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The canonical Wnt signaling antagonist DKK2 is an essential effector of PITX2 function during normal eye development

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Summary

Local control of cell signaling activity and integration of inputs from multiple signaling pathways are central for normal development but the underlying mechanisms remain poorly understood. Here we show that *Dkk2*, encoding an antagonist of canonical Wnt signaling, is an essential downstream target of the PITX2 homeodomain transcription factor in neural crest during eye development. Canonical Wnt signaling is ectopically activated in central ocular surface ectoderm and underlying mesenchyme in *Pitx2* and *Dkk2* deficient mice. General ocular surface ectoderm identity is maintained during development in *Dkk2* deficient mice but peripheral fates, including conjunctival goblet cells and eyelash follicles, are ectopically permitted within more central structures and eyelids are hypomorphic. Loss of DKK2 results in ectopic blood vessels within the periocular mesenchyme and PITX2 expression remains persistently high, providing evidence for a negative feedback loop. Collectively, these data suggest that activation of *Dkk2* by PITX2 provides a mechanism to locally suppress canonical Wnt signaling activity during eye development, a paradigm that may be a model for achieving local or transient inhibition of pathway activity elsewhere during embryogenesis. We further propose a model placing PITX2 as an essential integration node between retinoic acid and canonical Wnt signaling during eye development.

Keywords

homeobox; canonical Wnt signaling; mouse

INTRODUCTION

The use of cell-cell signaling to facilitate local induction, patterning, proliferation, differentiation, and even apoptosis is a universally employed strategy during development. Frequently, multiple distinct signaling pathways are active within close confines in a developing tissue or organ and integration of the various inputs is critical for normal development. Signaling integration is similarly important in adult tissue homeostasis,

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regeneration, and cancer yet specific molecular mechanisms for integrating inputs from distinct signaling pathways remain poorly understood.

The anterior segment of the mammalian eye consists of the iris, ciliary body, lens, cornea, outflow tract, conjunctiva, and eyelids, and provides an important model for understanding molecular mechanisms underlying development, including integration of cell-cell signaling pathways. Anterior segment development begins with induction of the lens from surface ectoderm by the optic vesicle followed by migration of neural crest into the space between the anterior rim of the optic cup and the newly formed lens vesicle, and the overlying ocular surface ectoderm (Cvekl and Tamm, 2004). Ultimately, the mature anterior segment develops from four embryonic lineages, including neural ectoderm (components of the ciliary body and iris), ocular surface ectoderm (lens, epithelial components of the cornea, limbus, conjunctiva, and the harderian and lacrimal glands, and eyelid epidermis), neural crest (corneal stroma and endothelium, conjunctival and eyelid mesenchyme), and mesoderm (schlemm's canal and blood vessel endothelium). Extensive and often reciprocal interactions occur between the four embryonic primordia and multiple signaling molecules are expressed in overlapping expression patterns, suggesting that integration of distinct signaling pathways is likely to be required for normal anterior segment development.

Local control of canonical Wnt/β-catenin signaling activity levels is essential for determining fates within the ocular surface ectoderm (OSE). Canonical Wnt/β-catenin signaling is initiated by binding of Wnt ligands to a heterodimeric cell surface receptor consisting of a frizzled (Fzd) subunit and an LRP5/6 subunit (Logan and Nusse, 2004). Receptor activation leads to stabilization of β-catenin protein, which translocates to the nucleus to activate target gene expression. Although components of the canonical Wnt/β-catenin signaling pathway, including ligands and receptors, are uniformly expressed throughout the OSE, expression of a β-catenin-responsive reporter transgene is undetectable within the presumptive cornea ectoderm but high in more peripheral tissues, including the early conjunctival epithelium and eyelid epidermis (Liu et al., 2003). Therefore, net pathway activity levels within the OSE are highly tissue specific and must be locally regulated. Local differences in pathway activity are functionally important since ectopic activation of β-catenin throughout newly specified ocular surface ectoderm blocks lens placode induction (Miller et al., 2006) and lacrimal gland outgrowth (Dean et al., 2005) while loss of canonical Wnt/β-catenin signaling permits ectopic lens fate differentiation within peripheral ocular surface ectoderm (Smith et al., 2005). However, the mechanism(s) leading to regional quantitative differences in canonical Wnt/β-catenin signaling activity levels within the ocular surface ectoderm despite uniform expression of Wnt ligands and receptors has not been uncovered.

Morphogenesis of the anterior segment is also critically dependent upon retinoic acid (RA) signaling originating from the anterior rim of the optic cup, the lens, and the OSE (Matt et al., 2005; Molotkov et al., 2006). Loss of RA synthesis results in eyelid defects and agenesis of the cornea, among other eye defects (Matt et al., 2005; Molotkov et al., 2006). Neural crest-derived periocular mesenchyme (POM) underlying the OSE is the primary immediate target of the RA signaling since mice with neural crest-specific deletions of the appropriate nuclear RA receptors exhibit the same phenotypes (Matt et al., 2005). Downstream effectors of RA signaling in the neural crest include key transcription factor genes required for anterior segment morphogenesis (Matt et al., 2005). One critical target of RA signaling in the neural crest is *Pitx2*, encoding a homeodomain transcription factor that is widely required for eye development and function (Matt et al., 2005). Global and neural crest-specific *Pitx2* knockout mice exhibit a severe ocular dysgenesis phenotype that overlaps significantly with that of RA mutant mice, including agenesis of the cornea, abnormal blood vessels, a dysgenic optic nerve, and coloboma (Evans and Gage, 2005; Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999). Heterozygous mutations in human *PITX2* result in a spectrum of anterior segment

defects, including microcornea, iris stroma hypoplasia, anterior synechiae, iris and lenticular attachments to the central posterior cornea (Peters Anomaly), and a high risk for early-onset open-angle glaucoma (Alward, 2000; Doward et al., 1999; Kulak et al., 1998; Semina et al., 1996). Despite its overall importance in anterior segment morphogenesis, no specific developmental pathways downstream of PITX2 have previously been identified.

We now show that *Dkk2*, encoding an extracellular antagonist of canonical Wnt/β-catenin signaling, is a critical downstream target of *Pitx2* in neural crest during eye development. Activation of *Dkk2* provides an essential mechanism for locally suppressing canonical Wnt/β-catenin signaling activity within corneal surface ectoderm and underlying mesenchyme, raising the possibility that Dickkopfs may be similarly deployed elsewhere when there is a need to locally or transiently suppress canonical Wnt/β-catenin signaling activity during development and tissue regeneration. Normal barriers to ocular blood vessel growth are lost and PITX2 expression levels are elevated in *Dkk2* deficient mice. The later phenotype provides evidence of an auto-regulatory loop between PITX2 and DKK2. Together with previous reports, our results identify *Pitx2* as an essential integration point between retinoic acid and canonical Wnt/β-catenin signaling during eye development, providing a mechanism for crosstalk between these two major signaling pathways that may be relevant elsewhere in development and disease.

MATERIALS AND METHODS

Mouse strains and generation of timed pregnant litters

Generation of $Pitx2^{null}$, $Pitx2^{flox}$, and $Dkk2^{null}$ mice was described previously (Gage et al., 1999; Lu et al., 1999). *Wnt1Cre* mice, which carry a transgene containing a Cre cassette under the control of the *Wnt1* promoter, were obtained from A. McMahon (Danielian et al., 1998). The *Wnt1Cre;* $Pitx2^{+/-}$ parental line was generated by mating *Wnt1Cre* and $Pitx2^{null}$ 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.

$Pitx2^{+/null}$ mice were mated to generate timed pregnancies carrying the *Pitx2* allelic series while $Dkk2^{+/null}$ mice were mated to generate timed pregnancies carrying the *Dkk2* allelic series. *Wnt1Cre;* $Pitx2^{+/-}$ mice were mated to $Pitx2^{flox/flox}$ mice to generate timed pregnancies for neural crest-specific *Pitx2* knockout mice. The morning after mating was designated as embryonic day 0.5. Embryos were collected 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 *Dkk2* (Li et al., 2005) using PCR-based methods. Embryos with a *Wnt1Cre;* $Pitx2^{flox/-}$ genotype were considered mutant, while $Pitx2^{flox/+}$embryos were used as controls.

Realtime PCR confirmation

Realtime RT-PCR was performed using TaqMan Gene Expression assays (Applied Biosystems; Foster City, CA). E12.5 $Dkk2^{+/+}$, $Dkk2^{+/null}$ and $Dkk22^{null/null}$ embryos were obtained from *Dkk2* null heterozygous matings. The eyes and surrounding periocular mesenchyme were microdissected and stored in RNAlater (Ambion; Cambridge, MA). Total RNA was extracted from homogenized tissue using RNAqueous-micro kit (Ambion; Cambridge, MA). cDNA was reverse transcribed from1–5μg total RNA by SuperScript III with random primers (Invitrogen; Carlsbad, CA) according to the manufacture's protocol. cDNA was diluted as 1 μg total RNA/100 μl final volume (10ng/ul). TaqMan Gene Expression Assays (Applied Biosystems; Foster City, CA) were performed with RealTime Ready master mix (Q.BIOgene; Solon, OH) in an iCycler thermal cycler (Bio-Rad; Hercules, CA) according

to the manufacture's protocol. Relative fold changes compared to wild type were calculated using $2^{-\Delta\Delta Ct}$ method and normalized to *Gpi1*. Standard error was computed from four samples of each genotype.

