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Retinoic acid signaling in perioptic mesenchyme represses Wnt signaling via induction of *Pitx2* and *Dkk2*

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Abstract

Morphogenesis during eye development requires retinoic acid (RA) receptors plus RA-synthesizing enzymes, and loss of RA signaling leads to ocular disorders associated with loss of *Pitx2* expression in perioptic mesenchyme. Several Wnt signaling components are expressed in ocular tissues during eye development including *Dkk2*, encoding an inhibitor of Wnt/ β -catenin signaling, which was previously shown to be induced by *Pitx2* in the perioptic mesenchyme. Here, we investigated potential cross-talk between RA and Wnt signaling during ocular development. Genetic studies using *Raldh1/Raldh3* double null mice deficient for ocular RA synthesis demonstrated that *Pitx2* and *Dkk2* were both down-regulated in perioptic mesenchyme. Chromatin immunoprecipitation and gel mobility shift studies demonstrated the existence of a DR5 RA response element upstream of *Pitx2* that binds all three RA receptors in embryonic eye. *Axin2*, an endogenous readout of Wnt/ β -catenin signaling, was up-regulated in cornea and perioptic mesenchyme of RA deficient embryos. Also, expression of *Wnt5a* was expanded in perioptic mesenchyme of RA deficient eyes. Our findings demonstrate excessive activation of Wnt signaling in the perioptic mesenchyme of RA deficient mice which may be responsible for abnormal development leading to defective optic cup, cornea, and eyelid morphogenesis.

Keywords

Retinoic acid; Wnt signaling; Perioptic mesenchyme; Cornea; Eyelid; *Raldh1*; *Raldh3*; *Pitx2*; *Dkk2*; *Axin2*; *Wnt5a*

Introduction

Retinoic acid (RA) is a vitamin A derivative essential for embryonic development. Vitamin A deficiency is known to cause various ocular malformations including retinal defects, microphthalmia, coloboma and vision impairment. Ocular morphogenesis during embryonic development is a multi-step process involving induction, proliferation, differentiation and migration of cells. The vertebrate eye is formed from neural ectoderm, surface ectoderm and perioptic mesenchyme, which is derived from neural crest cells. An essential function of perioptic mesenchyme is to contribute multiple mature cell lineages that are required for the normal ocular anterior segment and eyelid development (Le Lièvre and Le Douarin, 1975). In humans, defects in survival, migration and differentiation of perioptic mesenchymal cells can lead to anterior segment dysgenesis and increased risk of glaucoma (Gould et al., 2004). Also, mutation of human *PITX2* leads to Axenfeld-Rieger syndrome characterized by ocular anterior

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segment defects (Semina et al., 1996; Mears et al., 1998; Kozlowski and Walter, 2000; Vieira et al., 2006; Weisschuh et al., 2006).

A role for RA has been well established in various stages of eye development based upon the overlapping expression patterns of RA-synthesizing enzymes (retinaldehyde dehydrogenases; RALDHs) and retinoic acid receptors (RARs) in ocular tissues, combined with corresponding loss-of-function studies in mouse (Lohnes et al., 1994; Ghyselinck et al., 1997; Wagner et al., 2000; Mic et al., 2004; Matt et al., 2005; Molotkov et al., 2006; Matt et al., 2008). RA synthesis is carried out by three RALDH enzymes (encoded by *Raldh1*, *Raldh2*, and *Raldh3*) with ocular *Raldh1* expression limited to the dorsal retina and ocular *Raldh3* expression limited to the ventral retina (Duester, 2008). RA serves as a ligand for nuclear RA receptors (RAR α , RAR β , and RAR γ) which bind to DNA as heterodimers with retinoid X receptors (RXR α , RXR β , and RXR γ) (Mark et al., 2006). RA signaling is transduced when RA binds to the RAR component of RAR/RXR heterodimers that are bound to RA response elements (RAREs) of specific target genes (Mic et al., 2003). Loss of ocular RA synthesis in mouse embryos carrying mutations of both *Raldh1* and *Raldh3* does not disrupt eye dorsoventral patterning as originally proposed, but it does disrupt anterior segment morphogenesis leading to excessive perioptic mesenchyme growth associated with dysgenesis of cornea and eyelid and rotation of the optic cup along the dorsoventral axis (Matt et al., 2005; Molotkov et al., 2006). As *Raldh1* and *Raldh3* are expressed in the retina but not the perioptic mesenchyme, RA signaling for eye morphogenesis occurs in a paracrine fashion (Molotkov et al., 2006).

Besides RA signaling, many components of the Wnt signaling pathway are also expressed in ocular tissues. A role for Wnt signaling during embryonic eye development has been established in regulation of distinct processes including eye field specification, morphogenetic movements, proliferation, differentiation and apoptosis (Fuhrmann, 2008). In short, canonical Wnt/ β -catenin signaling is activated by binding of Wnt ligands to frizzled and LRP5/6 co-receptors followed by stabilization and translocation of β -catenin to the nucleus where it regulates transcription of target genes by interacting with TCF/LEF transcription factors (Van de Wetering et al., 1997; Logan and Nusse, 2004; Angers and Moon, 2009). Among the several Wnt ligands and signaling components expressed in the developing eye, *Dkk2* (an antagonist of canonical Wnt/ β -catenin signaling) is highly expressed in the perioptic mesenchyme, and recently it has been shown that *Dkk2* is a critical downstream target of *Pitx2* in neural crest derived perioptic mesenchyme (Gage et al., 2008). *Pitx2* encodes a homeodomain transcription factor which plays an essential role during ocular anterior segment patterning and development (Gage et al., 1999; Hjalt et al., 2000; Gage et al., 2005). Heterozygous mutations in human *PITX2* results in Axenfeld-Rieger syndrome characterized by anterior segment dysgenesis and high risk of developing glaucoma (Semina et al., 1996; Mears et al., 1998; Kozlowski and Walter, 2000; Vieira et al., 2006; Weisschuh et al., 2006). Global and neural crest specific *Pitx2* knockout mice exhibit a similar eye phenotype including abnormal anterior segment differentiation, vasculogenesis, eyelid defects and coloboma (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999; Evans and Gage, 2005). The eye defect of *Pitx2* knockout mice is quite similar to that reported for *Raldh1/Raldh3* and *RARb/g* double knockout mice (Matt et al., 2005; Molotkov et al., 2006; Matt et al., 2008). Previous studies in embryos lacking either ocular RA synthesis or RA receptors demonstrate that *Pitx2* is down-regulated in the perioptic mesenchyme (Matt et al., 2005; Matt et al., 2008; See and Clagett-Dame, 2009). The observation that *Pitx2* functions as an inducer of *Dkk2* in perioptic mesenchyme provides a new paradigm for inhibition of canonical Wnt signaling through a critical role of *Pitx2* during eye development (Gage et al., 2008).

Here, we investigate potential cross-talk between RA and Wnt signaling during eye development. We report that the *Pitx2* gene contains a DR5 RARE located 4.3 kb upstream. We also demonstrate that RA activity in the perioptic mesenchyme is required for expression

of not only *Pitx2* but also *Dkk2* which affects Wnt/ β -catenin signaling, and for repression of *Wnt5a*. RA inhibition of Wnt signaling in the perioptic mesenchyme provides a mechanism for proper anterior segment formation, eyelid development, and optic cup orientation along the dorsoventral axis.

