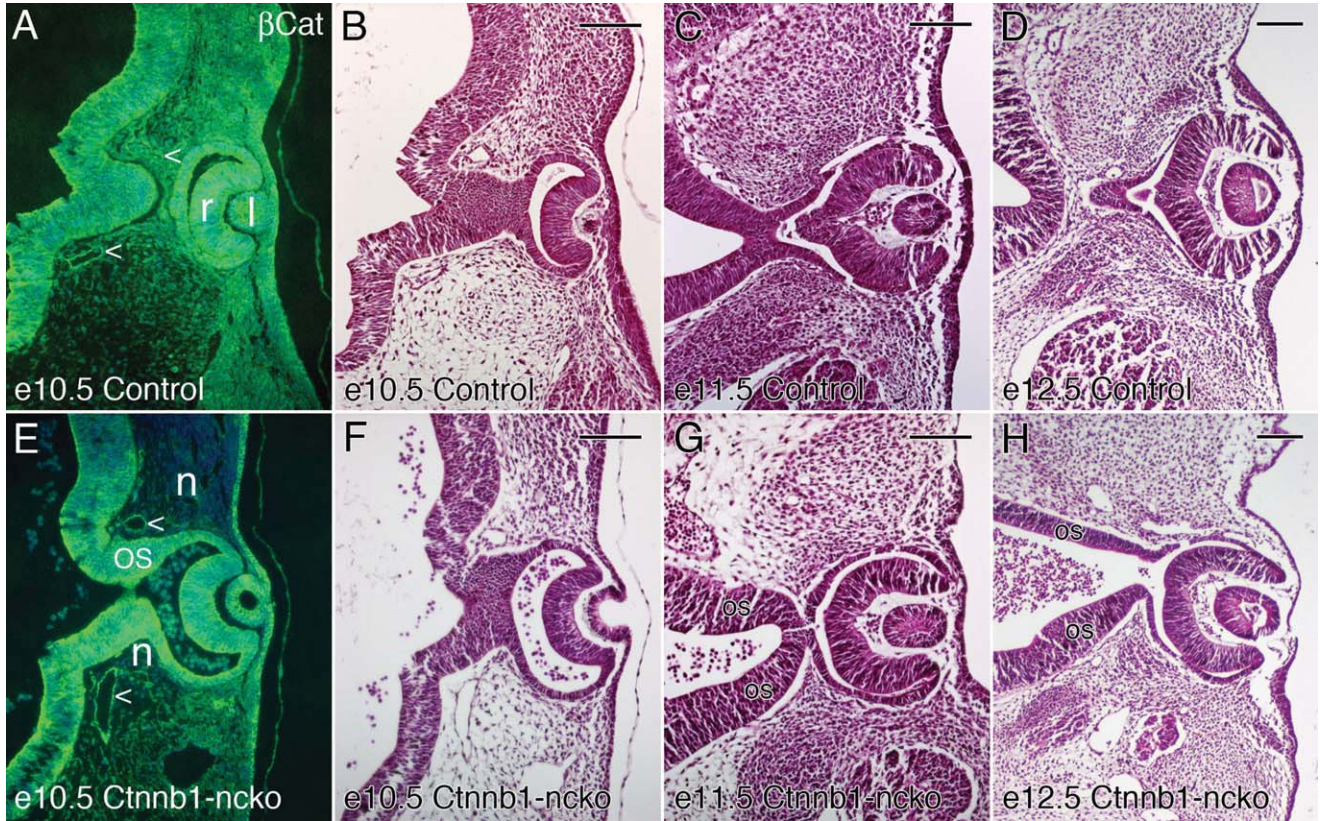


Fig. 1.

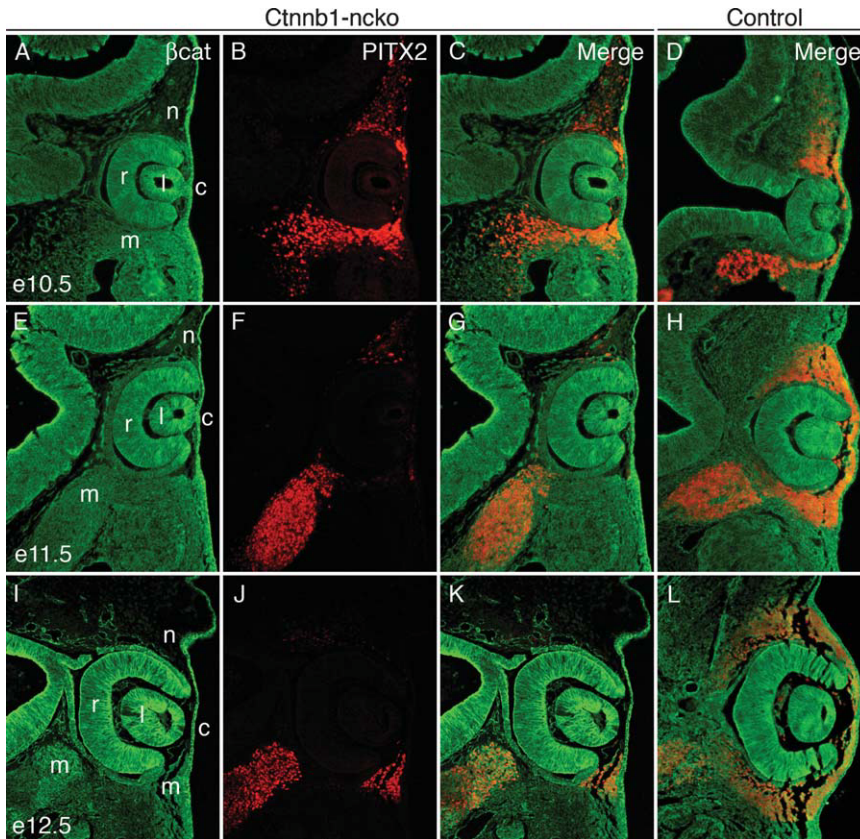


Fig. 2.