Primary cell isolation and ChIP assays

Eye primordia and surrounding mesenchyme microdissected from multiple wild type e12.5 embryos were pooled and cultured until primary mouse embryonic fibroblasts (MEF) were obtained as previously described (Crofford et al., 1994). ChIP assays were performed on the primary MEFs (passages 3 through 8) as previously described (Gummow et al., 2006). We used a goat polyclonal antibody specific for PITX2 (C-16; Santa Cruz Biotechnology; Santa Cruz, CA) for specific immunoprecipitation, and normal goat serum (Invitrogen; Carlsbad, CA), normal bovine IgG (ChIP-IT™ Mouse Control Kit; Active Motif; Carlsbad, CA), and water as negative controls. Immune complexes were captured using 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 primers as listed in Supplemental Table 1 online.

Promoter cloning and luciferase assays

A 4-kb genomic DNA fragment including 3.5-kb upstream of the *Dkk2* (ENSMUST 00000028031) transcription start site and 0.5-kb of untranslated first exon sequences was amplified using the Expand High Fidelity PCR System (Roche; Indianapolis, IN) and cloned into the pGL3-Basic vector (Promega; Madison, WI) using Sac I/Bam HI restriction sites built into the primers. Sequences of the primers used in amplification of the *Dkk2* promoter are available in Supplemental Table 1.

Transfections were carried out in Chinese hamster ovary (CHO) cells or human embryonic kidney (HEK293) cells using FuGENE6 (Roche; Indianapolis, IN) according to standard techniques. Cells were transfected with the *Dkk2* promoter-luciferase reporter plasmid (*Dkk2*-luc) and either an expression vector encoding one of the three PITX2 protein isoforms (Suh et al., 2002) or empty pcDNA3 (Invitrogen; Carlsbad, CA). For all conditions, the constitutively expressing renilla vector pPolIII-RL (Nybakken et al., 2005) was included as a control for transfection efficiency. Cells were lysed 48 hours after transfection, and luciferase and renilla levels assayed using the Dual-Luciferase Reporter Assay System (Promega; Madison, WI) according to the manufacturer's protocol. Normalized luciferase values were compared with the *Dkk2*-luc reporter plasmid transfected with empty pcDNA3 to calculate the extent of activation. Results are presented as relative activity and are expressed as the mean±s.e.m. of three experiments, each performed in triplicate. Co-transfection with empty pGL3-Basic together with PITX2 expression vectors did not result in activation of luciferase as compared to co-transfection with empty pcDNA3 (data not shown).

Embryo processing and histology

All embryos were fixed in 4% paraformaldehyde in PBS, washed, dehydrated, and processed for sectioning in either Paraplast Plus (McCormick Scientific; St. Louis, MO) or JB-4 plastic (Polysciences, Inc; Warrington, PA). Mounted paraffin sections for morphological analysis were dewaxed, rehydrated, and stained with hematoxylin and eosin or Periodic Acid Schiff Stain. Plastic sections were stained by the Lee's method.

Immunofluorescence

Paraffin sections were dewaxed and treated for antigen retrieval by boiling for 10 minutes in 10 mM citrate buffer (pH6.0). Sections were treated with Image-iT FX signal enhancer (Molecular Probes; Eugene, OR) for 30 minutes. Immunostaining was performed according

to standard protocols. Briefly, sections were stained with antibodies against cytokeratin 4, FoxC1, FoxC2 (Abcam; Cambridge, MA), cytokeratin 12 (kind gift form Winston Kao), Vegfr2 (Cell Signaling Technology; Danvers, MA), Vegfr3 (R&D Systems, Minneapolis, MN), Pax6, Filaggrin, and Loricrin (Covance; Berkley, CA). Treatment with the primary antibody was followed with biotinylated (Jackson Immuno Research; West Grove, PA) species-specific secondary antibodies. Signals were detected using tyramide signal amplification kits (Molecular Probes; Eugene, OR). Photography was performed on a Nikon Eclipse 800 fluorescence microscope.

In Situ Hybridization

Antisense riboprobes to *Pitx2* (Gage and Camper, 1997), *Dkk2* (gift from G. Hammer), and *Axin2* (gift from S. Camper) were generated and labeled with digoxigenin according to standard procedures. Paraffin sections were processed for *in situ* hybridization as previously described (Gage et al., 1999). All probes were incubated at 57°C.

RESULTS

Functional PITX2 in neural crest is required for *Dkk2* expression during eye development

We have previously used microarrays to compare global gene expression in e12.5 eye primordia taken from wild type, $Pitx2^{+/-}$, and $Pitx2^{-/-}$ mice as a means to begin understanding the molecular mechanisms underlying PITX2 function during eye development (Diehl et al., 2006). Further analysis of this data suggested *Dkk2*, encoding an antagonist of canonical Wnt signaling, as a gene whose expression is sensitive to *Pitx2* gene dose (data not shown). We confirmed this prediction using quantitative RT-PCR on total RNA taken from microdissected eye primordia of wild type, $Pitx2^{+/-}$, and $Pitx2^{-/-}$embryos (Fig. 1A). *Pitx2* is expressed in the periocular mesenchyme (POM) surrounding the optic cup and in emerging extraocular muscles of e12.5 wild type eye primordia (Fig. 1B). *Dkk2* mRNA is also expressed in the POM surrounding the optic cup but not in the extraocular muscles (Fig 1B) (Diep et al., 2004). In contrast, *Dkk2* expression is undetectable by RNA *in situ* hybridization in e12.5 global *Pitx2* knockout mice (Fig. 1B). *Dkk2* expression was similarly absent from neural crest-specific *Pitx2* knockout animals generated using our conditional $Pitx2^{flox}$ allele together with *Wnt1Cre* (Fig. 1B) (Evans and Gage, 2005). Based on these data, we concluded that *Dkk2* lies in a genetic cascade directly or indirectly downstream of *Pitx2* in neural crest during eye development.

Canonical Wnt signaling is deregulated in *Dkk2* and *Pitx2* mutant mice

The current model for DKK2 protein function is that it acts together with Kremen as an extracellular antagonist of canonical Wnt signaling (Mao and Niehrs, 2003). However, DKK2 may also have additional functions (Li et al., 2005). Therefore, we examined *Dkk2* mutant versus wild type littermate controls for expression of *Axin2*, a direct target of canonical Wnt signaling that is a useful surrogate for detecting up-regulation of the pathway (Jho et al., 2002; Lustig et al., 2002; Yan et al., 2001). *Axin2* expression is robustly enhanced in the conjunctiva, limbus, and cornea of *Dkk2* mutant eyes, as compared to wild type littermates (Fig. 1C). In each structure, *Axin2* expression is strongest in basal cells of the surface ectoderm as well as adjacent mesenchyme (Fig. 1C ii & iv). *Axin2* expression is also elevated in corneal stroma cells more distant from the surface ectoderm (Fig. 1C). *Axin2* expression is not noticeably affected in the corneal endothelium, sclera, anterior lens epithelia, and presumptive ciliary body and iris within the optic cup in eyes of *Dkk2* mutant mice (Fig. 1C). This is interesting because DKK2 is normally expressed either in precursors to these lineages (corneal endothelium and sclera) or in neighboring cells (anterior lens and presumptive ciliary body and iris). The sites of up-regulated canonical Wnt signaling, as measured by *Axin2* expression, correlate strongly with the most significant phenotypes in *Dkk2* mutant eyes (see below). Based

on this, we conclude that up-regulated canonical Wnt signaling is a major general mechanism contributing to the ocular phenotypes in these mice.

We also compared *Axin2* expression in wild type vs. *Pitx2*$^{-/-}$ eyes to determine whether loss of DKK2 downstream of PITX2 also led to up-regulated canonical Wnt signaling in these mice or was compensated by some other mechanism. In wild type eyes, *Axin2* is expressed within the surface ectoderm of the developing eyelids and conjunctiva but is excluded from the presumptive limbal and corneal epithelium (Fig. 1D). *Axin2* is also expressed in the underlying mesenchyme of the presumptive cornea, limbus, conjunctiva, and eyelid of wild type mice (Fig. 1D). These results agree well with previously reported sites of canonical Wnt signaling activity based on transgenic reporter expression (Liu et al., 2003;Miller et al., 2006;Smith et al., 2005). In contrast, *Axin2* is highly expressed throughout the ocular surface ectoderm in eyes of *Pitx2*$^{-/-}$ littermates, including the corneal epithelium and persistent lens stalk, when present (Fig. 1D). Therefore, local suppression of canonical Wnt signaling due to expression of *Dkk2* is likely to be a key mechanism by which PITX2 regulates anterior segment development in the eye.

PITX2 physically associates with and transactivates *Dkk2* promoter sequences

The mouse *Dkk2* 5′-flanking and promoter regions contain multiple potential PITX2 binding sites that can be identified either by using MatInspector or by manually searching for PITX2 response elements (TAATCC, TATTCC, CAATC, TGATCC, AAATCC), raising the possibility that PITX2 may directly regulate *Dkk2* expression (Fig. 2A). To test for physical interaction between PITX2 and *Dkk2* promoter sequences, we performed chromatin immunoprecipitation (ChIP) using an antibody specific for PITX2 on primary MEF's cultured from wild type e12.5 eye primordia. These cells maintain expression of *Pitx2* and *Dkk2* in culture (data not shown). PITX2 associates with elements located within the *Dkk2* promoter but not with unrelated sequences located elsewhere within the genome (Fig. 2B). Expression of a reporter plasmid under control of a 4-kb mouse *Dkk2* promoter/5′-untranslated fragment encompassing the regions that physically interact with PITX2 was significantly stimulated by co-transfection into CHO cells with expression vectors encoding any of the three PITX2 protein isoforms as compared to co-transfection of the empty expression vector (Fig. 2C). Expression from the empty reporter plasmid lacking inserted *Dkk2* sequences was unaffected by co-transfected PITX2 expression vectors (data not shown). Comparable results were also obtained after transfection into HEK293 cells (data not shown). Collectively, these data suggest that *Dkk2* is a direct target of PITX2 in ocular neural crest. Further experiments will be required to definitively identify the underlying PITX2 responsive site(s).