Materials and methods

Generation of *Raldh1*;*Raldh3* Double Null Mutant Embryos

Raldh1^{-/-};*Raldh3*^{-/-} double homozygous embryos were generated by crossing the two single mutant lines as previously described (Molotkov et al., 2006). Following mating, noon on the day of vaginal plug detection was considered embryonic day 0.5 (E0.5). Embryos were genotyped by PCR analysis of yolk sac DNA. All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Burnham Institute for Medical Research.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was performed according to the manufacturer's ChIP protocol (Active Motif, Carlsbad, CA). In separate experiments, ten E8.5 wild-type mouse embryos or eighteen E12.5 wild-type mouse eyes were dissected, pooled, and cross-linked with 1% formaldehyde at room temperature for 15 min; eye tissue included the whole eye and surrounding perioptic mesenchyme. Isolated nuclei (in 650 μ l of shearing buffer) from E8.5 mouse embryos were sonicated for twelve pulses of 10 sec each (Duty cycle-6, Output 30%) using a Branson Sonifier 450 (or in the case of nuclei from E12.5 eye samples for twenty pulses of 10 sec each at 40% power output) using a microtip probe from Misonix Digital Sonicator 4000 (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). Samples were sonicated on ice to shear DNA to an average size of 500 bp followed by centrifugation at 13,000 rpm for 10 min. At this point, a small portion of supernatant was stored as input control. For immunoprecipitation, 150 μ l of sheared chromatin mixed with 3 μ g of either anti-RAR α (sc-551, Santa Cruz Biotechnology), anti-RAR β (Affinity Bioreagents), anti-RAR γ (sc-550 Santa Cruz Biotechnology), or control IgG antibodies was used for each ChIP reaction incubated with 25 ml pre-blocked protein G-coated magnetic beads (Active Motifs, Carlsbad, CA) for 4 h at 4°C. Beads were washed and eluted DNA-protein complexes were reverse cross-linked and purified. The immunoprecipitated DNA was analyzed by PCR. ChIP analysis was performed at least in three independent experiments. PCR products were separated by 3% agarose gel electrophoresis and visualized using ethidium bromide staining. RARE specific and non-specific primer sequences for mouse *Pitx2*, *RAR β* , and *Raldh1* (*Aldh1a1*) genes used in this study were:

Pitx2 DR5a-F: 5'-CAAGATACTGGTCTGTTACCTTCC-3'

Pitx2 DR5a-R: 5'-GTTTCCGAATTACCTATCTGAGAGG-3'

Pitx2 DR5b-F: 5'-CATTTTAAGTCCCTCTCTGACAACC-3'

Pitx2 DR5b-R: 5'-GTGCAAGAGCCTGGTAATCCCT-3'

Pitx2 NS-F: 5'-GAAATTTGTTCCACTCTGGAGAACC-3'

Pitx2 NS-R: 5'-GGTAATGATGGGAAGGGGCTAATC-3'

RAR β DR5-F: 5'-TGGCATTGTTTGCACGCTGA-3'

RAR β DR5-R: 5'-CCCCCTTTGGCAAAGAATAGA-3'

RAR β NS-F: 5'-AGTACAGACCTTCCAAGAGTGCCT-3'

RAR β NS-R: 5'-GTCATGGGAAAGAGAGGTTGAGC-3'

Raldh1 DR5-F: 5'- TGCACACACACCCTTAGCACAG -3'

Raldh1 DR5-R: 5'- CAGGTGACAGGCTCAGCAAATTG -3'

Electrophoretic Gel Mobility Shift Assay (EMSA)

Sixteen wild-type E12.5 embryo eyes including surrounding perioptic mesenchyme tissue were dissected out and nuclear protein extracts were prepared as described (Dignam et al., 1983). Biotin-labeled double-stranded oligonucleotide probes containing RARE sequences (DR5a and DR5b) were bound to nuclear extracts. Binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Reaction mixtures were incubated for 20 min at room temperature. Binding reactions were subjected to EMSA on 6% non-denaturing polyacrylamide gels in 0.5 Tris-Borate-EDTA buffer, and detection was performed using a LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions. For supershift analysis, nuclear extracts were incubated with a 3 µg of anti-RAR α (sc-551, Santa Cruz Biotechnology), anti-RAR β (Affinity Bioreagents), or anti-RAR γ antibodies (sc-550 Santa Cruz Biotechnology) for 20 min on ice before adding probe. Sequences of wild-type and mutated oligonucleotides (underlined bases indicate mutations introduced) containing the DR5 RA response elements used in gel shift reactions are as follows (the complementary strand was also synthesized and annealed to the strand shown here which was biotin-labeled):

Pitx2 RARE DR5a WT: 5'-TTAGGTAATTCATTAGAAAGTCAATACAGAC-3'

Pitx2 RARE DR5a Mut: 5'-TTAGGTGATAAAATTAGAGAGAAAAATACAGAC-3'

Pitx2 RARE DR5b WT: 5'-TTTAAATCAGATCATCGAAGAGTCACCAGAAA-3'

Pitx2 RARE DR5b Mut: 5'-TTTAAATCGGAAAATCGAACAGAAACCAGAAA-3'

In situ Hybridization

To prepare cryosections for in situ hybridization, whole heads of E12.5 mouse embryos were dissected out, washed twice with 1xPBS (phosphate-buffered saline) and fixed in 4% paraformaldehyde (Sigma) for 2 h at 4°C. Embryo heads were then cryo-protected in 10% sucrose (Sigma) for 3 h followed by 30% sucrose overnight at 4°C. Heads were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), oriented, frozen and stored at -80°C. Heads were cryo-sectioned at 12 µm and processed for in situ hybridization as described (Wilkinson and Nieto, 1993) with the following digoxigenin-labeled antisense riboprobes: *Pitx2*, *Vax2*, *Dkk2*, *Axin2* and *Wnt5a*. Riboprobes were prepared as described previously (Wilkinson and Nieto, 1993).

TOPgal lacZ Detection

Mice carrying the TOPgal Wnt/ β -catenin reporter transgene which places *lacZ* (encoding β -galactosidase) under the transcriptional control of LEF/TCF response elements (DasGupta and Fuchs, 1999), was mated to our wild-type and *Raldh1*^{-/-};*Raldh3*^{-/-} double mutant mice to detect Wnt/ β -catenin activity in RA-deficient embryos. β -galactosidase activity was detected by staining with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) for 6 h at 37°C.

