

Localization of *Tfap2 β* , *Casq2*, *Penk*, *Zic1*, and *Zic3* Expression in the Developing Retina, Muscle, and Sclera of the Embryonic Mouse Eye

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Summary

Optic development involves sequential interactions between several different tissue types, including the overlying ectoderm, adjacent mesoderm, and neural crest mesenchyme and the neuroectoderm. In an ongoing expression screen, we identified that *Tfap2 β* , *Casq2*, *Penk*, *Zic1*, and *Zic3* are expressed in unique cell types in and around the developing eye. *Tfap2 β* , *Zic1*, and *Zic3* are transcription factors, *Casq2* is a calcium binding protein and *Penk* is a neurotransmitter. *Tfap2 β* , *Zic1*, and *Zic3* have reported roles in brain and craniofacial development, while *Casq2* and *Penk* have unknown roles. These five genes are expressed in the major tissue types in the eye, including the muscles, nerves, cornea, and sclera. *Penk* expression is found in the sclera and perichondrium. At E12.5 and E15.5, the extra-ocular muscles express *Casq2*, the entire neural retina expresses *Zic1*, and *Zic3* is expressed in the optic disk and lip of the optic cup. The expression of *Tfap2 β* expanded from corneal epithelium to the neural retina between E12.5 to E15.5. These genes are expressed in similar domains as Hedgehog (*Gli1* and *Ptch1*) and the Wnt (*Lef1*) pathways. The expression patterns of these five genes warrant further study to determine their role in eye morphogenesis: (J Histochem Cytochem 67: 863–871, 2019)

Keywords

cornea, extra-ocular muscles, eye, eyelid, optic cup, sclera

Introduction

Vertebrate eye development occurs as a series of highly coordinated induction events, with the lens vesicle, optic cup, optic stalk (OS), and precursor to the cornea all developing from the forebrain neuroectoderm.^{1,2} The onset of eye development occurs in tandem with the start of gastrulation, when the optic sulci are present in the anterior neural plate.^{1,2} The walls of the diencephalon then form the optic vesicles, which undergo coordinated invagination with the lens placode to form the lens vesicle and optic cup.^{1–3} The inner layer of the optic cup gives rise to the neural retina.³ In contrast to the inner layer, the outer layer of the optic cup matures into the retinal pigmented epithelium

(RPE) and OS. Elsewhere in the developing eye, the lens vesicle gives rise to the lens, whereas its overlying ectoderm matures into the cornea. In contrast, the iris, sclera, and ciliary body mature from the neural crest mesenchyme.^{1,4}

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Table 1. Primers Used to Clone and Generate Antisense in situ Probes for the Genes.

Gene	bp ^a	Forward Primer (5'→3')	Reverse Primer (5'→3')
<i>Casq2</i>	723	TCACACTGCAGGGACAGAAG	AGGCACTTGGGGATTCTTTT
<i>Penk</i> ^b	472	GAGACACCTTGGCCAACCTCT	ACAGCACAAAGCAGCATGTGA
<i>Zic1</i>	524	GGCAGCAAGCCTTTAGGTTTTT	GACTTTGGGACGAAATGTTTGC
<i>Zic3</i>	691	AAGTGCCGGGAAGTGAACAATA	TTGCCATTTTCAGAGGTGTGCTA

^aAmplicon size (bp).

^bWan et al.¹³

In an ongoing RNASeq screen in the laboratory focusing on skull morphogenesis, we determined that the expression of five genes (*Tfap2β*, *Casq2*, *Penk*, *Zic1*, and *Zic3*) were also expressed in the optic region. Transcription factor Activating Enhancer-Binding Protein 2 Beta (*Tfap2β*), an alternative splice form of *Tfap2*, is mainly expressed in epithelial and neural crest cell lineages during craniofacial and skeletal development.^{5,6} Conditional deletion of *Tfap2β* in mouse cranial neural crest results in postnatal ocular defects typified by opacity.⁷ The *Calsequestrin* (*Casq2*) is a Ca²⁺-binding protein in myocardial sarcoplasmic reticulum; *Casq2* variants lead to abnormal muscle development and cardiac function.⁸ *Casq2* expression is initially found in cardiac muscle, but later its expression domains include the myotome and cardiac muscle.⁹ *Proenkephalin* (*Penk*) gene encodes small endogenous opioid peptides, which are expressed in various tissues, including the nasal region, and *Penk* plays a critical role in cell proliferation and differentiation.^{10–13} *Zic1* and *Zic3* belong to zinc finger protein of the cerebellum family of transcription factors and are widely expressed, but spatially restricted, from gastrulation through organogenesis.^{14–16} *Zic1* and *Zic3* are expressed in peripheral margin of the optic cup.^{15,16}

Several signaling pathways are involved in the eye development. *Gli1* is expressed in the RPE, POM, and ventral eye cup.¹⁷ Canonical Wnt/ β -catenin pathway is active throughout retinal development.¹⁸ Interestingly, in *Tfap2β* null mice, the expression of DKK1 is reduced in corneal epithelium, implying that *Tfap2β* may be involved in the Wnt signaling pathway.¹⁸

While the role of *Tfap2β*, *Casq2*, *Penk*, *Zic1*, and *Zic3* are well documented in other organs, their specific roles in the developing eye are less apparent. In this study, we describe the complimentary, expression patterns of *Tfap2β*, *Casq2*, *Penk*, *Zic1*, and *Zic3* in the early stages of eye development. We also propose a pattern of expression for *Zic1*, and *Zic3* that is similar, but more restricted than those previously documented in the eye.^{15,16} Our expression data provide new genes that are expressed in discrete optic regions that will aid in understanding vertebrate eye development.