Dkk2 is required for normal eye development and homeostasis

We next tested the potential functional significance of this *Pitx2*-dependent cascade by examining eyes from *Dkk2*$^{-/-}$ (*Dkk2* mutant) animals (Li et al., 2005). Eyelids extend across the ocular surface and are fused shut in e18.5 wild type mice (Fig. 3A). In contrast, eyelids of e18.5 *Dkk2* mutant littermates are severely hypomorphic, leading to a lack of fusion and eyelids open at birth (EOB) (Fig. 3B–D). Despite the fact that the *Dkk2* expression pattern is symmetric, the morphological and molecular (see below) effects on eyelids are consistently more severe dorsally than ventrally. In addition, nascent ectopic hair follicles are readily apparent within the presumptive conjunctiva and limbal regions of e18.5 *Dkk2* mutant eyes (Fig. 3C,D).

EOB and ectopic growth of eyelashes would be expected to rapidly result in sequelae such as corneal irritation and subsequent corneal keratinization at postnatal time points, which would complicate interpretation of phenotypes identified after birth as developmental or disease response. Therefore, we focused our analysis of corneal development in response to loss of DKK2 on prenatal time points. The thickness of the corneal surface and limbal ectoderm is

moderately increased in e18.5 *Dkk2* mutant mice (3–5 cells thick) as compared to wild type littermates (2–3 cells thick). However, the mutant corneal surface ectoderm at this time point is otherwise morphologically unremarkable and readily distinguished from neighboring epidermis (Fig. 3E&F).

Keratinocytes of the wild type corneal stroma at e18.5 exhibit a characteristic wavy morphology and are arranged in a highly organized and laminated fashion across the entire width of the layer (Fig 3E). In contrast, corneal stroma cells in *Dkk2* mutant littermates have lost the characteristic wavy morphology and frequently have assumed a more stellate shape (Fig. 3F). Lamination is also disrupted, with the highest degree of disorganization occurring in cells of the anterior stroma located just beneath the corneal surface ectoderm (Fig. 3F). Finally, although the wild type corneal stroma is avascular by e18.5, ectopic small blood vessels invading the anterior corneal stroma are readily apparent in *Dkk2* mutant littermates (Fig. 3G&H). These early morphological alterations are consistent with gene expression changes also present at similar or earlier time points (see below). The corneal endothelium in *Dkk2* mutants and their wild type littermates appear indistinguishable at this time point (Fig. 3E&F). We conclude from these results that loss of DKK2 disrupts normal developmental of the corneal surface ectoderm and stroma.

By 3 weeks of age, eyelids of *Dkk2* mutant mice are thickened and fail to open fully (ptosis), and ectopic eyelashes (distichiasis) lie in contact with the cornea (Fig. 4A&B). Despite the external appearance owing to the eyelid defect, enucleated *Dkk2* mutant eyes are not microphthalmic (data not shown). Filamentous attachments span the anterior chamber of *Dkk2* mutant eyes by 3 weeks of age (Fig. 4C). These filaments consist of small blood vessels and associated pigmented cells that traverse the anterior chamber from the iris collarette to the corneal endothelium, a feature commonly associated with Peters Anomaly (Fig. 4D and (Doward et al., 1999)). Although the thickness of the corneal ectoderm in *Dkk2* mutant mice is similar to that of wild type littermates, the cellular morphology is now significantly different and PAS-positive goblet cells that would normally be limited to the conjunctival ectoderm extend well into the cornea (Fig. 4E&F). The anterior corneal stroma of *Dkk2* mutant eyes is heavily invested with small blood vessels, similar to wild type conjunctiva, and individual cells are highly disorganized relative to the wild type cornea (Fig. 4E&F). Fully developed ectopic eyelash cilia are present in the conjunctiva and limbus of *Dkk2* mutant eyes (Fig. 4H). However, ectopic cilia are never found in the cornea. By 12 weeks of age, an ectopic pigmented sheath surrounds the optic nerve of *Dkk2* mutant eyes after it exits the eye (Fig. 4I). This ectopic sheath consists of small blood vessels, pigmented cells, and other fibroblasts, and is contiguous with the choroidal capillaries (Fig. 4J).

General ocular surface ectoderm identity is not lost but corneal and conjunctival fates are interspersed in *Dkk2* mutant mice

Initial specification of the ectoderm-derived components of conjunctival and corneal epithelium from ocular surface ectoderm begins at around e12.5 in mice, which closely follows the initial activation of *Dkk2* in the neural crest ((Wolosin et al., 2004; Wolosin et al., 2002) and data not shown). To test the hypothesis that loss of DKK2 might grossly alter the initial cell fate decisions within the ocular surface ectoderm, we first analyzed cytokeratin 12 (cK12), a specific marker of corneal epithelium in wild type mice (Fig. 5A and (Moll et al., 1982; Tseng et al., 1982). cK12 expression is maintained in surface ectoderm overlying the cornea of e18.5 *Dkk2* mutant mice but expression is discontinuous and not present in every cell (Fig. 5A). No expansion into other regions of OSE was observed in *Dkk2* mutant eyes but ectopic cK12 expression is present in cells of the corneal stroma and endothelium, which are derived from mesenchyme (Fig 5A inset). These data suggest the partial retention of a corneal epithelium identity in these mice through e18.5. Expression of cytokeratin 4 (cK4), a specific

marker of conjunctival epithelium in wild type eyes ((Kurpakus et al., 1994) and Fig. 5A), is maintained in the conjunctiva of e18.5 *Dkk2* mutant eyes but is ectopically expanded into the corneal ectoderm (Fig. 5Aiv). Based on these data, we infer that specification of the corneal surface ectoderm fate is not completely lost in prenatal *Dkk2* mutant mice but that this cell population is also significantly biased towards a conjunctival fate, an observation that is consistent with the eventual appearance of goblet cells within the *Dkk2* mutant corneas. An alternative explanation would be that the conjunctivalization arises out of changes in the microenvironment due to the associated EOB phenotype. However, despite the frequency of EOB in the literature, we find no evidence of corneal conjunctivalization associated with these mice (see e.g. (Li et al., 2001; Luetteke et al., 1993; Mine et al., 2005; Tao et al., 2005; Vassalli et al., 1994; Zenz et al., 2003; Zhang et al., 2003)). Therefore, although we cannot formally exclude it, we do not favor this possibility as a likely mechanism underlying corneal conjunctivalization in *Dkk2* mutant mice.

Although our data suggested that overall OSE identity is maintained during development in the absence of DKK2, a previous report concluded that corneas in a second strain of *Dkk2* deficient mice undergo a developmental fate switch to epidermis (Mukhopadhyay et al., 2006). Therefore, we further analyzed our mice for expression of PAX6, which distinguishes OSE from surrounding head ectoderm fated to become epidermis (reviewed in (Cvekl and Piatigorsky, 1996)), as well as markers of mature, differentiated skin epidermis. PAX6 expression in *Dkk2* mutant eyes is indistinguishable from wild type littermates through e14.5 (data not shown). PAX6 expression is also indistinguishable from wild type littermates in OSE of e16.5 *Dkk2* mutant mice (Fig. 5B), indicating that overall OSE identity is maintained at least through late gestation. Interestingly, PAX6 protein is ectopically present in the corneal stroma and endothelium of *Dkk2* deficient mice at e16.5 (Fig. 5B), providing evidence of early molecular changes to corroborate morphological evidence of changes to the corneal stroma presented above. Filaggrin and loricrin are markers of the cornified cellular envelop in mature epidermis (Roop et al., 1984), including the outer covering of the eyelids in wild type mice, but are excluded from the conjunctiva, limbus, and cornea at e18.5 (Fig. 5C). The conjunctiva, limbal, and corneal ectoderm of $Dkk2^{-/-}$ eyes at e18.5 are also essentially devoid of filaggrin or loricrin staining (Fig. 5C). The rare exception occurs in very small patches of cells that are in direct contact with the tip of an eyelid, which likely irritates the cornea and activates a keratinization response even at this prenatal time point (Fig. 5C). Based on these results, we conclude that corneas in our strain *Dkk2* mutant mice do not undergo a developmental fate switch to epidermal skin. Although filaggrin and loricrin are subsequently activated throughout corneas of postnatal *Dkk2* mutant mice, we interpret this to be a disease response as the eyes undergo keratinization due to chronic corneal irritation and infection arising from the eyelid defects and ectopic eyelashes (data not shown).

Altered *Fox* gene expression contributes to eyelid defects and distichiasis in *Dkk2* mutant mice

Mutations in the genes for two forkhead transcription factors, FOXC1 and FOXC2 are associated with EOB in mice (Kidson et al., 1999; Kume et al., 2000; Smith et al., 2000) and heterozygous *Foxc2/FOXC2* mutations cause distichiasis in mice and humans (Fang et al., 2000; Kriederman et al., 2003). *Foxc1* is a target of RA signaling in the neural crest (Matt et al., 2005) and *Foxc2* is also expressed in the neural crest (Gage et al., 2005). We therefore hypothesized that altered FOXC1 and FOXC2 in neural crest might be a contributing mechanism to EOB and ectopic eyelashes in *Dkk2* mutant mice but no discernable differences in neural crest expression of these two proteins were identified in *Dkk2* mutant versus wild type littermates from e12.5–16.5 (Fig. 6A&B and data not shown). Unexpectedly, we found previously unreported expression of both proteins in the presumptive conjunctival ectoderm in wild type embryos, and FOXC1 expression extending into the surface ectoderm overlying

the eyelid (Fig. 6A&B, compare ii vs. iv in each). No FOXC1 or FOXC2 could be detected in the dorsal eyelid and conjunctival surface ectoderm of *Dkk2* mutant mice while expression of each protein is variably reduced in the ventral eyelid and conjunctiva (Fig. 6A&B). From these data, we infer that the previously reported requirements for FOXC1 and FOXC2 in eyelid development and suppression of ectopic eyelash cilia stems from their expression in ocular surface ectoderm, not the neural crest. The loss or reduction in these two proteins in the dorsal and ventral eyelids, respectively, is likely to contribute mechanistically to the EOB and distichiasis phenotypes in *Dkk2* mutant mice.