Results

Pitx2 expression is impaired in perioptic mesenchyme of *Raldh1*;*Raldh3* double null mutant

Raldh1^{-/-};*Raldh3*^{-/-} double null mutants exhibit dysgenesis of the ocular anterior segment caused by excessive invasion of perioptic mesenchyme, which leads to abnormal thickening of corneal stroma and premature closure of the eyelid folds (Matt et al., 2005; Molotkov et al., 2006). *Pitx2* is one of the key genes expressed in the neural crest derived perioptic mesenchyme

surrounding the optic cup and it plays an important role in anterior segment formation (Evans and Gage, 2005). Previous studies reported that *Pitx2* expression was reduced or absent in perioptic mesenchyme of RA deficient mice (Matt et al., 2005; Matt et al., 2008; See and Clagett-Dame, 2009). At E12.5, we show that *Pitx2* expression is down-regulated in the perioptic mesenchyme of our *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutants (Fig. 1A-B; n=6). This finding confirms that *Pitx2* expression is dependent on paracrine RA signals from *Raldh1* and *Raldh3* expressed in the retina. The loss of *Pitx2* expression in *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutants suggests *Pitx2* may be a RA target gene.

As a control, *Vax2* expression is shown in wild-type and *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutants (Fig. 1 C-D). *Vax2* is expressed in the ventral retina (Barbieri et al., 2002; Mui et al., 2002), and loss of RA synthesis does not alter its expression although the optic cup exhibits rotation along the dorsoventral axis which places the ventral retina deeper inside the head (Fig. 1D). Sections of the double mutant eye also show corneal mesenchyme thickening and collapse of the dorsal eyelid fold, while the ventral eyelid fold is still easily observed (Fig. 1B, D).

RA directly regulates *Pitx2* in vivo

We investigated whether *Pitx2* regulation by RA is direct or indirect. *In silico* analysis of the mouse *Pitx2* upstream regulatory region was performed to identify retinoic acid response elements (RAREs). Two putative DR5-type RAREs (direct repeat with 5 bp between) were located in the *Pitx2* upstream regulatory region at -4.3 kb (DR5a) and -3.6 kb (DR5b) relative to the ATG start codon (Fig. 2A). These DR5 elements are conserved and display a high degree of sequence homology between mouse, rat and human (Fig. 2B). To test if *Pitx2* expression is directly regulated by RAR binding to these DR5 RAREs *in vivo*, we performed chromatin immunoprecipitation (ChIP) using antibodies specific for RAR α , RAR β , and RAR γ on cross-linked DNA-protein complexes from either E8.5 whole embryos (Fig. 2C) or E12.5 eye tissues (Fig. 2D). ChIP analysis of both whole embryo and eye tissue reveals that all three RARs bind specifically with DR5a element located at -4.3 kb in *Pitx2* regulatory region, but no binding was observed using IgG control and non-specific primers for a region several kb away that does not contain a RARE. Interestingly, the DR5b element was not able to recruit any of the RA receptors in either embryo or eye indicating that only the DR5a element is functionally active. As a control, we also show that the *RAR β* gene, which is transcriptionally activated by RA via a well characterized DR5 RARE (Mendelsohn et al., 1991), recruited all three RARs in both embryo and eye ChIP (Fig. 2C-D). Also, the *Raldh1* (*Aldh1a1*) gene, which contains a DR5 RARE (Balmer and Blomhoff, 2005), recruited all three RARs in eye ChIP (Fig. 2D). Collectively, ChIP data provide evidence that *Pitx2* is a direct target of RA action in the developing eye.

We used an electrophoretic gel mobility shift assay (EMSA) to further examine the mouse *Pitx2* RAREs. Using a nuclear extract from wild-type E12.5 embryonic eyes, we found that a double-stranded 31-mer oligonucleotide probe containing the DR5a RARE located at -4.3 kb was shifted to a more slowly migrating species (Fig. 3, lane 2); a probe containing the DR5b RARE located at -3.6 kb was shifted much less efficiently than the DR5a probe (Fig. 3, lane 7). DR5a and DR5b probes with mutations introduced into the direct repeats exhibited no detectable shifted species (Fig. 3, lanes 3 and 8). Specificity of the DR5a shifted species for binding to RARs was demonstrated by inclusion of RAR antibodies in the gel shift reaction which resulted in supershifted species for RAR α , RAR β , and RAR γ antibodies (Fig. 3, lanes 4-6). Combined with the ChIP results demonstrating binding of DR5a but not DR5b to RARs *in vivo* (Fig. 2D), our findings support DR5a as a functional RARE for the *Pitx2* gene in embryonic eye and suggest that DR5b is ineffective as a RARE.

Retinoic acid interferes with *Dkk2* expression in perioptic mesenchyme

Dkk2 is a member of the dickkopf family of secreted proteins that acts as an antagonist of canonical Wnt signaling (Mao and Niehrs, 2003; He et al., 2004). During eye development, onset of *Dkk2* expression is first detected in the anterior segment perioptic mesenchyme at E11.5 which then extends to mesenchyme surrounding the optic cup and the developing cornea (Monaghan et al., 1999; Gage et al., 2008). Recently, *Dkk2* was identified as a direct target of *Pitx2* during anterior segment morphogenesis and evidence was presented for a role in negative regulation of canonical Wnt signaling activity which is essential for normal ocular morphogenesis (Gage et al., 2008). We examined E12.5 *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutants and wild-type littermate controls for expression of *Dkk2* in ocular tissues to ascertain the role of canonical Wnt signaling in perioptic mesenchyme. In the *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutant, *Dkk2* expression was consistently reduced in perioptic mesenchyme and cornea (Fig. 4A-B, n=6). As *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutant eyes completely lack RA signaling at E11.5 (Molotkov et al., 2006), the residual *Dkk2* expression we observe at E12.5 is likely due to an RA-independent mechanism. A reduction of *Dkk2* expression in RA deficient mice eyes suggests that increased Wnt signaling may contribute to the ocular defects observed in these mice.

Activated Wnt signaling in the developing eyes of RA deficient mice

Our *Dkk2* mRNA expression data suggests that activation of Wnt signaling may occur in the perioptic mesenchyme of RA deficient mice. To begin assessing active Wnt signaling in the eye, we used *Raldh1*; *Raldh3* null mice carrying the TOPgal transgene, a reporter for the canonical Wnt signaling pathway (DasGupta and Fuchs, 1999). TOPgal does not reflect all Wnt/ β -catenin signaling present in embryos (see *Axin2* data below), but it is a very useful marker for examining the eyelid front. Staining of E12.5 whole-mount embryos shows that TOPgal expression in wild-type eyes exists along a thin line around the entire optic cup that corresponds with the migrating front of the eyelid (Fig. 5A; n=6). However, the *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutant eye appears to be missing the dorsal eyelid fold, although a portion of the ventral eyelid fold may be present (Fig. 5B; n=6). Sagittal sections of these same eyes also show a loss of the eyelid front in the double mutant, and further demonstrate that TOPgal expression is up-regulated along the edge of the dorsal retina in the double mutant (Fig. 5C-D; n=6). Frontal sections of these eyes region reveal that wild-type eyes normally lack TOPgal expression in the perioptic mesenchyme (except for weak activity in the eyelid fold domains) and have very little expression in the dorsal retina, whereas in the double mutant TOPgal expression was positive in the perioptic mesenchyme near where the cornea should form and is much stronger in the dorsal retina (Fig. 5E-F, n=6). Previous studies reported that TOPgal expression is transiently active in the developing mouse retina, but such expression is independent of Wnt/ β -catenin signaling (Fuhrmann, 2008). However, TOPgal expression in the perioptic mesenchyme of the double mutant may reflect an increase in Wnt/ β -catenin signaling when RA signaling is lost (see *Axin2* data below).