Materials and Methods

Animals

Embryos were collected from timed pregnant CD-1 females purchased from Charles River (E12.5 and E15.5) or C57/Bl6J females (E10.5 and E16.5). The pregnant dams were euthanized by CO₂, and the embryos were collected via C-section on the appropriate day of pregnancy. We confirmed the stage of the embryos with morphology. All animal husbandry procedures and protocols were approved by and performed under the oversight of the University of Pittsburgh Institutional Animal Care and Use Committee.

Antisense RNA Probe Preparation

We homogenized E15.5 skulls and extracted the RNA using the Trizol reagent (Invitrogen, Waltham, MA) according to standard procedures. SuperScript Vilo (Invitrogen) was used to reverse transcribe the RNA into cDNA. Using the cDNA template, we amplified fragments of *Casq2*, *Penk*, *Zic1*, and *Zic3* using the primers in Table 1.¹³ The fragments were cloned into the dual-promoter TOPO-TA cloning kit (Thermo Fisher, Waltham, MA) and purified using the Plasmid Midi Kit (QIAGEN, Germantown, MD). To ensure that we cloned the appropriate genes, we sequenced all plasmids to verify sequence identity and orientation of each fragment prior to probe synthesis. Dr. Trevor Williams kindly provided the *Tfap2β* clone.¹⁹ All antisense digoxigenin-labeled RNA probes were transcribed from linearized clones using the appropriate RNA polymerase.

Histology and Section in situ Hybridization

Embryos were fixed in 4% paraformaldehyde at 4C overnight and embedded and infiltrated in paraffin using standard protocols. The embryos were cut in 8- μ m sections and adjacent sections were placed on TESPA (triethoxysilane)-coated Superfrost Plus slides. Sections for in situ hybridization were selected based on the histology in the neutral fast red stained adjacent sections. For nuclear fast red staining, the slides were deparaffinized,