DKK2 is required to regulate PITX2 expression during corneal stroma differentiation

Initial PITX2 expression is high throughout the mesenchyme of the nascent anterior segment but expression progressively diminishes in cells of the differentiating cornea (Fig. 7) (Berry et al., 2006;Hjalt et al., 2000). *Pitx2* has previously been identified as a direct target of canonical Wnt signaling in cell culture (Briata et al., 2003;Kioussi et al., 2002) and the timing and location of the reduction in PITX2 expression levels coincides well with the increase in canonical Wnt signaling that we documented in *Dkk2* mutant mice. Therefore, we examined PITX2 expression in wild type versus *Dkk2* mutant littermates in order to test the hypothesis DKK2-mediated suppression of canonical Wnt signaling might normally be required for down modulation of PITX2 expression levels. PITX2 expression in wild type versus *Dkk2* mutant eyes is indistinguishable at e12.5, which is shortly after activation of *Dkk2* (Fig. 7A&B). PITX2 expression is also not noticeably different in the corneal endothelium and mesenchymal cells of the emerging iridocorneal angles in eyes from either genotype at e16.5 (Fig 7C&D). In contrast, while PITX2 expression in the corneal stroma of e16.5 wild type eyes is significantly lower than at earlier time points, as expected, high PITX2 expression persists in cells within the corneal stroma of *Dkk2* mutant littermates (Fig. 7E&F). These data are consistent with the idea that functional DKK2 and the associated suppression of canonical Wnt signaling are required for the normal reduction of PITX2 expression levels in the differentiating corneal stroma. In addition, they provide further evidence of early molecular changes occurring in parallel with the morphological chances within the mutant corneal stroma reported above.

DISCUSSION

Dkk2 is required within the ocular anterior segment for normal “cross-talk” between neural crest and ocular surface ectoderm

Ocular surface ectoderm is set aside from surrounding head ectoderm fated to become epidermis during the initial stages of eye development and subsequently further specified into lens and the epithelial components of the cornea, limbus, conjunctiva, lacrimal gland, and eyelid (reviewed in (Chow and Lang, 2001; Wolosin et al., 2004)). Ligands and receptors required for canonical Wnt/β-catenin signaling are expressed uniformly throughout the OSE yet the pathway is normally active only in peripheral structures and is apparently suppressed in central structures such as the presumptive corneal ectoderm (Liu et al., 2003). Although the underlying mechanism(s) has remained unclear, these regional differences in canonical Wnt/β-catenin signaling activity play a critical role in subdivision of the multipotent OSE into discrete mature fates as genetic or pharmacologic activation of the pathway blocks development of central structures such as lens and lacrimal gland development while ablation or inhibition of the pathway permits ectopic activation of the lens differentiation program in more peripheral regions (Dean et al., 2005; Miller et al., 2006; Smith et al., 2005). Mature fates within the OSE are governed by cues from the underlying mesenchyme, as demonstrated by tissue recombination experiments (Ferraris et al., 1994; Ferraris et al., 2000; Pearton et al., 2005). However, the identity of the specific molecule(s) required has remained unknown

Based on our results, we conclude that DKK2 expressed from the underlying neural crest mesenchyme is an essential regulator of OSE differentiation through its ability to locally suppress canonical Wnt/β-catenin signaling activity within the central OSE. This blockade of pathway activity is essential for promotion of corneal fates and inhibition of conjunctival fates within the central OSE since loss of DKK2 and the accompanying activation of pathway signaling results in intermingling of corneal and conjunctival fates both developmentally and post-natally. DKK2 function is also required to prevent ectopic specification of eyelash cilia within the conjunctiva and limbus, as well as for normal eyelid growth. Although more difficult to quantify since detectable levels of canonical Wnt/β-catenin signaling are normally present in OSE fated to become these conjunctiva and eyelids, the underlying mechanism(s) is likely related to net pathway activity levels, which are dys-regulated in *Dkk2* mutant mice. Therefore, it appears that development within the OSE requires graded canonical/β-catenin signaling levels ranging from outright suppression in centrally located structures to increasing activity in more peripheral structures.

Loss of DKK2 also results in developmental defects with in the mesenchyme, particularly within the corneal stroma and ocular blood vessels (see below). Therefore, DKK2 has autocrine effects on the neural crest in addition to paracrine functions in the OSE. Consistent with this interpretation, both autocrine (e.g. PAX6, cK12, PITX2) and paracrine (e.g. FOXC1, FOXC2) gene expression changes are present in *Dkk2* mutant eyes. Given that *Axin2* expression is also affected in both layers, the simplest model to account for these gene expression changes is that they are all a direct consequence of increased canonical Wnt signaling. However, we recognize that other, more complicated alternatives cannot be excluded based on current data and further work will be required to distinguish among the possibilities.

The local action of DKK2 induced by the optic cup and lens (see below) provides an effective solution for achieving specific, local suppression of canonical Wnt signaling within the central OSE and underlying mesenchyme, while at the same time permitting signaling levels to remain higher outside the zone of greatest DKK2 influence. Interestingly, DKK1 is also expressed in eyelid mesenchyme during the relevant timeframe, and genes encoding SFPR1 and particularly SFRP2 are highly expressed within the lens placode and early lens vesicle at the time when low/absent canonical Wnt signaling is required for lens development (Chen et al., 2004; Chow and Lang, 2001; Diep et al., 2004; Fjeld et al., 2005). Therefore, expression of secreted antagonists at specific developmental time points appears to be a general mechanism for locally suppressing canonical Wnt signaling and permitting normal differentiation of structures from the ocular surface ectoderm during eye development. This paradigm may also serve as a general model for other occasions where normal development or tissue regeneration through activation of stem or other precursor cells requires local or transient suppression of canonical Wnt/β-catenin signaling activity.

It should be noted that our results and conclusions differ in key ways from a previous report on a distinct strain of *Dkk2* knockout mice, which concluded that ocular surface ectoderm had undergone a developmental fate switch to epidermis based in part on postnatal expression of filaggrin and loricrin (Mukhopadhyay et al., 2006). We also find post-natal expression of these proteins in our mice but view this as a disease response to chronic corneal irritation arising from distichiasis and EOB. Importantly, EOB was not previously reported but is central to interpretation of the post-natal effects. Consistent with this interpretation, we observe no prenatal expression of either protein. In addition, we see no evidence for eyelash cilia in the cornea, which would be expected with a developmental fate switch to epidermis. Finally, we document much broader up-regulation of canonical Wnt signaling in both the ocular surface ectoderm and the underlying mesenchyme. This variation may result from mouse strain differences or our *Axin2* expression assay may have been more sensitive that the transgenic reporter used in the previous report.

A role for DKK2 in ocular blood vessel homeostasis

Ocular blood vessels in wild type mice observe strict boundaries beyond which growth does not occur. In the anterior segment, small blood vessels form a rich network within the mesenchyme underlying the limbal epithelium but do not penetrate into the cornea. Similarly, a network of small blood vessels grows centripetally from the outer to the inner iris stroma but does not cross the pupil. Finally, choroidal capillary blood vessels overlaying the retinal pigmented epithelium grow up to the scleral canal posterior to the optic nerve head but do not extend onto the optic nerve itself. Blood vessels in *Dkk2*-deficient eyes violate all three of these boundaries, identifying a role for DKK2 in establishing boundaries during normal ocular blood vessel growth and homeostasis.

Activation of canonical Wnt signaling has recently been identified as an important mechanism for promoting angiogenesis. Vascular endothelial cells express components representing all levels of the pathway, including SFRP's and DKK's (Goodwin et al., 2006). Expression of Wnt ligands or stabilized forms of β-catenin stimulates proliferation and the formation of capillary-like networks by vascular endothelial cells *in vitro* (Masckauchan et al., 2005; Wright et al., 1999). Specific mechanisms underlying Wnt function include transcriptional activation of genes for additional angiogenic factors, such as VEGF's (Longo et al., 2002; Skurk et al., 2005) and Interleukin-8 (Masckauchan et al., 2005), as well as β-catenin-mediated modulation of VEGF receptor activity (Skurk et al., 2005). Treatment of vascular endothelial cells with the Wnt-binding extracellular antagonist SFRP1 blocks β-catenin-dependent activation of gene transcription (Goodwin et al., 2006) and suppresses cell proliferation (Duplaa et al., 1999).

The strong correlation between up-regulated *Axin2* expression and ectopic blood vessel growth into corneal stroma in *Dkk2*-deficient mice strongly implicates activated canonical Wnt signaling as the underlying mechanism. DKK2 expression in the emerging wild type corneal stroma may act as a firewall to suppress local canonical Wnt signaling levels, making the resulting environment less stimulating or even non-permissive to blood vessel growth. We have not yet sought to correlate increased canonical Wnt signaling with ectopic blood vessel growth from the iris or onto the optic stalk because we have focused the current work primarily on prenatal time points. However, parallel mechanisms likely contribute to regulation of blood vessel growth and homeostasis at these sites as well. Mutations leading to activation of canonical/β-catenin signaling have been identified in several retinal diseases sharing the common thread of defective retinal vasculature development or function (Berger et al., 1992; Black et al., 1999; Chen et al., 1993; Ohlmann et al., 2004; Robitaille et al., 2002; Toomes et al., 2004; Xu et al., 2004). Therefore, a role for DKK2 in regulating ocular blood vessel development or homeostasis through modulation of canonical Wnt signaling is highly consistent with this emerging field.