As a more reliable marker of Wnt/ β -catenin signaling in perioptic mesenchyme, we examined mRNA expression of *Axin2*, an gene that is induced by the Wnt/ β -catenin pathway (Jho et al., 2002). *Axin2* mRNA was detected in the eyelid folds of E12.5 wild type eyes, but was very low in the perioptic mesenchyme and cornea directly adjacent to the optic cup (Fig. 6A; n=6). For the *Raldh1*^{-/-};*Raldh3*^{-/-} double null mutant, *Axin2* expression was significantly increased in the perioptic mesenchyme of the cornea and the collapsed dorsal eyelid fold, and expression was increased in the perioptic mesenchyme of the ventral eyelid fold (Fig. 6B; n=6).

Wnt5a encodes a Wnt ligand whose expression is detected in the eyelid folds of the developing mouse eye (Liu et al., 2003). We also observed expression of *Wnt5a* in the eyelid folds of E12.5 wild-type eyes, and we found that *Raldh1*^{-/-};*Raldh3*^{-/-} double mutant eyes exhibit an expansion

of *Wnt5a* expression in the ventral eyelid mesenchyme, but not in the remnant of the dorsal eyelid fold (Fig. 6C-D; n=6). As the dorsal eyelid fold is still observable in this section of the double mutant, it appears that the *Wnt5a*-positive dorsal eyelid domain has moved much further ventrally than normal (Fig. 6D). In addition to functioning in the non-canonical Wnt pathway (Angers and Moon, 2009), *Wnt5a* may also activate or inhibit Wnt/ β -catenin signaling depending on the cellular context (Mikels and Nusse, 2006). Thus, our observation of increased expression of both *Wnt5a* and *Axin2* expression in the mesenchyme around the ventral eyelid fold of RA deficient eyes (compare Figs. 6B and 6D) suggests a possible role for *Wnt5a* in stimulating the observed increase in ocular Wnt/ β -catenin signaling.

Discussion

Previous genetic studies on the role of RA in eye development using *Raldh* gene knockout or RA receptor mutants demonstrated the individual contribution of RA synthesizing enzymes (Fan et al., 2003; Matt et al., 2005; Molotkov et al., 2006) and RA receptors (Lohnes et al., 1994; Ghyselinck et al., 1997; Matt et al., 2008) crucial for ocular morphogenesis. During embryonic eye development, RA signaling controlled by *RAR β* and *RAR γ* expressed in the perioptic mesenchyme is required for normal morphogenetic movements necessary for invagination of the optic cup, closure of the choroid fissure and mesenchymal growth to correctly generate the cornea and eyelids. RA produced in the neural retina, the retinal pigmented epithelium and the corneal ectoderm by *Raldh1* and *Raldh3* is secreted and then acts in a paracrine fashion on the perioptic mesenchyme to regulate cell survival during anterior eye formation. Absence of RA signaling results in down-regulation of *Pitx2*, *Foxc1* and *Eya2* encoding transcription factors within perioptic mesenchyme required for proper anterior segment morphogenesis. Previous studies on RA deficient models (including *Raldh* or *RAR* knockout mice as well as vitamin A deficient rats) suggest *Pitx2* is a key effector of RA signaling in the neural crest derived perioptic mesenchyme (Matt et al., 2005; Matt et al., 2008; See and Clagett-Dame, 2009). Global and neural crest specific *Pitx2* mutant mice exhibit corneal and eyelid dysgenesis, iris stroma, sclera, thickening of mesenchyme between the lens and cornea, coloboma, and defects in the optic nerve (Evans and Gage, 2005). Importantly, mutations in human *PITX2* lead to Axenfeld-Rieger syndrome, a congenital disorder characterized by ocular anterior segment dysgenesis and increased risk of glaucoma (Semina et al., 1996; Kozlowski and Walter, 2000; Weisschuh et al., 2006). In this study, we present evidence that *Pitx2* in the perioptic mesenchyme is transcriptionally regulated directly by RA via a conserved DR5 RARE located 4.3 kb upstream of the *Pitx2* promoter. Gel-shift and chromatin immunoprecipitation (ChIP) studies in embryonic eye tissue demonstrate that all three RARs are able to recruit to this RARE *in vivo*, identifying *Pitx2* as a direct RA-inducible gene. An earlier study on F9 embryonal carcinoma (EC) cells did not identify *Pitx2* as a RA responsive gene in their microarray experiments (Eifert et al., 2006). Also, *Pitx2* expression in lateral plate mesoderm of early embryos does not require RA signaling (Niederreither et al., 2001). However, taking into account both expression studies in RA-deficient embryos as well as our RAR ChIP findings in perioptic mesenchyme, it is clear that ocular *Pitx2* expression is dependent upon RA signaling for induction and most likely a direct target of RA signaling.

In the present study, we investigated the integrated role of RA and Wnt signaling during embryonic eye development. Several RA and Wnt signaling (canonical and non-canonical) components including enzymes, ligands and receptors are expressed in developing ocular tissues and their mutual interaction in various stages of eye development cannot be neglected. A recent study on the role of Wnt signaling in eye development identified *Dkk2* as an important downstream effector of *Pitx2* function in perioptic mesenchyme and suggested a *Pitx2*-*Dkk2* auto-regulatory feedback mechanism for proper ocular anterior segment development (Gage et al., 2008). In our genetic study using *Raldh1^{-/-};Raldh3^{-/-}* double mutants, *Dkk2* expression was severely reduced in the perioptic mesenchyme and developing cornea, suggesting up-

regulation of Wnt/ β -catenin signaling may have occurred. We also demonstrated an increase in *Axin2* expression in the perioptic mesenchyme, cornea, and ventral eyelid fold of *Raldh1*^{-/-};*Raldh3*^{-/-} double mutants. As *Axin2* expression is a well-established endogenous readout of the Wnt/ β -catenin signaling pathway (Jho et al., 2002), our findings provide strong evidence that a loss of RA signaling in *Raldh1*^{-/-};*Raldh3*^{-/-} double mutants results in an up-regulation of Wnt/ β -catenin signaling in mesenchymal ocular tissues. Our findings suggest a model in which cross-talk between RA and Wnt signaling is required for eye morphogenesis with *Pitx2* being the intermediary (Fig. 7).

Earlier studies on the role of Wnts in mouse eye development showed that *Wnt5a* is expressed in the eyelid fold perioptic mesenchyme (Liu et al., 2003). We observed that *Wnt5a* expression was up-regulated in RA deficient eyes at E12.5. Although *Wnt5a* has mostly been associated with non-canonical Wnt signaling (Slusarski et al., 1997), *Wnt5a* may also activate or inhibit Wnt/ β -catenin signaling depending on the context (Mikels and Nusse, 2006). As we observe an increase of both Wnt/ β -catenin signaling (*Axin2* expression) and *Wnt5a* expression in the ventral eyelid and surrounding perioptic mesenchyme in RA deficient eyes, we suggest that *Wnt5a* may activate Wnt/ β -catenin signaling in the eye. However, as *Wnt5a* may down-regulate Wnt/ β -catenin signaling under some circumstances (Torres et al., 1996), our observation of increased *Wnt5a* expression in the same ventral eyelid region where increased *Axin2* expression is observed may indicate a response by the RA-deficient eye to dampen the increased Wnt/ β -catenin signaling seen in RA-deficient eyes. Our studies have not ruled out that excessive *Wnt5a* expression may result in excesses in both canonical and non-canonical Wnt signaling, especially as abnormal dorsoventral rotation of the optic cup may be the result of aberrant non-canonical Wnt signaling which is known to effect morphogenetic movements (Angers and Moon, 2009).