**Fig. 1.** Loss of  $\beta$ -catenin protein and analysis of early eye morphogenesis in *Ctnnb1-ncko* mice. **A, E:** Eye sections of e10.5 control and *Ctnnb1-ncko* littermates were stained for detection of  $\beta$ -catenin by immunofluorescence (green) and imaged with DAPI (blue) to visualize nuclei. Note how the mesoderm-derived endothelial cells of blood vessels (<) stand out in *Ctnnb1-ncko* mice due to the efficient loss of  $\beta$ -catenin in surrounding neural crest. **B–D:** Eye sections of control embryos at the indicated ages were stained by H&E to visualize eye morphogenesis. **F–H:** Eye sections of *Ctnnb1-ncko* littermates of wild type embryos in B–D were stained by H&E to visualize eye morphogenesis. r, retina; l, lens; n, neural crest; <, blood vessel; os, optic stalk. Scale bar = 100  $\mu$ m.

**Fig. 2.** PITX2 protein expression in *Ctnnb1-ncko* versus control eyes during early eye development.  $\beta$ -catenin (green) and PITX2 (red) protein expression were detected by double immunofluorescence on eye sections taken from *Ctnnb1-ncko* and control littermates at the indicated time points. Overall PITX2 expression is comparable in the two genotypes at e10.5 but is rapidly reduced (e11.5) and then lost (e12.5) specifically in neural crest cells of *Ctnnb1-ncko* eyes. Mesoderm expression of PITX2 is unaffected in the mutants. r, retina; l, lens; c, cornea; n, neural crest; m, mesoderm.



confirm comprehensive loss of  $\beta$ -catenin in these cells (data not shown). The overall pattern of PITX2 expression in eyes of *Ctnnb1-ncko* embryos, including in neural crest lacking  $\beta$ -catenin, is indistinguishable from control littermates at e10.5, although levels of PITX2 expression sometimes appear diminished in individual neural crest cells (Fig. 2A–D). PITX2 expression is still detectable but dramatically reduced in neural crest of *Ctnnb1-ncko* mice compared to control littermates at e11.5 (Fig. 2E–H). By e12.5, PITX2 expression is undetectable in neural crest of *Ctnnb1-ncko* embryos but high-level expression is observed in neural crest surrounding eyes of control littermates (Fig. 2I–L). Mesoderm expression appears to be unaffected in *Ctnnb1-ncko* eyes at any timepoint examined (Fig. 2).

We considered the possibility that intact canonical Wnt/ $\beta$ -catenin signaling could be required for survival of neural crest and that the apparent loss of PITX2 expression might result from the death of these cells. In addition to *Pitx2<sup>lox</sup>*, mutant animals also carried the *R26R* reporter allele, allowing us to fate map neural crest following Cre-mediated activation of *lacZ* (Soriano, 1999). Neural crest expressing *lacZ* are abundant in *Ctnnb1-ncko* eyes throughout the relevant developmental stages (Fig. 3A,D). Consistent with this finding, TUNEL assays detected no differences in apoptotic cell death in mutant versus control littermate eyes (Fig. 3B,C,E,F). Altogether, these data indicate that maintenance of PITX2 expression in neural crest during eye development requires intact canonical Wnt/ $\beta$ -catenin signaling.

### $\beta$ -Catenin and LEF1 Bind *Pitx2* Promoter Sequences in Developing Periocular Mesenchyme

A functional TCF/LEF-responsive element is located 200-bp 5' to the promoter for the *Pitx2a* and *Pitx2b* mRNA isoforms (Fig. 4A) (Kioussi et al., 2002). This element is conserved between mice and humans, and is active in cells lines from the anterior pituitary gland (Kioussi et al., 2002). A second functional TCF/LEF-responsive element is present within an enhancer located 3' to the

*Pitx2* gene and is required for in vivo enhancer activity within Rathke's pouch, the embryonic precursor to the anterior pituitary gland (Ai et al., 2007). However, the potential relevance of these elements to *Pitx2* expression in periocular mesenchyme has not been explored.

To determine whether either of the two previously identified TFC/LEF-responsive elements are occupied during eye development, we performed chromatin immunoprecipitation on periocular mesenchyme derived from e12.5 embryos. These assays revealed that *Pitx2* promoter sequences containing the TCF/LEF-responsive element are significantly enriched over background levels in samples immunoprecipitated with antibodies specific to either  $\beta$ -catenin or LEF1 (Fig. 4B). In contrast, neither sequences spanning the TCF/LEF-responsive element located within the 3' enhancer, nor sequences within a *Pitx2* intron that are not associated with a potential TCF/LEF site, were enriched by immunoprecipitation with the specific antibody (see Supp. Fig. S1, which is available online). These data indicate that the TCF/LEF-responsive element within the 5' *Pitx2* promoter sequences is physically occupied by  $\beta$ -catenin and LEF1 in periocular mesenchyme and suggest that canonical Wnt signaling acts directly through this site to maintain *Pitx2* expression in these cells during development.