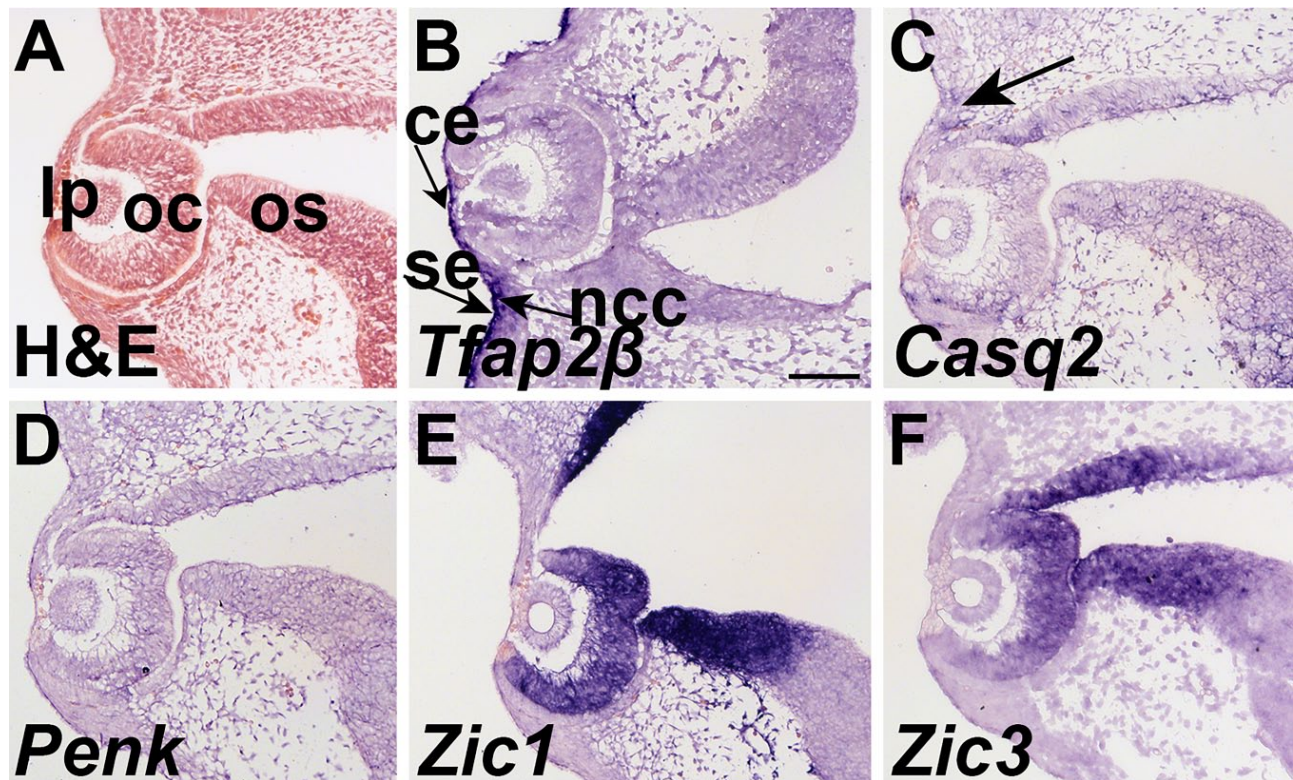


Figure 1. *Tfap2 β* , *Casq2*, *Penk*, and *Zic1* are expressed in and surrounding the eye at E10.5. Transverse sections through the eye region at E10.5 hybridized to (B) *Tfap2 β* , (C) *Casq2*, (D) *Penk*, (E) *Zic1*, and (F) *Zic3*. (A) H&E staining shows the histology of the ocular region. (B) *Tfap2 β* is expressed in the mesenchyme and epithelium. *Tfap2 β* is expressed in the eyelid epithelium and is also present in the condensed mesenchyme of the future eyelid. *Tfap2 β* expression is found in the superficial and corneal epithelium. (C) *Casq2* is expressed in a subset of the mesenchyme near the lid (black arrow). *Casq2* expression is also peppered through the optic stock and cup. (D) *Penk* expression is absent from the ocular region at E10.5. (E) *Zic1* is expressed in the optic cup, and the lateral edge of the optic stock. (F) *Zic3* is also expressed in the optic cup and stalk. Scale bar in A = 100 μ m and applies to all. Abbreviations: lp, lens placode; oc, optic cup; os, optic stalk; ce, corneal epithelium; se, superficial epithelium; ncc, neural crest cells.

rehydrated, and stained in 0.1% nuclear fast red (Sigma, St. Louis, MO). Following staining, the slides were dehydrated, mounted in Richard-Allen Scientific Mounting Media (Fisher) and coverslipped. The section in situ hybridization were performed according to standard protocols and detected with BM Purple.¹³ The development reaction was stopped after appropriate signal had developed and the slides were counterstained with eosin, dehydrated, mounted in Richard-Allen Scientific Mounting Media (Fisher), and coverslipped. We observed very little variation in the expression domains within the three biological replicates that we analyzed; the images are representative of the three individuals for each stage and probe.

Microscopy

Histological and section in situ images were collected on a Zeiss Axioskop A1 microscope with the Mrc3 camera and Zen imaging software. Image composites of the entire optic region at E12.5 and E15.5 were stitched

together using the automatic settings in Zen software. The figures were assembled in Adobe Photoshop.

Results

Tfap2 β , *Casq2*, *Penk*, and *Zic1* are Expressed in Distinct Spatial Domains at E10.5

At E10.5, the optic cup has been induced and the lens placode is being induced and the neural crest cells are being excluded between the corneal epithelium and the optic cup.²⁰ We observed the expression of *Tfap2 β* expressed in the superficial and corneal epithelium as well as the neural crest cells adjacent to the eye (Fig. 1B). *Casq2* expression is found peppered through the neuroepithelium, as well as in the mesenchyme that will be invaded by the eyelid epithelia bud. At E10.5, we observed little expression of *Penk* (Fig. 1C and D). Both *Zic1* and *Zic3* are robustly expressed in the OS and cup (Fig. 1E and F).