Interaction between RA and Wnt signaling pathways have previously been reported during embryogenesis. A recent study demonstrated that loss of RA in *Raldh2* null embryos increases Wnt/ β -catenin signaling during body axis extension by de-repressing *Wnt8a* and *Wnt3a* (Zhao and Duester, 2009). This finding supports earlier studies in vitamin A deficient quail embryos (Olivera-Martinez and Storey, 2007), suggesting RA/Wnt interaction during trunk development is conserved. Another relevant example is the recent demonstration of deregulated RA signaling in the early developing eyes of Wnt co-receptor *Lrp6* mutant embryos that exhibit complete loss of *Raldh1* expression and ectopic expression of *Raldh3* (Zhou et al., 2008). Several putative TCF/LEF binding sites were identified in the *Raldh1* and *Raldh3* promoter regions indicating the possibility of direct Wnt-mediated regulation of RA synthesis during early eye development (Zhou et al., 2008).

In conclusion, our studies here demonstrate that RA signaling is initially required to activate *Pitx2* expression within the perioptic mesenchyme around the optic cup which in turn induces *Dkk2* to locally suppress Wnt signaling in this tissue. Such cross-talk between ocular RA and Wnt signaling may be required for proper formation of the anterior segment as well as normal optic cup orientation and eyelid development.

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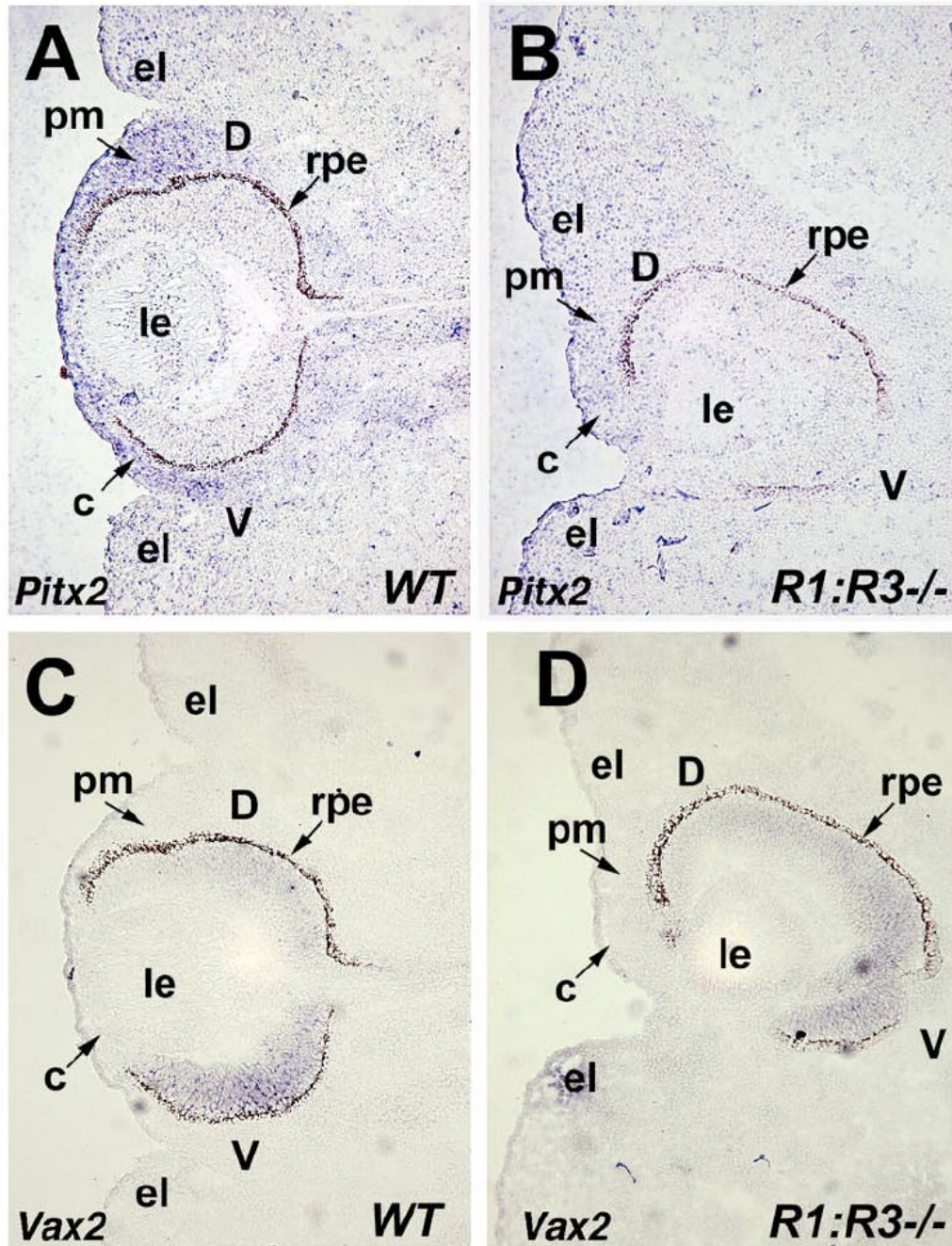


Fig. 1. Ablation of *Raldh1* and *Raldh3* impairs *Pitx2* expression in perioptic mesenchyme. (A-B) In situ hybridization with digoxigenin-labeled *Pitx2* antisense riboprobe on frontal sections through the eye of E12.5 wild-type (WT) and double null (*Raldh1*^{-/-};*Raldh3*^{-/-}) embryos. Note that *Pitx2* expression in the perioptic mesenchyme of *R1;R3*^{-/-} embryo is severely reduced compared to WT control indicated by arrow. (C-D) *Vax2* shows normal expression pattern in the ventral retina of wild-type (WT) compared to double null (*Raldh1*^{-/-};*Raldh3*^{-/-}) eyes, however note the dorsoventral rotation of the optic cup in the *R1;R3*^{-/-} double mutant. c, cornea; d, dorsal retina; el, eyelid fold; le, lens; pm, perioptic mesenchyme; rpe, retinal pigment epithelium; v, ventral retina.