### Transient Expression of *Pitx2* Is Sufficient to Rescue Initiation of Anterior Segment Development

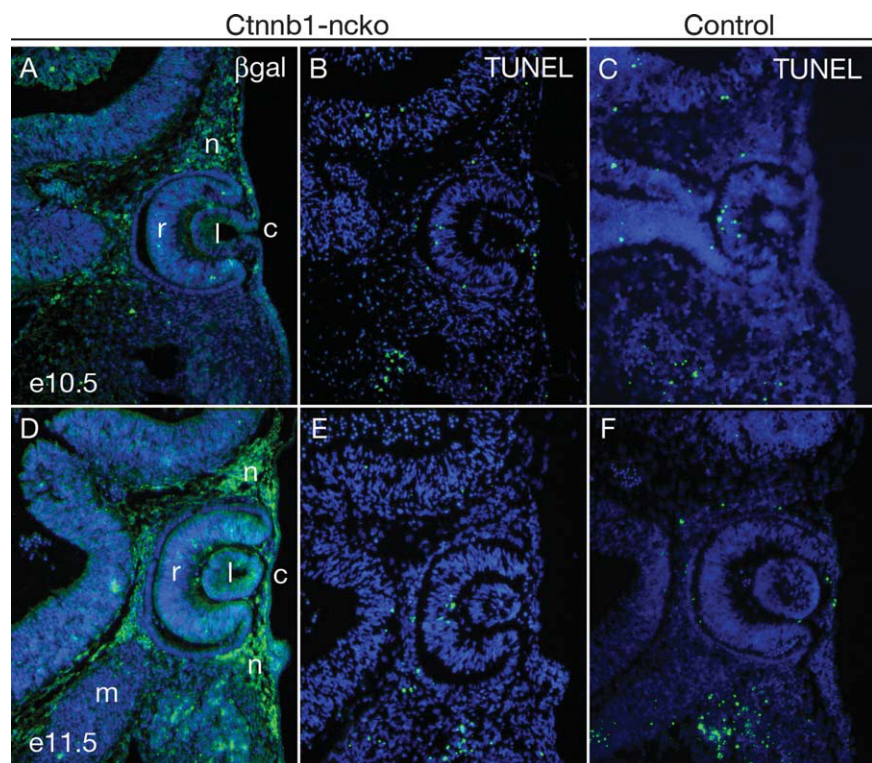
The optic stalk of wild type eyes elongates as development proceeds and the optic cup remains in intimate contact with the overlying surface ectoderm (Fig. 5A). In contrast, global or neural crest-specific *Pitx2* knockout mice exhibit severe defects including a thickened and substantially shortened the optic stalk, as well as displacement of the optic cup to a midline position substantially removed from the overlying ocular surface ectoderm (Fig. 5B) (Gage et al., 1999; Kitamura et al., 1999; Evans and Gage, 2005). We have previously hypothesized that displacement of the

optic cup in *Pitx2* mutant mice results primarily from the foreshortened optic stalk "pulling" the optic cup towards the midline and away from the surface ectoderm (Evans and Gage, 2005). However, optic cups of *Ctnnb1-ncko* mice are appropriately positioned directly beneath the overlying ocular surface ectoderm, despite the fact that morphogenesis of the optic stalk appears to be disrupted at least as severely in these animals as in *Pitx2* mutant mice (Fig. 5C). This raised the possibility that transient expression of *Pitx2* in *Ctnnb1-ncko* mice is sufficient to activate a genetic program required to anchor the optic cup/lens to the overlying ocular surface ectoderm.

We tested this hypothesis by performing temporal knockouts of *Pitx2* (*Pitx2-<sup>tko</sup>*) in mice carrying our conditional *Pitx2<sup>lox</sup>* allele (Gage et al., 1999) and *UBC-CreER* (Gruber et al., 2007). The Cre-ER fusion protein required to delete *Pitx2* is ubiquitously expressed by this transgene but is functionally inactive except in the presence of tamoxifen (Gruber et al., 2007). In animals with the genotype *UBC-CreER;Pitx2<sup>lox/lox</sup>*, injection of timed pregnant dams with tamoxifen resulted in complete loss of PITX2 protein within 24 hr (see Supp. Fig. S2, which is available online). We, therefore, injected timed pregnant dams from the appropriate matings with tamoxifen at e10.5, which permitted normal activation but resulted in the loss of all PITX2 protein by e11.5, a time course similar to that observed in *Ctnnb1-ncko* mice.

In contrast to global or neural crest-specific *Pitx2* knockout eyes (Fig. 5B and data not shown), the eyes of *Pitx2* mutant mice are located peripherally, similar to, although not identical to the eyes of wild type and *Ctnnb1-ncko* mice (Fig. 5D). The corneal mesenchyme of e14.5 *Pitx2-<sup>tko</sup>* eyes is significantly thicker than that found in wild type or *Ctnnb1-ncko* eyes, an early manifestation of the severe corneal defects found in these mice at later time points (data not shown). The *Pitx2-<sup>tko</sup>* eyes are connected to the lateral diencephalon by an optic stalk that is thickened and shortened relative to wild type eyes (Fig. 5A vs. 5D), a defect that appears similar to what we have reported previously for global



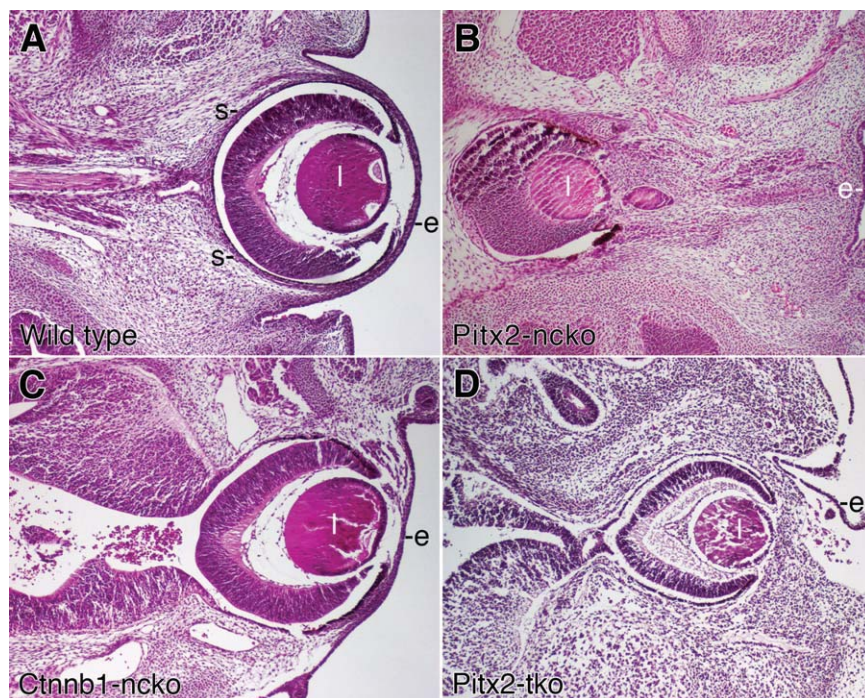


**Fig. 3.**  $\beta$ -catenin is not required for survival of ocular neural crest. **A,D:** Survival of neural crest mesenchyme in *Ctnnb1-ncko* eyes at the indicated time points is demonstrated by using immunofluorescence to detect  $\beta$ -galactosidase expressed following Cre-mediated activation of the R26R reporter allele. **B,C,E,F:** Apoptotic cells in *Ctnnb1-ncko* and control littermates at the indicated time points as detected by TUNEL assay. No difference in TUNEL-positive cells is observed between the two genotypes. r, retina; l, lens; c, cornea; n, neural crest; m, mesoderm. Note: The image in A is taken from a section adjacent to the image in Figure 2A and the image in D is taken from a section adjacent to the image in Figure 2E.