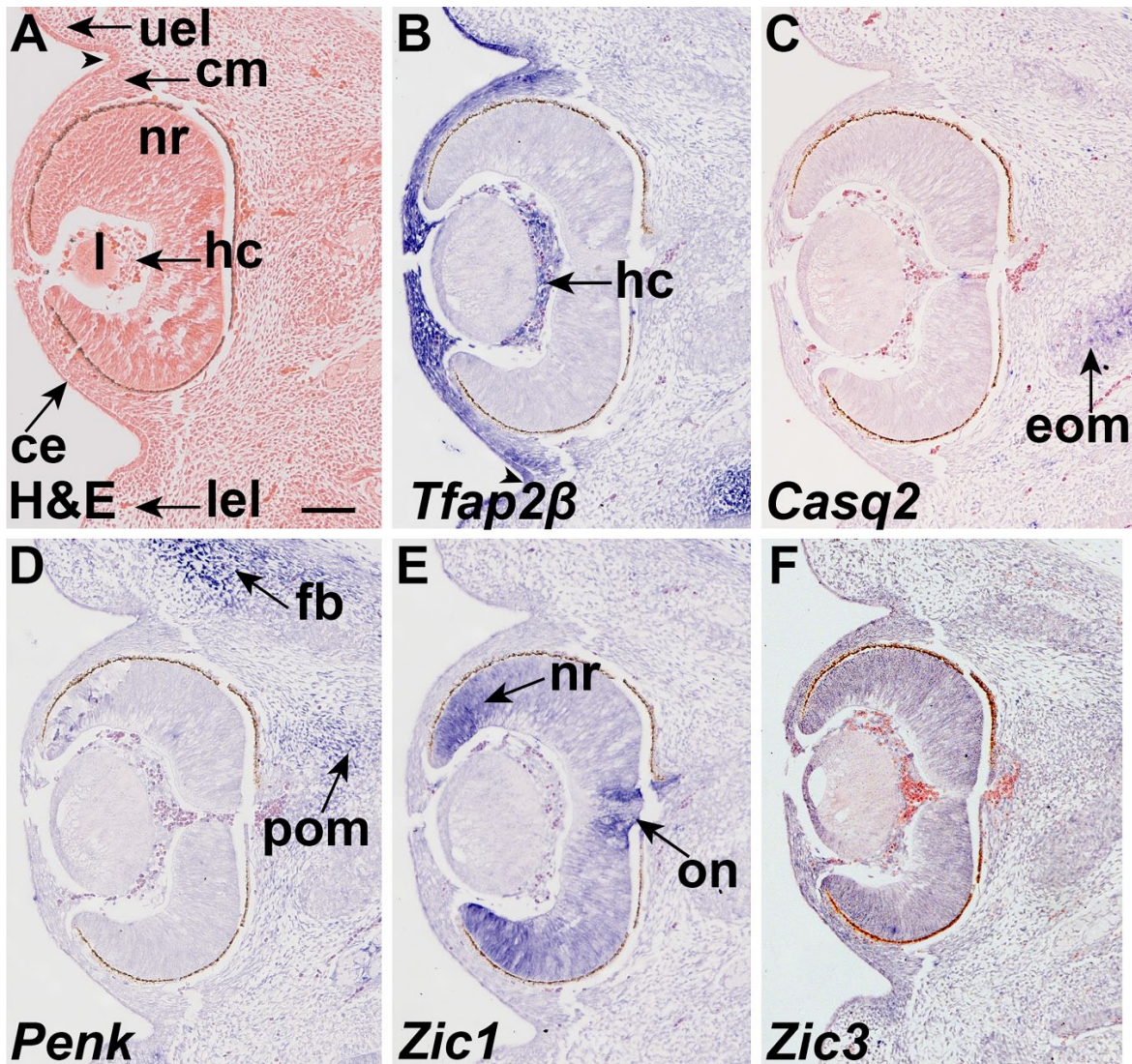


Figure 2. *Tfap2 β* , *Casq2*, *Penk*, and *Zic1* are expressed in and surrounding the eye at E12.5. Coronal sections of an E12.5 embryo hybridized to antisense probes to (B) *Tfap2 β* , (C) *Casq2*, (D) *Penk*, (E) *Zic1*, and (F) *Zic3*. (A) Nuclear fast red staining shows the histology of the ocular region. (B) *Tfap2 β* is expressed in the mesenchyme and epithelium. *Tfap2 β* is expressed in the eyelid epithelium and is absent from the base of the groove (arrowhead). *Tfap2 β* expression is found in the corneal epithelium and mesenchyme, as well as tissues within the hyaloid cavity. (C) *Casq2* is expressed in the extrinsic ocular muscles (black arrow). (D) *Penk* is expressed in periocular mesenchyme (arrow) as well as frontal bone primordium (arrow). (E) *Zic1* is expressed in the neural retina (black arrow), and the optic disk surrounding the optic nerve (arrow). (F) *Zic3* is expressed in neural retina (nr) at E12.5. Scale bar in A = 100 μ m and applies to all. Abbreviations: ce, corneal epithelium; cm, corneal mesenchyme; eom, extrinsic ocular muscles; fb, frontal bone primordia; hc, hyaline cavity; l, lens; lel, lower eye lid bud; nr, neural retina; on, optic nerve; pom, peri-ocular mesenchyme; uel, upper eye lid bud.

Tfap2 β , *Casq2*, *Penk*, and *Zic1* are Expressed in Distinct Spatial Domains at E12.5

At E12.5, when most of the tissues of the eye are present. *Tfap2 β* is expressed in the corneal epithelium and mesenchyme. The tissue in the hyaline cavity expresses *Tfap2 β* , but it is not expressed in the optic cup of the retina (Fig. 2B). *Tfap2 β* is expressed in the epithelium of the eyelids and is

absent from the base of the groove (Fig. 2B, arrowhead). *Casq2* expression was detected in the extrinsic ocular muscles (Fig. 2C). *Penk* is expressed in periocular mesenchyme as well as frontal bone primordium (Fig. 2D) but is absent from the globe and associated tissues. *Zic1* is expressed in the neural retina and optic nerve (Fig. 2E). *Zic3* is not expressed in tissues surrounding the eye at E12.5 (Fig. 2F).