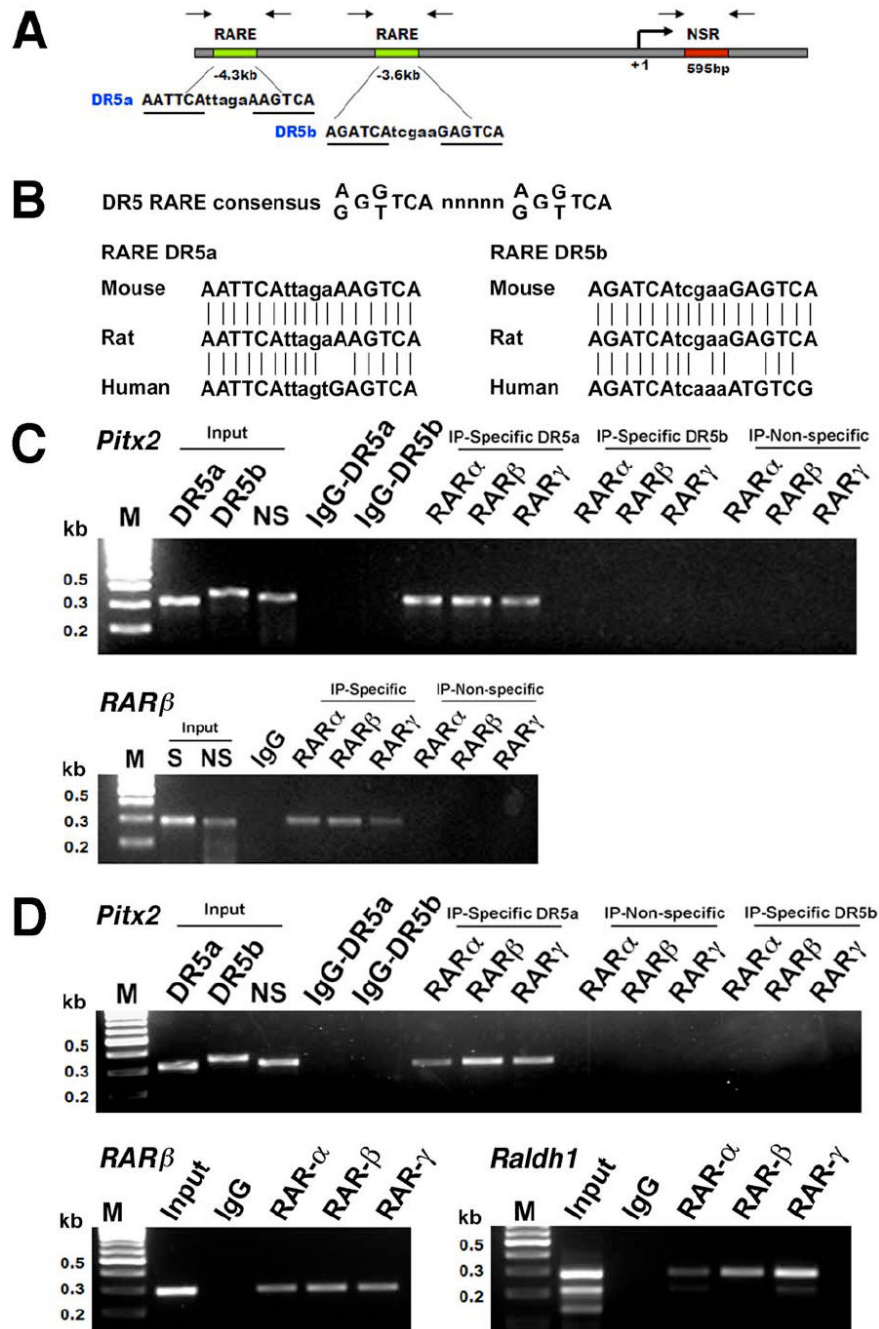


Fig. 2. Recruitment of retinoic acid receptors to the mouse *Pitx2* promoter *in vivo*. (A) Schematic representation of the mouse *Pitx2* upstream region containing two putative DR5 retinoic acid response elements (RARE) located 3.6 and 4.3 kb upstream of transcription start site. Arrows indicate the location of primer pairs used to amplify the RARE-containing regions and a non-specific negative control region (NSR) located 595 bp downstream for chromatin immunoprecipitation (ChIP) assay. (B) Nucleotide sequence alignment of conserved DR5a and DR5b RAREs in mouse, rat, and human *Pitx2* genes. ChIP assay was performed using (C) E8.5 mouse embryos and (D) E12.5 eyes. The cross-linked protein-DNA complexes were immunoprecipitated with anti-RAR- α , RAR- β , and RAR- γ antibodies. Mouse IgG was used

as a negative control. Input material and immunoprecipitates were analyzed by PCR with specific primers flanking the RARE and non-specific primers located several kb away for the mouse *Pitx2*, *RAR β* , and *Raldh1* promoters. S: RARE specific primers, NS: Non-specific primers.

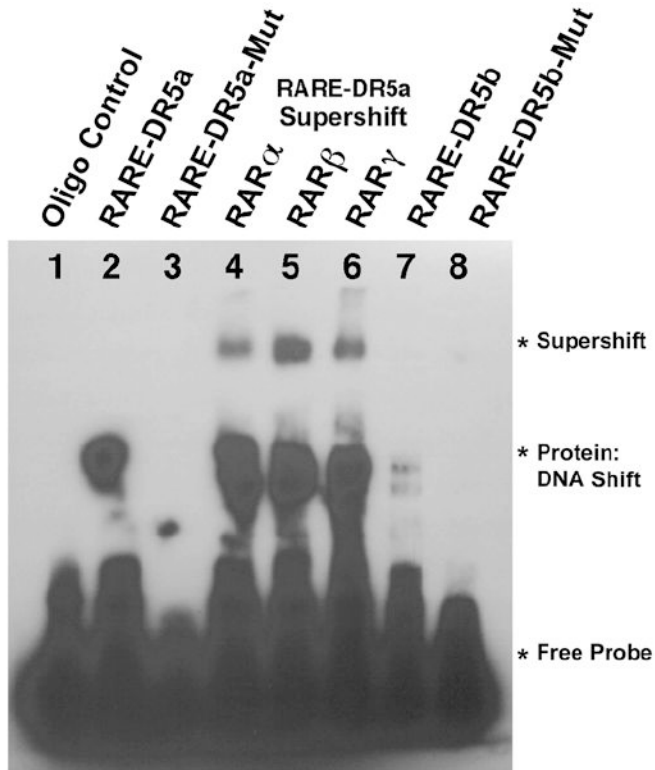


Fig. 3.

Electrophoretic gel mobility shift assays on the mouse *Pitx2* RAREs. (Lane 1) DR5a 31-mer probe incubated without nuclear extract added. (Lanes 2-3) DR5a and DR5a-mutant probes, respectively, incubated with E12.5 eye nuclear extract. (Lanes 4-6) DR5a probe supershifted by adding RAR antibodies along with the E12.5 eye nuclear extract. (Lanes 7-8) DR5b and DR5b-mutant probes, respectively, incubated with E12.5 eye nuclear extract.