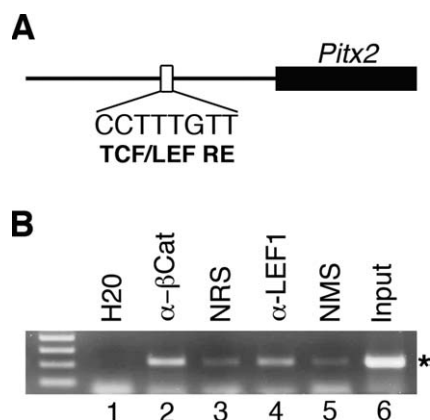
and neural crest-specific *Pitx2* knock-out eyes (Gage et al., 1999; Evans and Gage, 2005). The observation that *Ctnnb1-ncko* and *Pitx2-tnko* eyes are located peripherally, despite significant defects in development of the optic stalk, indicates that the elongation of the optic stalk alone cannot determine placement of the optic cup and lens. Rather, these data suggest a model wherein early expression of PITX2 protein at e10.5–11 also activates a program of adhesion that facilitates a stable interaction between the anterior optic cup, the lens, the anterior segment mesenchyme, and the overlying ocular surface ectoderm.

### Other Regulators of Anterior Segment Development Do Not Depend on Canonical Wnt Signaling in Ocular Neural Crest

*Foxc1*, *Foxc2*, and *Tfap2b* each encode transcription factors that are essen-



**Fig. 5.** Comparison of eye phenotypes in *Pitx2* and *Ctnnb1* mutant mice. Transverse sections of eyes from e14.5 embryos with the indicated genotypes were stained with hematoxylin and eosin. Images of each genotype were taken at the same magnification. l, lens; e, surface ectoderm.



**Fig. 4.**  $\beta$ -catenin and LEF1 physically associate with *Pitx2* promoter sequences. **A:** Schematic illustrating location of conserved TCF/LEF-responsive element located 200-bp upstream of the 5' *Pitx2* promoter. Modified from Kioussi et al. (2002). **B:** ChIP analysis on primary cultures of mesenchyme grown from wild type e12.5 eye primordia with specific antibodies, normal control sera, water, and sheared input DNA. Specific PCR product (\*) is significantly enriched in samples immunoprecipitated with rabbit anti- $\beta$ -catenin and mouse anti-LEF1 antibodies as compared with the normal rabbit serum (NRS) and normal mouse serum (NMS), respectively, indicating physical association of  $\beta$ -catenin and LEF1 with the *Pitx2* promoter sequences in cells derived from periocular mesenchyme.



tial in the ocular neural crest for normal anterior segment development and function. We analyzed the expression of these genes in *Ctnnb1-ncko* mice to determine whether their expression in neural crest might require intact canonical Wnt/ $\beta$ -catenin signaling and because the results could provide insight into the extent to which initial patterning of the anterior segment might be disrupted in these mice.

The forkhead transcription factor gene, *Foxc1*, is required for anterior segment development in mice, and mutations in human *FOXC1* are a second genetic cause of Axenfeld-Rieger Syndrome (Mears et al., 1998; Lehmann et al., 2000, 2003). Like *Pitx2*, retinoic acid signaling is also required to activate *Foxc1* expression in the ocular neural crest (Matt et al., 2005, 2008; Molotkov et al., 2006). FOXC1 protein is normally expressed in neural crest of the periorbital mesenchyme and in the surface ectoderm of the palpebral conjunctiva and palpebral epidermis in the eyelid (Fig. 6A,C) (Kume et al., 2000; Gage et al., 2008). This expression pattern within the anterior segment is unchanged in *Ctnnb1-ncko* mice at e12.5 and e14.5, suggesting that *Foxc1* expression within these structures of the eye is not dependent on canonical Wnt signaling (Fig. 6B,D). However, FOXC1 protein levels are diminished in mesenchyme adjacent to the optic cup and normally fated to become sclera in the *Ctnnb1-ncko* mutants. However, the sclera is absent in *Ctnnb1-ncko* eyes. Therefore, it is not clear whether this loss of *Foxc1* expression is a direct result of inactivating canonical Wnt signaling in these cells or an indirect result of losing scleral specification, which requires *Pitx2* expression in neural crest (Evans and Gage, 2005).

Mutations in *Foxc2/FOXC2* cause distichiasis (extra eyelashes) in mice and humans, as well as additional anterior segment defects in mice (Smith et al., 2000; Kriederman et al., 2003). In developing wild type eyes, FOXC2 protein is initially expressed in the neural crest surrounding the optic cup, but expression in corneal neural crest is down-regulated by e12.5 and beyond (Fig. 6E,G) (Kidson et al., 1999; Gage et al., 2008). FOXC2 is also expressed in surface ectoderm of

the palpebral conjunctiva (Fig. 6E,G) (Gage et al., 2008). These expression patterns are conserved in both the neural crest and palpebral conjunctiva of *Pitx2* mice, suggesting that initial patterning of anterior segment structures is not completely lost in these mutants and *Foxc2* does not appear to be regulated by canonical Wnt signaling (Fig. 6F,H).