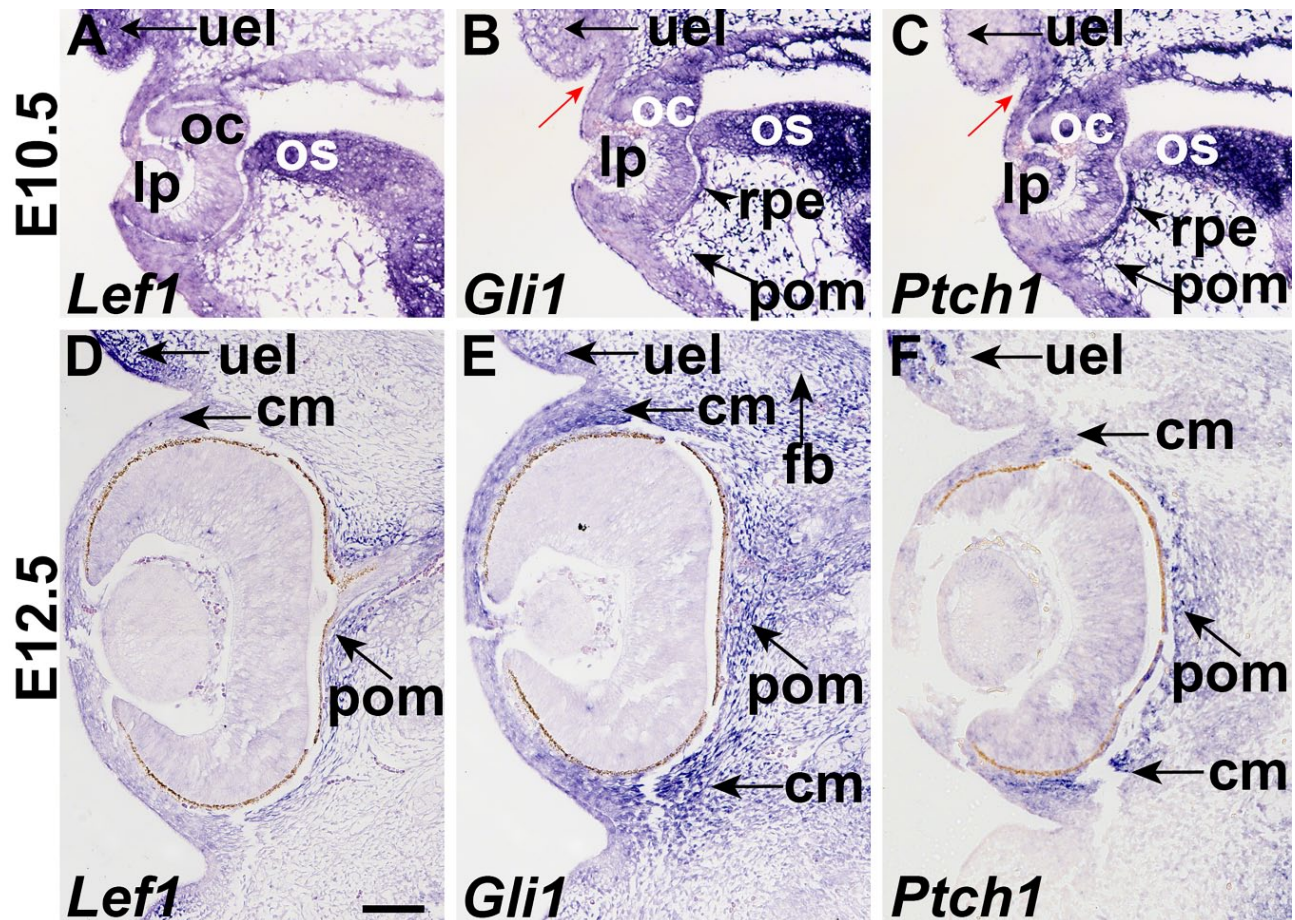


Figure 3. Hh and Wnt signaling is found in the periocular mesenchyme. Section in situ hybridization of (A, D) *Lef1*, (B, E) *Gli1*, and (C, F) *Ptch1* in coronal sections of E10.5 (A-C) and E12.5 (D-F) skull. Refer to Figs. 1 and 2 for histology of adjacent sections. (A) *Lef1* is expressed in the upper eye lid (uel), lens placode (lp), optic cup (oc), and optic stalk (os). (B) *Gli1* is expressed in the invaginating eyelid epithelium (blue arrow), and the condensed mesenchyme adjacent to it. In addition, *Gli1* expression is found in the optic cup (oc), optic stalk (os), retinal pigmented epithelium (rpe), and periocular mesenchyme (pom). (C) *Ptch1* is expressed in early eyelid (blue arrow) and is excluded from the adjacent condensed mesenchyme. *Ptch1* expression is found in the optic cup, optic stalk, pigmented retinal epithelium and periocular mesenchyme (arrow). (D) *Lef1* was expressed in the eyelid bud mesenchyme (arrow), and periocular mesenchyme adjacent to the optic nerve (arrow). (E) *Gli1* is expressed in the eyelid (arrow), corneal and periocular mesenchyme as well as the frontal bone. (F) *Ptch1* is expressed in the eyelid, periocular and corneal mesenchyme (arrow). Scale bars in A = 100 μ m and applies to all. Abbreviations: cm, corneal mesenchyme; fb, frontal bone primordia; lp, lens placode; oc, optic cup; os, optic stalk; pom, peri-ocular mesenchyme; rpe, pigmented retinal epithelium; uel, upper eye lid bud.