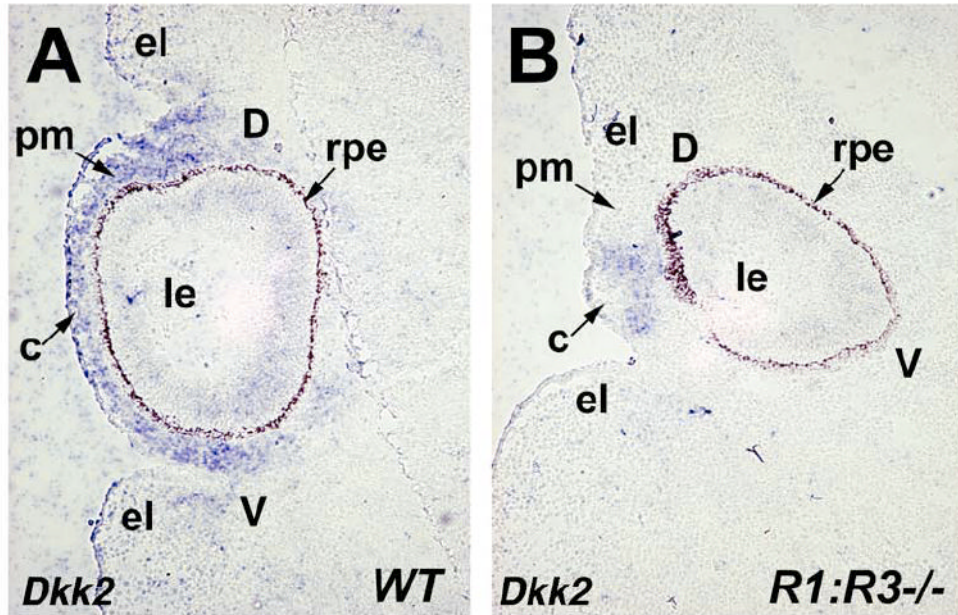


Fig. 4.

Loss of *Raldh1* and *Raldh3* interferes with *Dkk2* expression in perioptic mesenchyme. (A-B) In situ hybridization with *Dkk2* antisense riboprobe on frontal sections through the eye of E12.5 wild-type (*WT*) and double null (*Raldh1*^{-/-};*Raldh3*^{-/-}) embryos. Note that *Dkk2* expression in the perioptic mesenchyme of *R1;R3*^{-/-} embryo (B) is severely reduced compared to WT control (A) indicated by arrow. c, cornea; d, dorsal retina; el, eyelid fold; le, lens; pm, perioptic mesenchyme; rpe, retinal pigment epithelium; v, ventral retina.

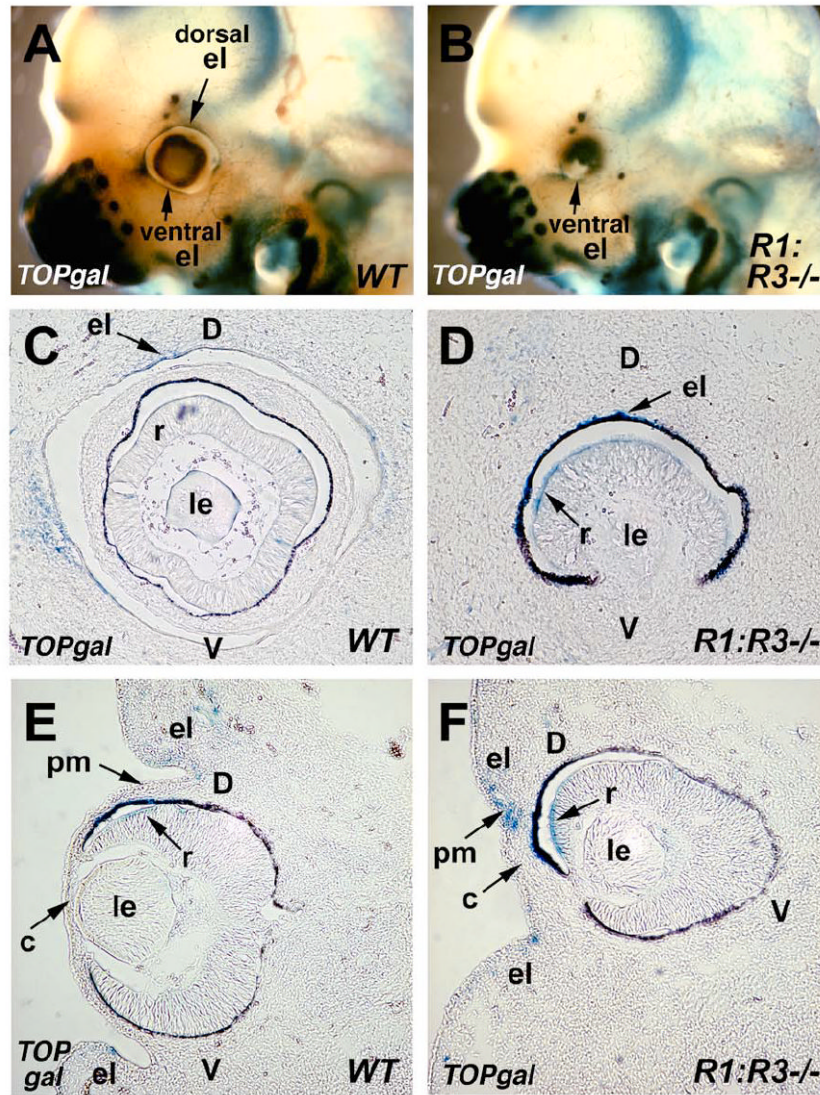


Fig. 5.

Activation of the *Wnt* reporter TOPgal in wild-type and RA deficient mouse eyes. (A-B) Staining of whole-mount heads of E12.5 wild-type (*WT*) and double null (*Raldh1^{-/-};Raldh3^{-/-}*) embryos carrying the TOPgal reporter. Arrows indicate TOPgal expression in both the dorsal and ventral eyelid folds of WT, but only in the ventral eyelid fold of the mutant. (C-D) Sagittal sections through the eyes of TOPgal stained E12.5 wild-type (*WT*) and double null (*Raldh1^{-/-};Raldh3^{-/-}*) embryos showing the loss of the normal eyelid folds in the mutant, and ectopic TOPgal expression in the retina. (E-F) Frontal sections showing that the mutant lacks the dorsal eyelid domain, but retains the ventral eyelid domain, and also showing that the mutant has increased TOPgal expression in a central domain of perioptic mesenchyme located where the cornea would normally develop and nearby in the retina. c, cornea; d, dorsal retina; el, eyelid fold; le, lens; pm, perioptic mesenchyme; r, retina; v, ventral retina.