*Tfap2b* encodes an AP-2 family transcription factor whose expression from e12.5–14.5 marks neural crest of the corneal, limbal, and conjunctival mesenchyme, as well as surface ectoderm of the presumptive conjunctiva and eyelid (Fig. 6I,K). These expression patterns are retained in *Ctnnb1-ncko* mice (Fig. 6J,L), providing further evidence that initial patterning of anterior segment structures is not completely lost in these mutants.

## DISCUSSION

Inappropriately low or high levels of PITX2 activity lead to anterior segment defects in mice and humans (Semina et al., 1996; Kozlowski and Walter, 2000; Priston et al., 2001; Holmberg et al., 2004), indicating that tight control of *Pitx2* in time and space is a prerequisite for normal eye development and function. Similar control of *Pitx2* expression is required for normal development of organs such as the pituitary gland, craniofacial structures, and heart but the underlying mechanisms regulating expression in any of these structures are not well defined. In the present study, we extend our understanding of the molecular requirements leading to normal *Pitx2* expression during eye development, information that may have relevance in other tissues and organs.

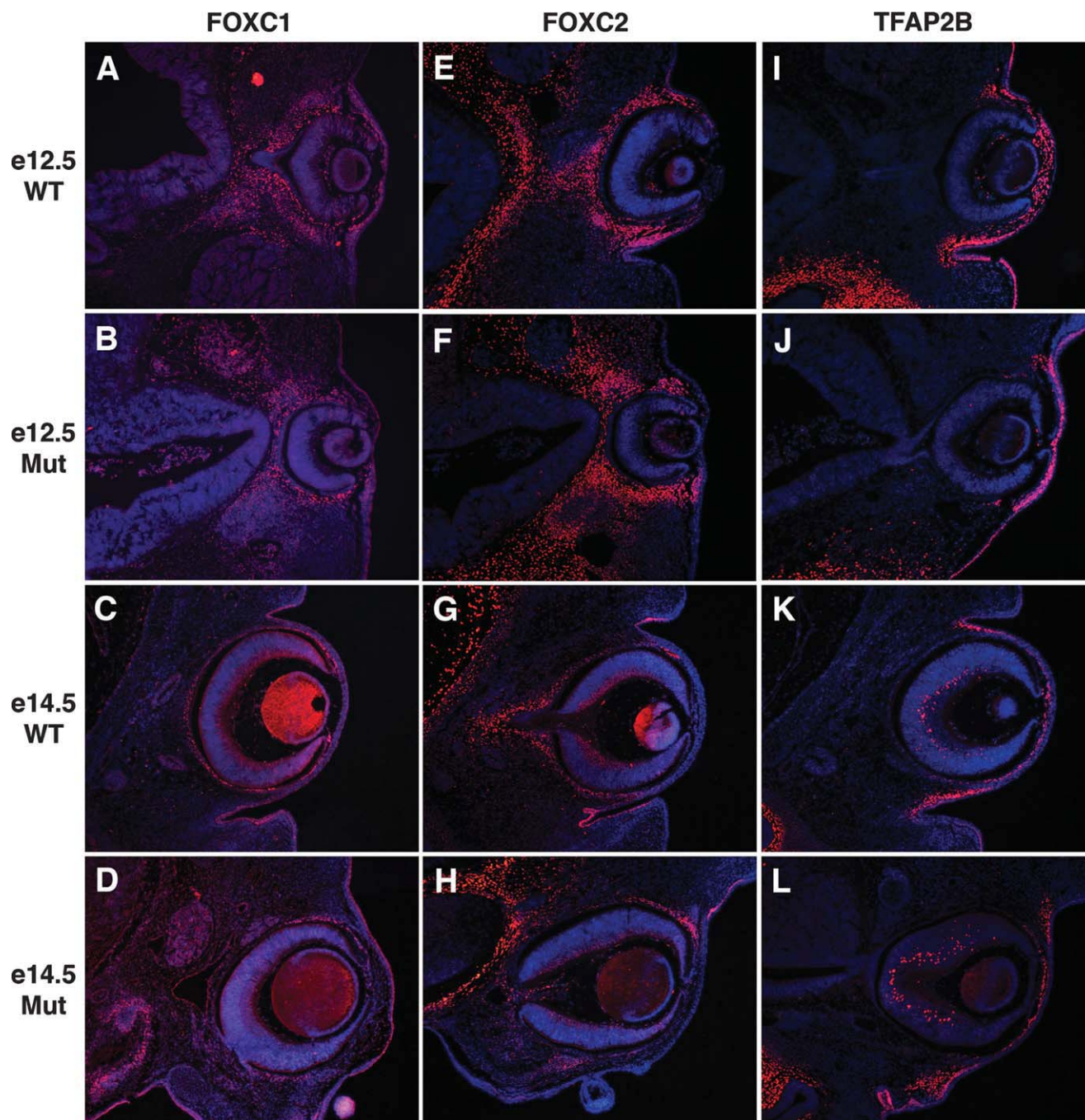
### Normal *Pitx2* Expression in Ocular Neural Crest Is the Net Response to Inputs From Multiple Signaling Pathways

*Pitx2* is not expressed in neural crest migrating to the eye primordia. Rather, retinoic acid directly activates *Pitx2* expression in neural crest after arrival of these cells in the developing eye field (Fig. 7) (Matt et al., 2005, 2008; Molotkov et al., 2006; Kumar and Duester, 2010). The anterior optic cup, lens vesicle, and ocular surface

ectoderm are the sources of the retinoic acid, providing an effective means for stimulating *Pitx2* activation only within neural crest adjacent to the developing eye. The action of retinoic acid on the neural crest is direct as demonstrated by neural crest-specific ablation of the appropriate nuclear receptors (Matt et al., 2005, 2008). Recently, a DR5 RA response element that binds all three RA receptors in the developing eye was identified upstream of *Pitx2*, supporting the idea that *Pitx2* is a direct RA target (Kumar and Duester, 2010). The competence of neural crest to activate *Pitx2* in response to retinoic acid appears to be transient and ends within days of the stage when *Pitx2* is normally first expressed (See and Clagett-Dame, 2009). Therefore, a second mechanism likely underlies persistent *Pitx2* expression within the neural crest, a fact that fits well with the data presented herein.