Wnt and *Hedgehog* Signaling Effectors Have Restricted Expression Patterns Throughout E10.5–E12.5

Since we observed that *Tfap2 β* , *Casq2*, *Penk*, and *Zic1* genes expressed in discrete anatomical regions of the developing eye, we next determined if their expression domains overlapped with signaling pathways that are required for eye development. *Lef1* is a transcriptional target of Wnt signal pathway, and we used its expression domain to define where active Wnt signaling is occurring. At E10.5, *Lef1* is mainly expressed in the upper eye lid (uel) and os (Fig. 3A).

The expression of *Lef1* is maintained in the eyelid bud mesenchyme at E12.5. The expression of *Lef1* in OS develop into the corneal and periocular mesenchyme (Fig. 3D).

To identify where active Hedgehog (Hh) signaling is occurring we assayed the expression of *Gli1*, a transcription factor that is a target and activator of the pathway, and *Ptch1*, a Hh co-receptor, that is a target and inhibitor of the pathway. At E10.5, *Gli1* and *Ptch1* are broadly expressed in lens placode (lp), optic cup (oc), os, rpe, and periocular mesenchyme (pom). *Gli1* is expressed in mesenchyme of upper eye lid (uel), while *Ptch1* is in surface mesenchyme of upper eye lid (uel). At E12.5, *Gli1* is