A PITX2-DKK2 auto-regulatory loop is required for normal ocular anterior segment development

Our data establish that *Dkk2* is an important downstream effector of *Pitx2* function in neural crest during eye development. *Dkk2* expression is lost in both global and neural crest-specific *Pitx2* knockout mice. The ability of PITX2 to physically interact with sequences within the *Dkk2* promoter and to transactivate a reporter construct linked to these sequences is consistent with the idea that *Dkk2* is a direct transcriptional target of PITX2. The functional relevance of this relationship is established by demonstration that *Dkk2* mutant mice share many elements in common with *Pitx2/PITX2* phenotypes in mice and humans, including altered corneal thickness, iridocorneal adhesions, Peters anomaly, and altered blood vessel development (Alward et al., 1998; Asai-Coakwell et al., 2006; Doward et al., 1999; Evans and Gage, 2005; Gage et al., 1999; Kitamura et al., 1999; Kulak et al., 1998; Lu et al., 1999; Semina et al., 1996). Collectively, these observations are significant because they identify *Dkk2* and

canonical Wnt/β-catenin signaling as the first functionally important gene/regulatory pathway downstream of PITX2 during normal anterior segment development and the suggest that dys-regulation of this network is a contributing mechanism underlying *Pitx2/PITX2* mutant phenotypes in mice and humans. However, the increased severity of the phenotype in *Pitx2* vs. *Dkk2* knockout eyes strongly implies the existence of additional essential regulatory genes and signaling networks lying downstream of PITX2 during eye development.

Our data also establish that DKK2 is required for normal reductions in PITX2 protein levels within the developing corneal stroma. This feedback presumably occurs though moderation or suppression of canonical Wnt/β-catenin activity within the neural crest since this pathway is dys-regulated in neural crest of *Dkk2* mutant eyes and has previously been implicated in *Pitx2* transcription (Kioussi et al., 2002) and mRNA stability (Briata et al., 2003) in other cell types *in vitro*. The ability of DKK2 to negatively feedback on *Pitx2* gene expression or mRNA stability by modulating Wnt signaling provides an attractive means for achieving/maintaining correct PITX2 protein function levels, which is critical for normal eye development and function. The idea that PITX2 protein function levels are important in development of the eye, face, and other organs was first suggested by demonstration that gain- or loss-of-function *PITX2* mutations cause Axenfeld-Rieger Syndrome (ARS) (Amendt et al., 1998; Berry et al., 2006; Kozlowski and Walter, 2000; Lines et al., 2002; Priston et al., 2001) and was further supported by *Pitx2* gene dose experiments in mice (Diehl et al., 2006; Gage et al., 1999; Holmberg et al., 2004). In particular, persistently high-level *Pitx2* expression in transgenic mice leads to ARS-like phenotypes, confirming that the ability to moderate PITX2 function levels is critical for normal eye development (Holmberg et al., 2004). It will be important to determine whether inhibition of canonical Wnt/β-catenin signaling plays a similar feedback role in other organs where correct PITX2 activity levels are critical, including during craniofacial and heart development. DKK2-mediated feedback might also provide for a more sophisticated means of sensing and responding to small variations in PITX2 levels during development or in response to environmental cues such as stress and fluctuations in intraocular pressure in the mature eye. However, this hypothetical ability of DKK2 feedback to “fine tune” PITX2 levels must be limited since mutations leading to even apparently modest reductions in PITX2 function have been identified in ARS patients (Kozlowski and Walter, 2000; Kulak et al., 1998; Lines et al., 2002).

Pitx2 integrates retinoic acid and canonical Wnt signaling during ocular anterior segment development

Ocular neural crest has recently been identified as the key tissue orchestrating developmental responses to retinoic acid (RA) signaling in early eye development. The optic cup and lens are the primary sources of RA during this period and ablation of the relevant RA-synthesizing enzymes results in eye defects that largely phenocopy those in global and neural crest-specific *Pitx2* knockout mice (Matt et al., 2005; Molotkov et al., 2006). Neural crest is the primary target of the RA signaling since neural crest-specific deletion of *Rarb* and *Rarg*, encoding RA-activated nuclear receptor transcription factors, produces an analogous phenotype (Matt et al., 2005). *Pitx2* expression is severely reduced in mice lacking either RA synthesis or the RA-activated nuclear receptors, identifying PITX2 as a key effector of RA signaling in the neural crest (Fig. 8) (Matt et al., 2005). As we have now demonstrated, PITX2 –dependent activation of *Dkk2* in neural crest results in local suppression of canonical Wnt signaling in both the neural crest and ocular surface ectoderm. Collectively, these data identify *Pitx2* as an essential integration node between RA signaling from the optic cup and canonical Wnt signaling within the ocular surface ectoderm and adjacent neural crest (Fig. 8). This model also predicts that RA and Wnt signaling may combinatorially or synergistically regulate *Pitx2* expression. Indeed, it will be particularly interesting to determine whether canonical Wnt/β-catenin

signaling is required to initially activate *Pitx2* within the neural crest or simply plays a modulatory role.

Examples of functional interactions between RA and Wnt signaling during development have been reported previously (Easwaran et al., 1999; Hecht et al., 2000; Prinos et al., 2001; Verani et al., 2007). A particularly relevant example is the recent demonstration that the activation of *Dkk1* and subsequent inhibition of Wnt signaling underlie the ability of RA signaling to induce a neuronal differentiation program in mouse embryonic stem cells (Verani et al., 2007). In addition, RA and Wnts interact during establishment of vertebral patterning along the anterior-posterior axis in part by synergistically regulating transcriptional expression of *Cdx1* (Prinos et al., 2001). Additional examples of RA/Wnt interactions can be found in breast and colon cancer (Szeto et al., 2001; Tice et al., 2002a; Tice et al., 2002b). Although there are exceptions, the underlying molecular mechanisms in most cases remain poorly understood. Therefore, our results fit well within an emerging theme in development and cancer, and suggest that PITX2 may be playing a role in integrating RA and Wnt signaling elsewhere during development and in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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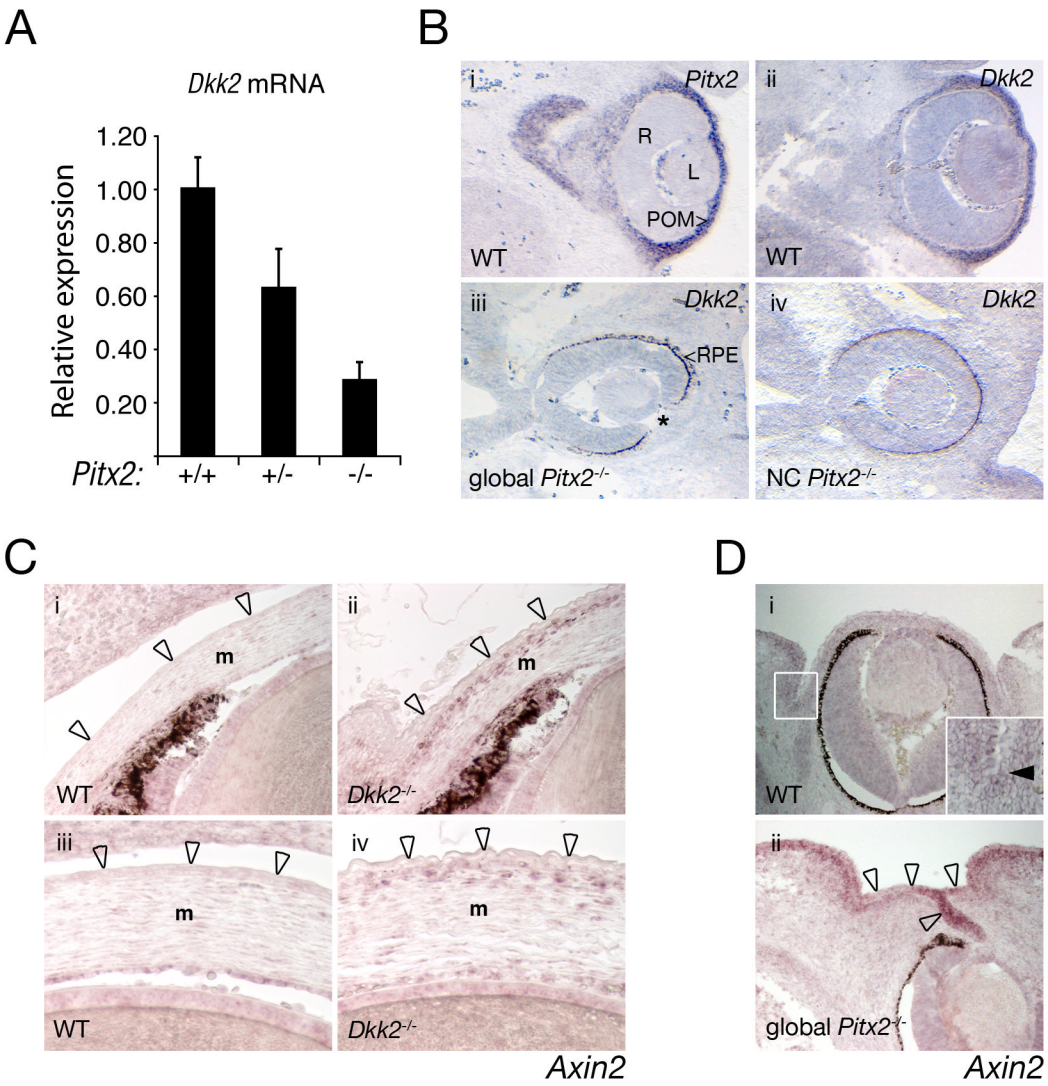