We tested the potential requirements for canonical Wnt signaling activity on *Pitx2* expression in ocular neural crest. Unlike retinoic acid signaling, canonical Wnt/ $\beta$ -catenin signaling is not required for activation of *Pitx2*, as initial expression in *Ctnnb1-ncko* mice is comparable to control littermates. In contrast, *Pitx2* expression rapidly diminishes and is soon lost in ocular neural crest of *Ctnnb1-ncko* mice. We conclude that intact canonical Wnt/ $\beta$ -catenin signaling is required for maintenance of *Pitx2* expression in neural crest but is not sufficient for activation in these cells (Fig. 7). The action of Wnt signaling appears to be direct as a functional TCF/LEF site within the *Pitx2* promoter is occupied by LEF-1 and  $\beta$ -catenin in ocular neural crest. Collectively, we propose a model in which a serial code of signaling pathways is required for normal overall *Pitx2* expression in ocular neural crest, with retinoic acid required for activation and canonical Wnts subsequently required for maintained expression (Fig. 7). As with retinoic acid, the ocular surface ectoderm and optic cup are likely to be the sources of Wnt ligands (Liu et al., 2003).

Modulation of canonical Wnt/ $\beta$ -catenin signaling activity levels also mediates net local PITX2 protein levels over time in the developing and



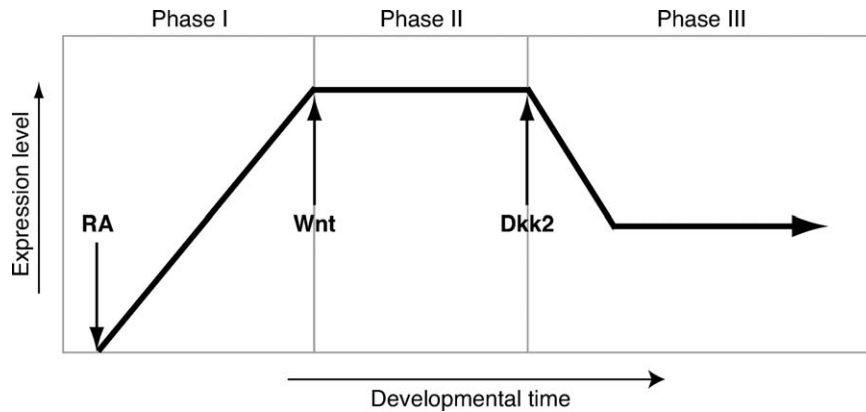
**Fig. 6.** Additional transcription factor expression in *Ctnnb1-ncko* versus control eyes. Eye sections of control and *Ctnnb1-ncko* littermates at the indicated developmental stages were immunostained to detect expression of the transcription factors, FOXC1 (A–D), FOXC2 (E–H), or TFAP2B (I–L). For each, expression in neural crest mesenchyme and surface ectoderm was independent of  $\beta$ -catenin function in the neural crest.

mature eye (Fig. 7) (Gage et al., 2008). Most Axenfeld-Rieger cases linked to *PITX2* arise via a haploinsufficiency mechanism, although an apparent gain-of-function mutation has also been reported (Kozlowski and Walter, 2000; Priston et al., 2001). Over-expression of *PITX2* also results in features of Axenfeld-Rieger in mice, including corneal opacifica-

tion, corneal hypertrophy, and irido-corneal adhesions (Holmberg et al., 2004). Therefore, the ability to ultimately control and fine-tune *PITX2* protein levels through modulation of canonical Wnt signaling activity is likely critical for normal eye development in humans and mice. Additional negative regulators of canonical Wnt signaling are expressed in the develop-

ing and mature eye (Chow and Lang, 2001; Chen et al., 2004; Diep et al., 2004; Fjeld et al., 2005). Although their effects on *PITX2* expression levels have not been tested, it may be that the net effects of *DKK2* and additional negative regulators on canonical Wnt signaling activity provide a key mechanism for providing temporal and spatial control over *PITX2* protein





**Fig. 7.** Model for regulation of *Pitx2* expression in the anterior segment by retinoic acid and Wnt signaling. Phase I: *Pitx2* expression is initiated by retinoic acid signaling produced by the anterior optic cup, anterior lens vesicle, and surface ectoderm. Phase II: *Pitx2* expression is maintained by canonical Wnt signaling produced by the surface ectoderm. Phase III: *Pitx2* expression levels are reduced to lower steady-state levels due to inhibition of canonical Wnt signaling activity by DKK2.

levels once the *Pitx2* gene is initially activated by retinoic acid.

It appears that complex regulation is a general feature of *Pitx2* expression in multiple tissues, as initiation and maintenance of asymmetric *Pitx2* expression in the left lateral plate mesoderm is also dependent upon a two-step process (Shiratori et al., 2001). Regulation of expression at multiple steps and, potentially by divergent mechanisms in different lineages as development proceeds, likely provides advantages over more simple forms of regulation. In the eye, RA signaling activates *Pitx2* expression within a large pool of neural crest cells that are subsequently fated to generate multiple mature tissues over the course of eye development, including corneal stroma, corneal endothelium, and trabecular meshwork. Local and temporal differences in the signaling environment may determine whether *Pitx2* expression is maintained, and at what levels, conditions that likely have a profound effect on fate decisions undertaken by individual cells.