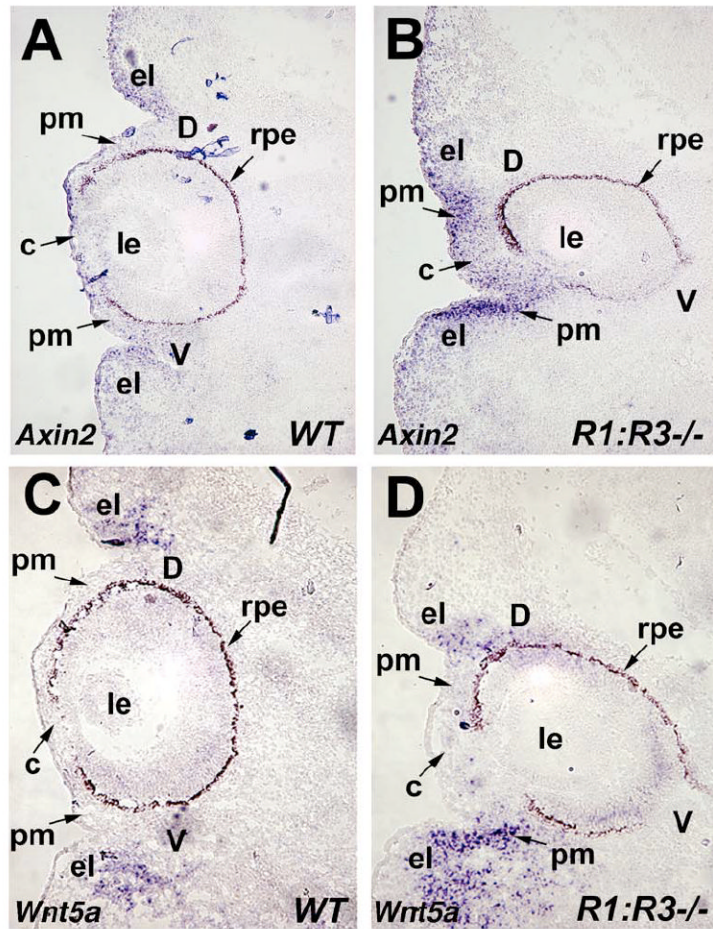


Fig. 6. Enhanced expression of Wnt markers in mouse eyes deficient for RA synthesis. (A-B) In situ hybridization with *Axin2* antisense riboprobe on frontal sections through the eye of E12.5 wild-type (WT) and double null (*Raldh1*^{-/-};*Raldh3*^{-/-}) embryos. Note that *Axin2* expression in the underlying perioptic mesenchyme, cornea, and ventral eyelid fold of *R1;R3*^{-/-} embryo is greatly increased compared to WT control indicated by arrow. (C-D) *Wnt5a* mRNA expression in frontal sections of E12.5 wild-type (WT) and double null (*Raldh1*^{-/-};*Raldh3*^{-/-}) eyes. The *Wnt5a* expression domain in the ventral eyelid fold and perioptic mesenchyme of *R1;R3*^{-/-} embryo is increased significantly compared to the wild-type control. c, cornea; d, dorsal retina; el, eyelid fold; le, lens; pm, perioptic mesenchyme; rpe, retinal pigment epithelium; v, ventral retina.

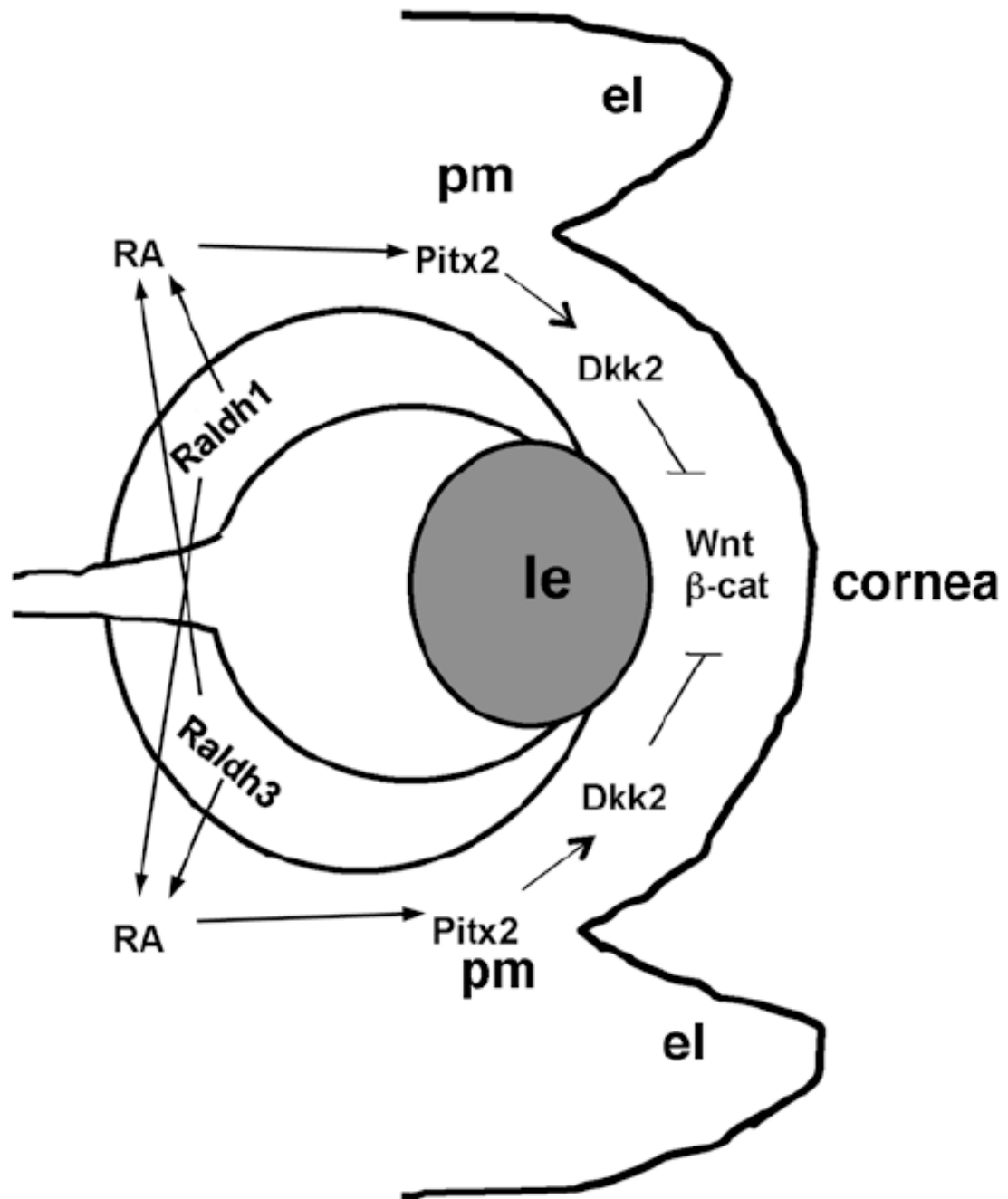


Fig. 7.

Model for cross-talk between RA and Wnt signaling during eye morphogenesis. Our findings demonstrate that RA synthesized by the dorsal (Raldh1) or ventral (Raldh3) retina is secreted and acts directly with nuclear RA receptors to induce *Pitx2* in the perioptic mesenchyme; elimination of both enzymes is necessary to observe ocular defects as RA generated by either Raldh1 or Raldh3 can provide RA to both dorsal and ventral perioptic mesenchyme (Molotkov et al., 2006). Our model further suggests that a major downstream target of RA-induced *Pitx2* is *Dkk2* whose induction in the perioptic mesenchyme antagonizes Wnt/ β -catenin signaling to allow proper morphogenesis of the perioptic mesenchyme, eyelids, and cornea. el, eyelid fold; le, lens; pm, perioptic mesenchyme; RA, retinoic acid.