Figure 1. *Dkk2* is regulated by *Pitx2* gene dose and suppresses canonical Wnt signaling in the developing eye

(A) *Dkk2* expression levels correlate strongly with *Pitx2* gene dose in TaqMan Gene expression assays performed on total RNA isolated from microdissected eye primordia of e12.5 *Pitx2* embryos of the indicated genotypes. Values are derived from N=4 samples/genotype. (B) RNA *in situ* hybridization to detect *Pitx2* or *Dkk2* expression in e12.5 wild type and *Pitx2* mutant eyes. *Dkk2* (i) and *Pitx2* (ii) are both expressed in periocular mesenchyme of wild type eyes but *Dkk2* is completely undetectable in eyes of global (iii) or neural crest-specific (iv) *Pitx2* knockout embryos. (C) *Axin2* expression by RNA *in situ* hybridization in eyes from e15.5 wild type and $Dkk2^{-/-}$ littermates. *Axin2* is highly upregulated in ocular surface ectoderm (open arrowheads) and underlying mesenchyme (m) within the iridocorneal angle (ii) and central cornea (iv) of mutant eyes. (D) *Axin2* expression by RNA *in situ* hybridization in eyes from e13.5 wild type and $Pitx2^{-/-}$ littermates. *Axin2* expression ceases at the border between conjunctival and corneal ectoderm in wild type eyes (insert i, closed arrowhead) but extends throughout the ocular surface ectoderm in *Pitx2*−/− eyes (ii, open arrowheads). *Key*: L, lens; R, retina; POM, periocular mesenchyme; RPE, retinal pigmented epithelium; *, presumptive cornea.

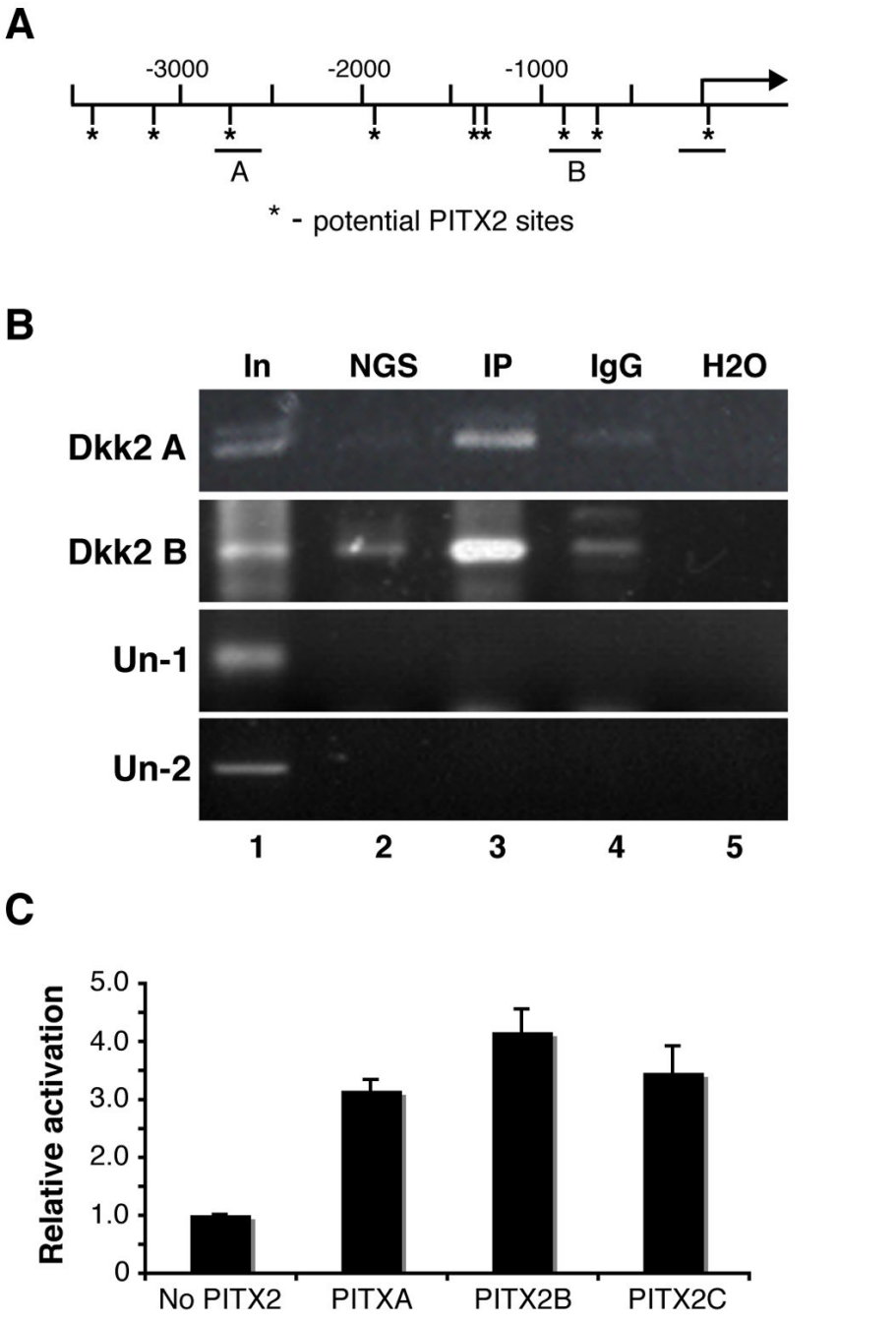

Figure 2. PITX2 physically associates with *Dkk2* promoter sequences and transactivates a linked reporter

(A) Schematic of *Dkk2* genomic sequences spanning from −3,500 to +500 base pairs from the transcription start site illustrating position of potential PITX2 binding sites and regions amplified by *Dkk2* primer sets A and B. (B) ChIP analysis on primary cultures of mesenchyme grown from wild type e12.5 eye primordia with an anti-PITX2 antibody showed that PITX2 specifically interacts with elements of the *Dkk2* promoter but not unrelated sequences. (C) Reporter assay using a 4-kb *Dkk2* fragment including 3.5-kb of promoter sequences and 0.5-kb of untranslated 1st exon sequences linked to luciferase. CHO cells were transfected with

the reporter plasmid and an empty expression vector or vector encoding one of the three PITX2 isoforms. Error bars represent s.e.m.

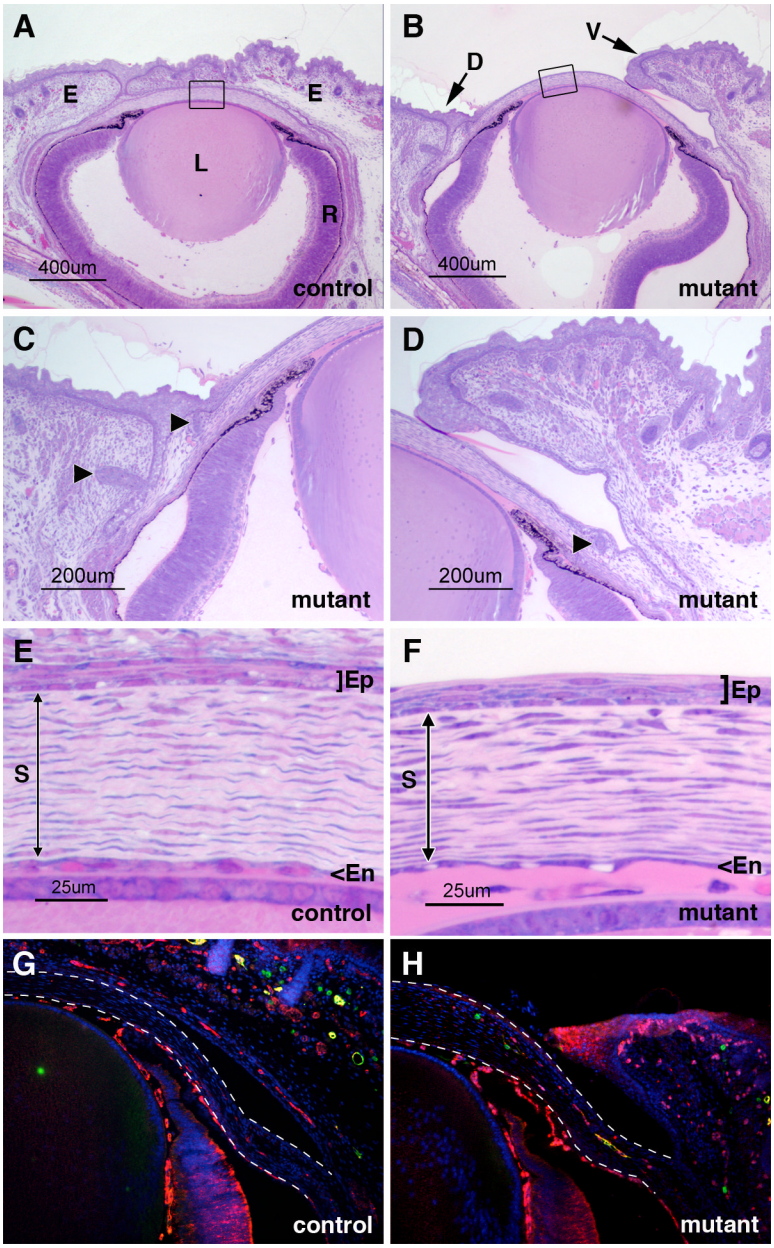

Figure 3. Prenatal *Dkk2* mutant eyes are defective in ocular anterior segment development

Coronal JB-4 plastic (A-F) and paraffin (G,H) sections taken from e18.5 littermates with genotypes as marked. (A,B) Eyelids of *Dkk2*$^{null/null}$ mutant embryos are hypomorphic compared to wild type littermate controls, with the dorsal lid (D) always more severely affected than the ventral lid (V). (C,D) *Dkk2* mutant embryos have frequent ectopic eyelash follicles within the presumptive conjunctiva and limbus (arrowheads). (E,F) High magnification central cornea images taken from boxed regions in panels A&B, respectively. The distinctive wavy morphology and highly ordered lamellar organization of emerging keratinocytes in the wild type corneal stroma is replaced by more stellate-shaped cells and loss of regular organization in presumptive stroma cells of *Dkk2* mutant eyes, and the mutant corneal epithelium is moderately thickened. (G,H) VEGFR2 (red) and VEFGR3 (green) immunofluorescence highlights the avascular corneal stroma of the wild type littermate compared to the presence of ectopic blood vessels penetrating the anterior corneal stroma of *Dkk2* mutant eyes. Note:

for examples from each genotype, the dashed lines outline the inner and outer curvature of the cornea. Key: L, lens; R, retina; box, central cornea; E, eyelids; Ep, corneal epithelium; S, corneal stroma; En, corneal endothelium. All images are oriented with dorsal to the left.