Inputs from other signaling pathways, in addition to retinoic acid and Wnts, are likely to contribute to the code required to achieve appropriate temporal and spatial *Pitx2* expression levels during eye development. Hedgehog appears to be a third component of the signaling code required to maintain *Pitx2* expression during eye development. At e12.5, *Pitx2* expression is largely equivalent between *Ihh*<sup>-/-</sup> eyes and those of control littermates

(Dakubo et al., 2008). By e13.5, however, *Pitx2* expression is lost from mesenchyme located at the posterior and periphery of *Ihh*<sup>-/-</sup> eyes, establishing that hedgehog signaling is required to maintain *Pitx2* expression within the subset of periocular mesenchyme fated to contribute the scleral coating (Dakubo et al., 2008). Notably, hedgehog signaling is not sufficient to activate *Pitx2* expression indicating that this step is mediated by retinoic acid or another signaling pathway. Therefore, in posterior periocular mesenchyme, loss of hedgehog and canonical Wnt signaling has similar consequences, suggesting that the two pathways may act in parallel, a possibility that should be experimentally tested. However, hedgehog signaling is not required to maintain *Pitx2* expression in the anterior segment, indicating that there are clear differences in the requirements for the two pathways, depending upon the cellular position within the eye primorium (Dakubo et al., 2008). This provides further evidence that overall regulation of *Pitx2* expression in these cells is accomplished by a multistep mechanism. TGF- $\beta$  signaling has also been implicated in *Pitx2* expression within anterior segment neural crest but the functions of this pathway with respect to early development are unclear (Ittner et al., 2005).

The requirement for intact canonical Wnt signaling activity in ocular neural crest is not shared by several other key transcription factors in an-

terior segment development. The inclusion of *Foxc1* in this group is particularly surprising since it shares many features with *Pitx2*, including a dependence on retinoic acid signaling for its activation and Indian hedgehog for persistent expression in peripheral and posterior mesenchyme (Matt et al., 2005, 2008; Molotkov et al., 2006; Dakubo et al., 2008). Alterations in *Foxc1/FOXC1* gene dose lead to Axenfeld-Rieger Syndrome in humans and cause anterior segment defects in mice. This indicates that achieving appropriate FOXC1 activity levels is a prerequisite for normal eye development and, similar to PITX2, they are likely subject to tight temporal and spatial control(s) (Mears et al., 1998; Lehmann et al., 2000; Smith et al., 2000). However, despite the implication of common regulatory mechanisms suggested by these functional similarities, it does not appear that a requirement for canonical Wnt signaling activity for maintenance of *Foxc1/FOXC1* expression is among the similarities.

### Roles for Canonical Wnt Signaling in the Neural Crest During Early Eye Morphogenesis

Canonical Wnt signaling has the capacity to control many functions in neural crest fated to contribute ocular tissues, including induction, migration, cell survival, cell proliferation, and differentiation. Our data suggest that intact canonical Wnt signaling is not required for migration of neural crest to the eye primordia, as these cells populate early *Ctnnb1-ncko* eyes at a level comparable to control eyes. Similarly, intact canonical Wnt signaling appears to be dispensable for early eye morphogenesis from the neural and surface ectoderm, as the optic cup and lens vesicle are grossly normal in *Ctnnb1-ncko* mutants at e10.5. Therefore, we conclude that the earliest stages of eye development do not require competence of the neural crest to respond to canonical Wnt signaling.

In contrast, *Ctnnb1-ncko* eyes begin to exhibit morphological differences from control littermates by e11.5, when deviations in the cornea, orientation of the optic cup, and the optic



stalk first become apparent. These phenotypes are enhanced over the next several days and are followed by agenesis of the sclera and eyelids. The initial emergence of these phenotypes correlates strongly with the timing of loss of *Pitx2* expression. In addition, these phenotypes encompass many of the phenotypes observed in *Pitx2* mutant mice (Gage et al., 1999; Evans and Gage, 2005). Collectively, these data suggest that maintenance of *Pitx2* expression is an important early functional target of canonical Wnt signaling activity in the neural crest during eye development.

Development of the cornea, optic cup, and optic stalk are complex processes and must be regulated by many factors and pathways. Therefore, it was somewhat surprising that none of the other key regulators of eye development that we tested were dependent on canonical Wnt signaling for their expression. This was likely due to the fact that we only tested a small number of genes that had previously been identified as important transcriptional regulators of anterior segment development, comparable to *Pitx2*. We expect that canonical Wnt signaling regulates the expression and function of additional genes in the ocular neural crest that are required for eye development, and that these will be revealed in the future using more open-ended, unbiased approaches. Discovery of these genes and ascertaining their functions are prerequisites for a more complete understanding of eye development.

### ***Pitx2* Expression Facilitates Cohesion Between Tissues During Early Anterior Segment Morphogenesis**

The optic cups in global and neural crest-specific *Pitx2* mutant eyes are displaced a considerable distance from the surface ectoderm (Gage et al., 1999; Evans and Gage, 2005), whereas eyes of *Ctnnb1-ncko* mice are located in the wild type position at the periphery of the head, immediately beneath the surface ectoderm. Transient expression of *Pitx2* in temporal knockout mice also largely rescues the displaced optic cup and lens phenotypes of global and neural crest-specific *Pitx2* mutant mice,

permitting them to remain localized beneath the surface ectoderm in association with the presumptive corneal epithelium. Collectively, these data suggest that early *Pitx2* expression in the neural crest activates a mechanism for bonding anterior segment elements together, maintaining the position of these elements relative to each other as the head grows rapidly in size during development. This bonding mechanism likely works in concert with the *Pitx2*-dependent signaling that is required for optic stalk extension (Evans and Gage, 2005).

The molecular mechanism(s) contributing to this process remains to be determined. At an earlier stage of eye development, during formation of the optic cup and lens vesicle, contractile filopodia tether the presumptive lens to the retina via a Cdc42- and IRSp53-dependent mechanism, providing a method for coordinated evagination between the two epithelial layers (Chauhan et al., 2009). This raises the possibility that early expression of *Pitx2* may activate similar connections between the anterior optic cup, lens, neural crest, and surface ectoderm during the subsequent phase of eye development. Alternatively, *Pitx2* may activate expression of adhesive extracellular matrix molecules, such as integrins or collagens that bond together the various tissue components. Further work will be required to identify the underlying molecular mechanism(s).