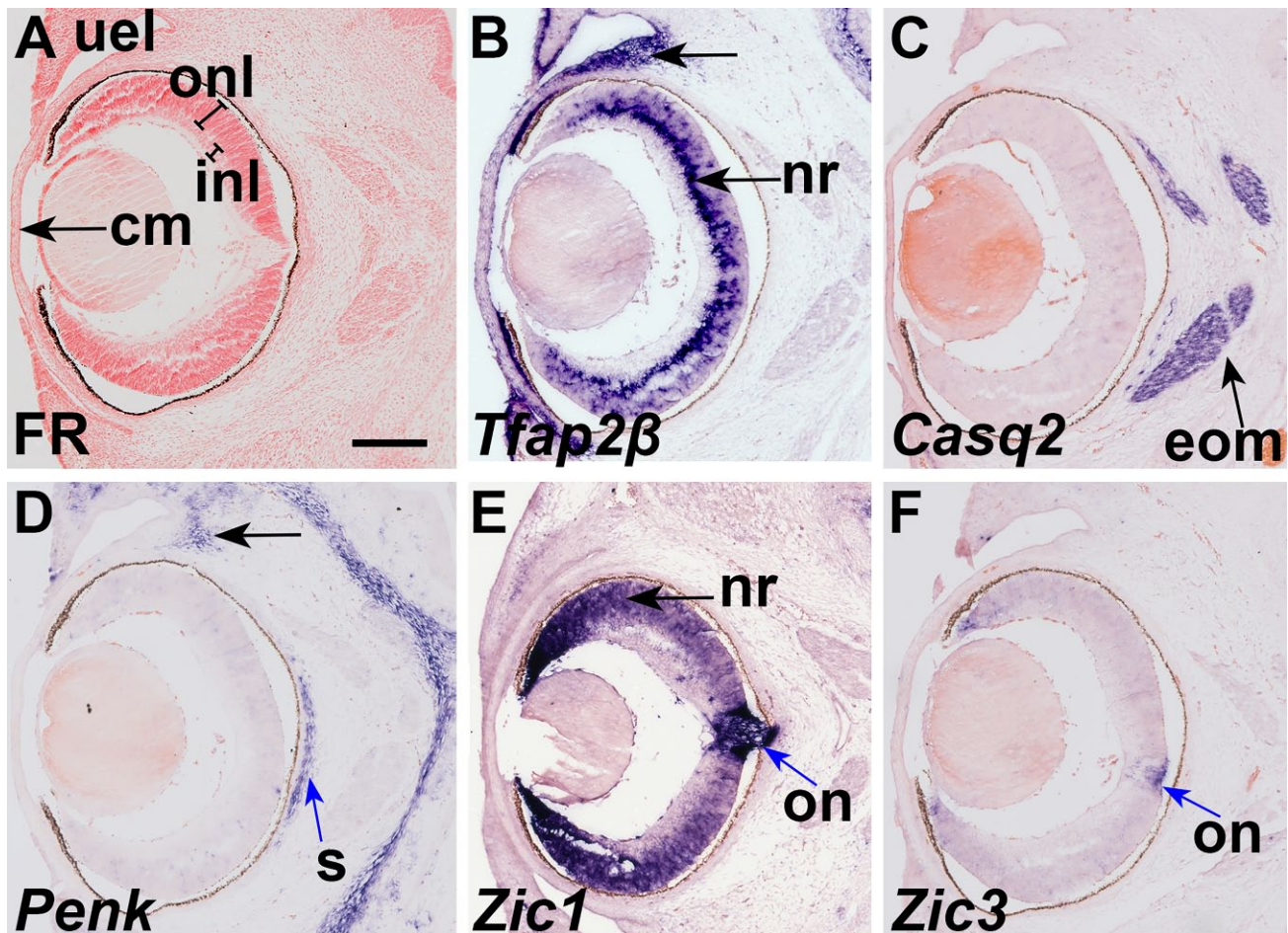


Figure 4. *Tfap2 β* , *Zic1*, *Zic3*, *Penk*, and *Casq2* expression define the neuronal, skeletogenic, and muscular cells in the E15.5 optic region. In situ hybridization of E15.5 coronal sections with probes to (B) *Tfap2 β* , (C) *Casq2*, (D) *Penk*, (E) *Zic1*, and (F) *Zic3*. (A) Nuclear fast red staining of an adjacent section. (B) *Tfap2 β* is expressed in the epithelium of the early eyelid (arrow), innermost layer of the outer layer of the neural retina (arrow), and the bulbar conjunctiva (arrow). (C) *Casq2* is expressed in the extrinsic ocular muscles (arrow). (D) *Penk* is expressed in the sclera (blue arrow) and bulbar conjunctiva (black arrow). (E) *Zic1* is expressed in the neural retina (arrow), strong in the optic nerve (blue arrow) and optic disk. (F) *Zic3* is expressed in the optic nerve (blue arrow). Scale bars = 100 μ m. Abbreviations: cm, corneal mesenchyme; eom, extrinsic ocular muscles; inl, inner nuclear layer; on, optic nerve; onl, outer nuclear layer; nr, neural retina; s, sclera; uel, upper eye lid bud.

broadly expressed in the eyelid bud mesenchyme, corneal mesenchyme, and periocular mesenchyme as well as the frontal bone primordium (Fig. 3E). *Ptch1* is expressed in a few cells in the lens, the early eyelid bud and periocular mesenchyme (Fig. 3F).

Tfap2 β , *Casq2*, *Penk*, *Zic1*, and *Zic3* Have Discrete Expression Patterns at E15.5

At E15.5, *Tfap2 β* was still expressed in the epithelium surrounding the eyelid, and bulbar conjunctiva mesenchyme adjacent to the corneal epithelium. *Tfap2 β* is expressed in innermost layer of the outer nuclear layer of the retina, and a few cells in the outermost layer of the neural retina (Figs. 4B and 5B). *Tfap2 β* is expressed

in the corneal endothelium at E15.5 (Figs. 4B and 5B). *Casq2* expression is continues to be expressed in the extrinsic ocular muscles (Figs. 4C and 5C). *Casq2* is also expressed in the skeletal muscle surrounding the femur (Fig. 5D). *Penk* is detected in the sclera adjacent to the pigmented retinal epithelium as well as the perichondrium of the nasal cavity and sparsely through the bulbar conjunctiva (Figs. 4D and 5E; Wan et al.¹³). *Zic1* expression is detected in the outer nuclear layer of the retina, especially in the optic disk and the lip of the optic cup (Figs. 4E and 5F). We detected *Zic3* expression at E15.5, only expressed in the optic nerve and lip of the optic cup (Figs. 4F and 5G).

Between E15.5 and E17.5, the eyelid buds extend over the cornea and fuse together at the midline.^{21,22} This