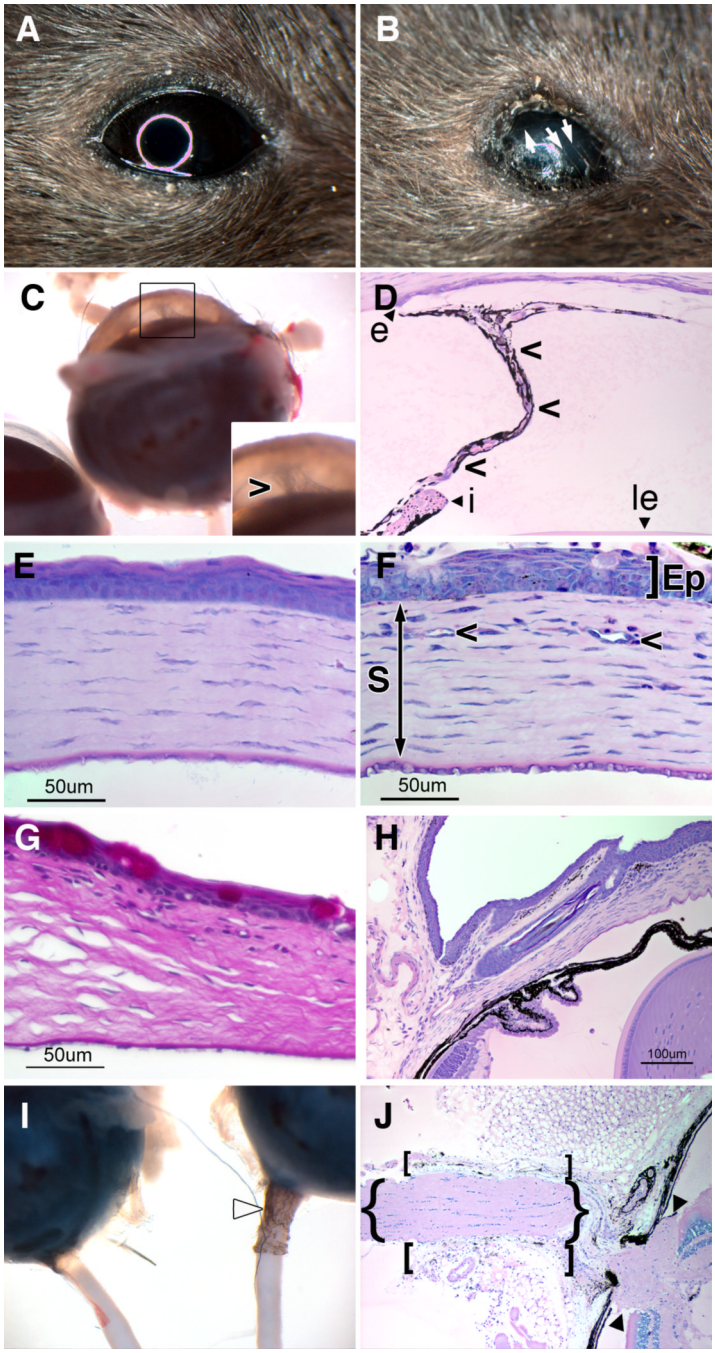

Figure 4. Postnatal *Dkk2* mutant eyes have additional defects

All samples are of 3-week-old eyes, except where noted. Position of normal eyelashes in wild type littermate (A) compared to ectopic eyelashes lying across cornea in *Dkk2*$^{null/null}$ eye (B). Note also periorbital edema and ptosis in mutant eye. (C) *Dkk2*$^{null/null}$ eyes have iridocorneal attachments (box and inset). (D) Histologic section of *Dkk2*$^{null/null}$ eye showing iridocorneal attachments consisting of ectopic small blood vessels spanning anterior chamber from iris collarette (i) to central corneal endothelium (e). Keratinocytes in a mature wild type corneal stroma (E) have a characteristic appearance and are highly organized while in corneas of *Dkk2*$^{null/null}$ mice (F) the presumptive stroma cells (S) are disorganized and numerous small blood vessels (<) populate the anterior corneal stroma. In addition, the morphology of mutant

corneal epithelium cells (Ep) is altered relative to wild type and apparent goblet cells are present. (G) Periodic acid schiff staining confirms the presence of mucin-containing goblet cells in *Dkk2*$^{null/null}$ corneas. (H) Fully developed ectopic eyelash cilia are present in the limbus and conjunctiva of *Dkk2*$^{null/null}$ eyes. (I) Ectopic pigmented sheath extending onto optic nerve (open arrowhead) of a *Dkk2*$^{null/null}$ eye at 12 weeks of age. Wild type control littermate eye is on left. (J) Plastic section of ectopic sheath ([]) and optic nerve ((Evans and Gage, 2005)) showing small capillaries and associated pigment cells, and continuity with the choroidal capillaris.

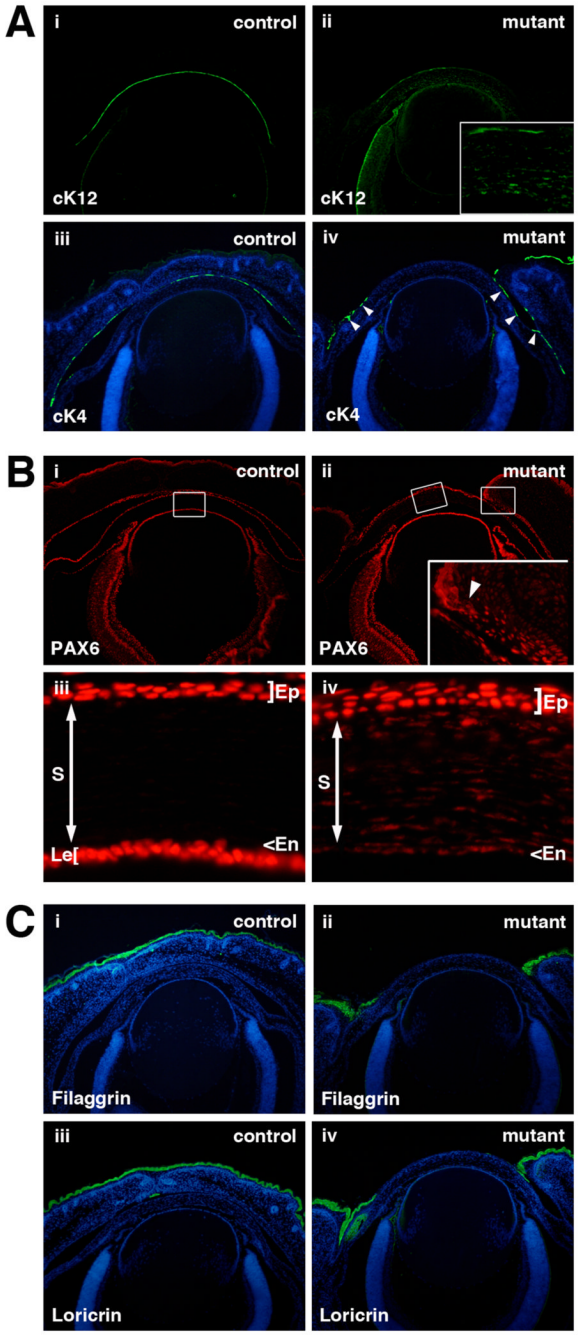

Figure 5. Expression of lineage-specific markers shows prenatal bias of corneal surface ectoderm towards a conjunctival fate but maintenance of an overall OSE identity in *Dkk2* mutant eyes

(A) Expression of the lineage-specific markers cytokeratin 12 (corneal epithelium) and cytokeratin 4 (conjunctival epithelium) in eyes from e18.5 wild type control and *Dkk2*$^{-/-}$ littermates by immunofluorescence. (B) Expression of the general OSE marker PAX6 in e16.5 wild type control and *Dkk2*$^{-/-}$mutant littermate eyes by immunofluorescence. Magnified central corneas from wild type (iii) and *Dkk2* mutant (iv) eyes (boxed regions in panels i & ii, respectively). Nuclear PAX6 expression, a signature of all ocular surface ectoderm, is maintained in all components of the ocular surface ectoderm in *Dkk2* mutant eyes (e.g. inset in panel ii). Note: non-nuclear epidermis label (*) is non-specific background. Key: Ep, corneal

epithelium; S, corneal stroma; En, corneal endothelium; Le, anterior lens epithelium (not present in panel ii). (C) Expression of mature epidermis markers Filaggrin and Loricin in expression in eyes from e18.5 wild type control and *Dkk2*$^{-/-}$ littermates by immunofluorescence.