## **EXPERIMENTAL PROCEDURES**

### **Mouse Strains and Generation of Timed Pregnant Litters**

Generation of alleles for Cre-mediated inactivation of *Ctnnb1* (*Ctnnb1<sup>tm2Kem</sup>*) (Brault et al., 2001) and *Pitx2* (*Pitx2<sup>lox</sup>*) (Gage et al., 1999), or Cre-mediated activation of a lacZ reporter (*R26R<sup>lox</sup>*) was described previously (Soriano, 1999). Transgenic mice expressing Cre under the control of the *Wnt1* promoter (*Wnt1-Cre*) were obtained from A. McMahon (Danielian et al., 1998). Transgenic mice expressing a tamoxifen-inducible form of Cre (Cre-ER<sup>T2</sup>) under the control of the ubiquitously expressed *UBC* promoter

(*Ubc-CreER<sup>T2</sup>*) were obtained from The Jackson Laboratories (Bar Harbor, ME) (Gruber et al., 2007; Ruzankina et al., 2007). The *Wnt1-Cre; Ctnnb1<sup>+tm2Kem</sup>* parental line was generated by mating *Wnt1-Cre* and *Ctnnb1<sup>tm2Kem</sup>* mice. The *Ubc-CreER<sup>T2</sup>; Pitx2<sup>+null</sup>* parental line was generated by mating *Ubc-CreER<sup>T2</sup>* and *Pitx2<sup>null</sup>* mice. Mice were maintained as separate breeding colonies at the University of Michigan. 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.

*Wnt1-Cre; Ctnnb1<sup>+tm2Kem</sup>* mice were mated to *Ctnnb1<sup>tm2Kem/tm2Kem</sup>; R26R<sup>lox/lox</sup>* mice to generate timed pregnancies for neural crest-specific *Ctnnb1* knockout mice, designating the day after mating as embryonic day 0.5. Embryos were collected by caesarian section at the appropriate stages, after euthanasia of the mother. The resulting embryos were genotyped for *Cre* (Gage et al., 2005), *Ctnnb1* (Brault et al., 2001), or *R26R* (Soriano, 1999) using PCR-based methods. Embryos with a *Wnt1-Cre; Ctnnb1<sup>tm2Kem/tm2Kem</sup>* genotype were considered mutant, while *Ctnnb1<sup>+tm2Kem</sup>* or *Ctnnb1<sup>tm2Kem/tm2Kem</sup>* embryos lacking the *Wnt1-Cre* transgene were used as controls.

*Ubc-CreER<sup>T2</sup>; Pitx2<sup>+null</sup>* mice were mated to *Pitx2<sup>lox/lox</sup>; R26R<sup>lox/lox</sup>* mice to generate timed pregnancies for temporal-specific *Pitx2* knockout mice, designating the day after mating as embryonic day 0.5. A single intraperitoneal injection of tamoxifen (Sigma, St. Louis, MO; T5648-1G) suspended in corn oil at a dose of 100  $\mu$ g per gram of body weight was administered to the pregnant dam at noon on e10.5. Embryos were collected on either e11.5 or e14.5 by caesarian section after euthanasia of the mother. The resulting embryos were genotyped for *Cre* (Gage et al., 2005), *Pitx2* (Gage et al., 1999), or *R26R* (Soriano, 1999) using PCR-based methods. Embryos with a *Ubc-CreER<sup>T2</sup>; Pitx2<sup>lox/null</sup>* genotype were considered mutant. Embryos with the genotype *Pitx2<sup>+lox</sup>* were considered wild type controls while embryos with

the genotype *Pitx2<sup>fllox/null</sup>* were considered heterozygous controls.

## Embryo Processing and Histology

All embryos were fixed in 4% paraformaldehyde in PBS, washed, dehydrated, and processed for sectioning in Paraplast Plus (McCormick Scientific; St. Louis, MO). Mounted paraffin 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 min in 10 mM citrate buffer (pH 6.0). Sections were treated with Image-iT FX signal enhancer (Molecular Probes; Eugene, OR) for 30 min. Immunostaining was performed according to standard protocols. Briefly, sections were incubated with primary antibodies against FoxC1, FoxC2 (Abcam; Cambridge, MA), TFAP2B (Novus Biologicals; Littleton, CO),  $\beta$ -catenin (Santa Cruz Biotechnology; San Diego, CA),  $\beta$ -galactosidase (kind gift from Tom Glaser), and PITX2 (Hjalt et al., 2000). Treatment with the primary antibody was followed with biotinylated species-specific secondary antibodies (Jackson Immuno Research; West Grove, PA). Signals were detected using tyramide signal amplification kits (Molecular Probes; Eugene, OR). Photography was performed on a Nikon Eclipse 800 fluorescence microscope.

## TUNEL Assay

Terminal dUTP nick end labeling (TUNEL) was performed using an in situ Cell Death Detection kit (Roche, Indianapolis, IN) per manufacturer protocol.

## Primary Cell Isolation and Chromatin Immunoprecipitation Assays

Eye primordia and surrounding mesenchyme microdissected from multiple wild type e12.5 embryos were pooled and cultured until primary mouse fibroblasts (MEF) were obtained as previously described (Crofford et al., 1994). CHIP assays were performed on

the MEFs (passages 3 through 8) as previously described (Gummow et al., 2006). Specific immunoprecipitations were performed using a rabbit polyclonal antibody generated against  $\beta$ -catenin (Santa Cruz; Santa Cruz, CA) or a mouse monoclonal antibody generated against LEF1 (Millipore; Temecula, CA). Parallel control immunoprecipitations were performed using normal rabbit serum (Vector; Burlingame, CA) or normal mouse serum (Millipore), respectively. Immune complexes were captured using a mix of protein A and protein G beads (Active Motif; Carlsbad, CA) and eluted DNA fragments were purified with a QIAquick Spin Kit (Qiagen; Valencia, CA). Purified DNA was analyzed by PCR using region-specific *Pitx2* genomic primers as described below. Water and sheared input DNA were used as negative and positive controls, respectively. Sequences spanning the TCF/LEF site within the 5' *Pitx2* promoter were amplified using the forward and reverse primers 5'-TTA GAA GGG GTT TGA CGC AC-3' and 5'-CCA CTA GGG CTG AGA AGT GA-3', respectively. Sequences spanning the TCF/LEF site within the 3' enhancer were used as described previously (Ai et al., 2007).

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