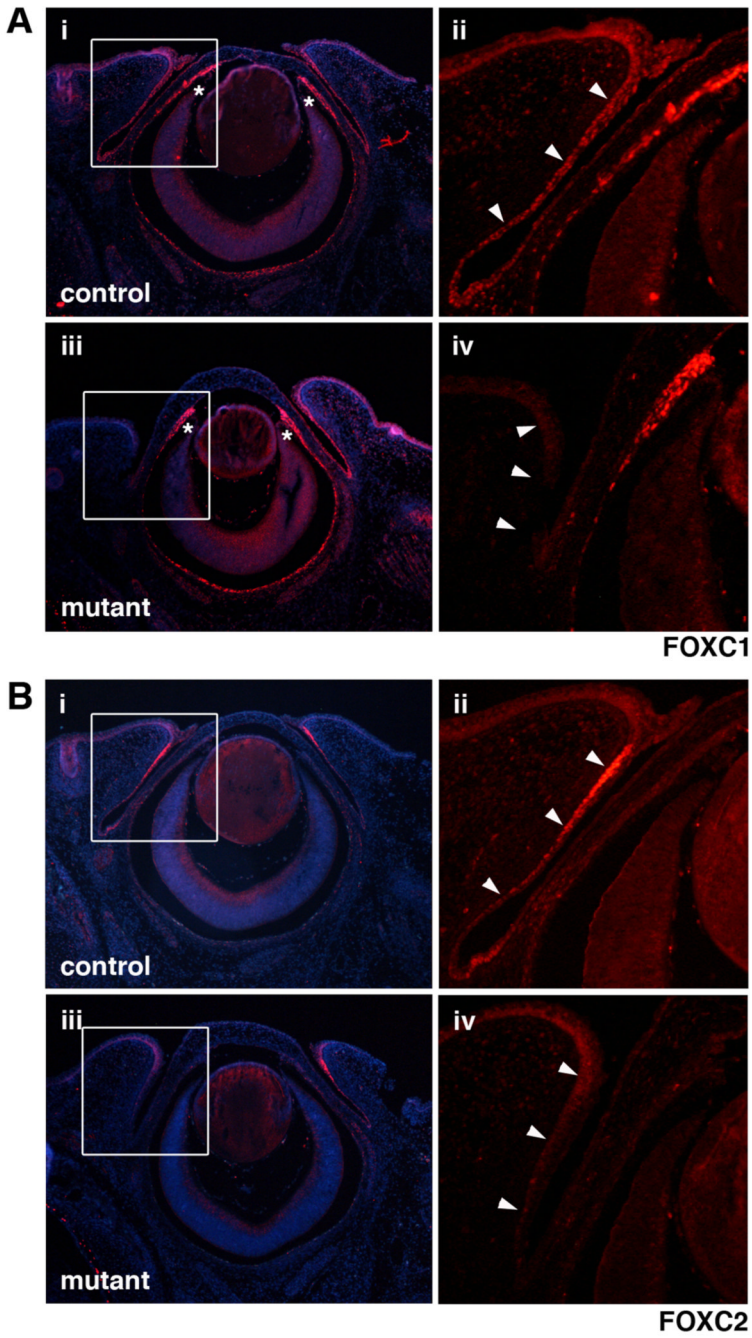

Figure 6. Altered FOXC protein expression in wild type versus *Dkk2* mutant eyes

Detection of FOXC1 (A) and FOXC2 (B) in e15.5 wild type control (i) and *Dkk2* mutant (ii) littermate eyes by immunofluorescent histochemistry. For each, boxed areas in panels i and iii are enlarged in panels ii and iv, respectively. Iridocorneal angles (*) are marked in each low power view while the curvature of dorsal conjunctival ectoderm (arrowheads) is marked in each high power view. Note: weak signal from e.g. eyelid epidermis and lens is a background characteristic of this FOXC2 antibody that does not correspond with the RNA *in situ* hybridization pattern (data not shown).

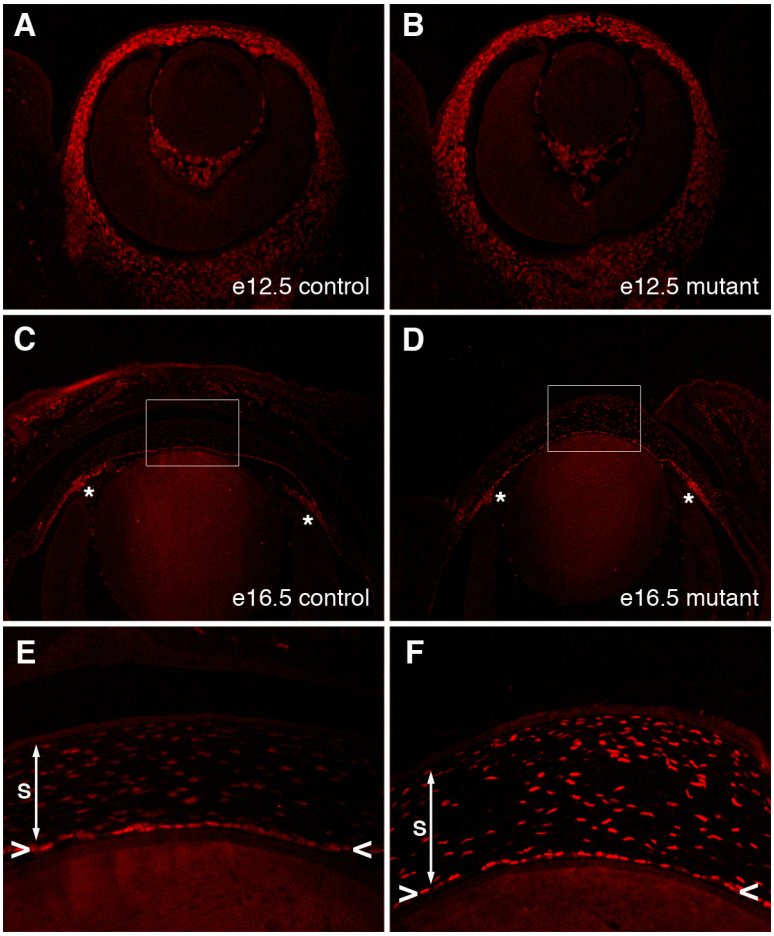

Figure 7. Persistent PITX2 expression in *Dkk2* mutant eyes

(A,B) PITX2 immunofluorescence intensity is indistinguishable in e12.5 wild type (control) vs. *Dkk2*$^{-/=}$ eyes. (C,D) PITX2 immunofluorescence in e16.5 wild type (control) vs. *Dkk2*$^{-/-}$ (mutant) eyes. Staining intensity in the presumptive iridocorneal angles (*) is indistinguishable between the two genotypes. (E,F) Enlargements of boxed areas in C and D, respectively. High-level PITX2 staining intensity persists in the differentiating corneal stroma (S) of mutant but not wild type eyes. Staining intensity in the corneal endothelium (><) is not noticeably different between the two genotypes. Note: Sections were photographed with invariant camera settings and exposure times to facilitate comparison of relative PITX2 expression levels.

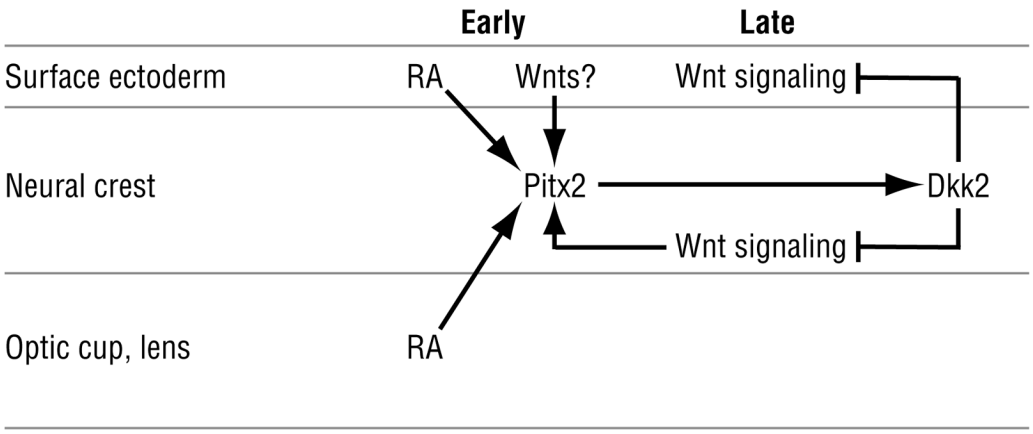

Figure 8. Model for PITX2-dependent integration of retinoic acid and canonical Wnt signaling during ocular anterior segment development

Pitx2 expression requires retinoic acid produced by the surface ectoderm, optic cup, and lens (Matt et al., 2005). *Dkk2* expression in neural crest is activated downstream of *Pitx2* and DKK2 acts to locally suppress canonical Wnt signaling in the neural crest and overlying ocular surface ectoderm. The persistently elevated PITX2 protein levels in *Dkk2*-deficient mice are consistent with a role for canonical Wnt signaling in contributing to net *Pitx2* expression levels, either through stimulation of *Pitx2* transcription (Kioussi et al., 2002) or enhancement of mRNA stability (Briata et al., 2003). Several Wnt genes are highly expressed in the overlying ocular surface ectoderm (Liu et al., 2003), suggesting this as the likely source of the Wnt ligands.