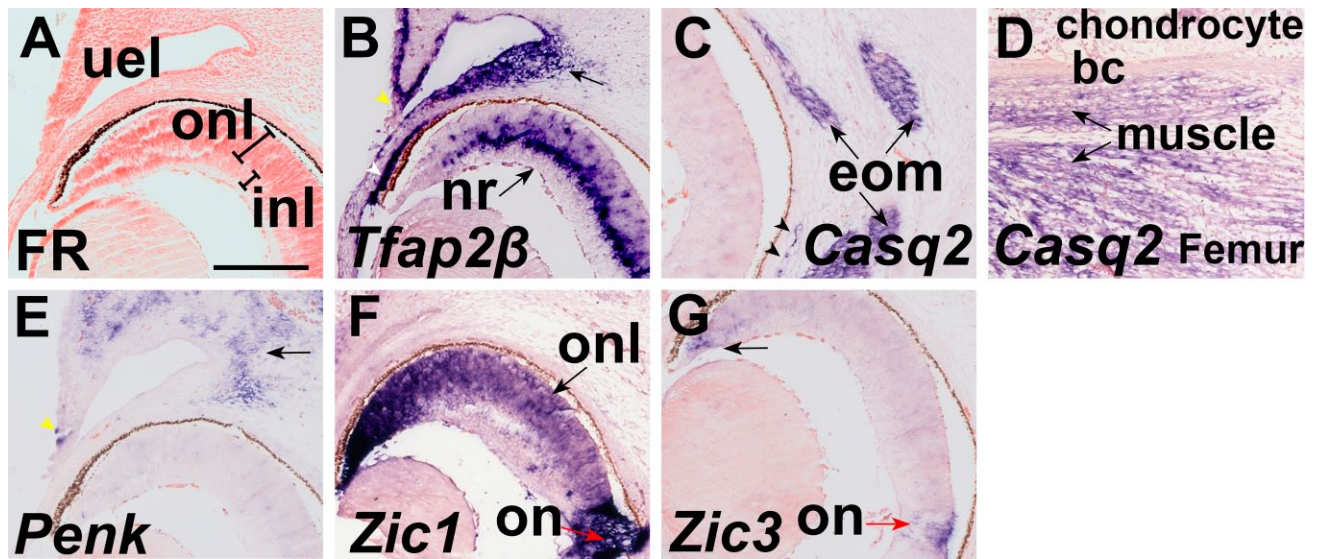


Figure 5. High power images of *Tfap2 β* , *Zic1*, *Zic3*, *Penk*, and *Casq2* expression at E15.5 (B) *Tfap2 β* , (C) *Casq2*, (D) *Penk*, (E) *Zic1*, and (F) *Zic3* in coronal sections of E15.5. (A) Nuclear fast red staining. (B) *Tfap2 β* is the ectoderm adjacent to the periderm cells in the eyelid bud (white arrowhead). *Tfap2 β* is expressed in the innermost layer of the outer nuclear layer of the neural retina, and some cells in the outermost layer (arrow). *Tfap2 β* is expressed in the corneal endothelium (white arrowhead). (C) *Casq2* is expressed in the extrinsic ocular muscles (arrow) and vessels (black arrowheads). (D) *Penk* is expressed in the mesenchyme of the eyelid. The distal eyelid ectoderm also expresses *Penk*. (E) *Zic1* is expressed in the neural retina (arrow) and has more intense expression in the optic nerve. (F) *Zic3* is expressed in the optic nerve (red arrow). Scale bar in A = 200 μ m and applies to all. Abbreviations: bc, bone collar; eom, extrinsic ocular muscles; inl, inner nuclear layer; on, optic nerve; onl, outer nuclear layer; nr, neural retina; uel, upper eye lid bud.

process is accomplished by the extension and fusion of the periderm at the leading edge of the eyelid bud.^{21,22} *Penk* and *Tfap2 β* is found in the epithelium underlying the periderm (yellow arrowhead, Fig. 5B and D).

Discussion

In this study, we have performed an in situ hybridization expression analysis of *Tfap2 β* , *Casq2*, *Penk*, *Zic1*, and *Zic3* in the mouse eye at E12.5 and E15.5. We found that these five genes have a complimentary expression pattern to each other, suggesting that their functions may be coordinated in normal eye development. *Tfap2 β* expression is found in the outer nuclear layer of the neural retina. In line with its function in cardiac muscle *Casq2* expression is found in the external ocular muscles from E12.5 to E15.5. *Zic1* was consistently expressed in the outer nuclear layer of the neural retina, including the lip of the optic cup between E12.5 and E15.5. Both *Zic3* and *Penk* expand their region of expression between E12.5 and E15.5. The expression patterns here suggest that these genes may play a role in the eye development in a stage-dependent manner.

We chose to study the location of signaling pathways using the expression of *Lef1* for Wnt/ β -catenin signaling, because it is an epithelial transcription factor that is the target of activated Wnt signaling. The expression

domains of *Lef1*, *Gli1*, and *Ptch1* at E10.5 suggests that the eyelid morphogenesis may share similarities to organs that develop as the result of epithelial and mesenchymal interactions. Similar to the tooth bud, we observed a central region of Hh signaling that is found in an invaginating epithelium, adjacent to the *Lef1* domain. This epithelium is adjacent to a highly condensed mesenchyme. More work is needed to confirm if other morphogenetic and signaling processes are shared between the eyelid and tooth bud.

Our expression data show that *Tfap2 β* , *Zic1*, and *Zic3* have complimentary and overlapping expression domains within the neural retina. *Tfap2 β* is expressed in the outer layer of neural retina and is excluded from the lip of the optic cup. *Zic1* is found in the entire neural retina and the enriched in the lip of the optic cup, while *Zic3* is only found in the lip of the optic cup. These complementary and overlapping expression patterns suggest that these transcription factors cooperate in the development of the neural retina. These data support the hypothesis that the domains of *Zic1*, *Zic3*, and *Tfap2 β* define the medial-lateral axis of the neural retina.

Tfap2 β (Figs. 1B and 2B) and *Lef1* expression (Fig. 3A and D) partially overlap in and the developing eyelid mesenchyme and corneal epithelium at both E10.5 and E12.5. In *Tfap2 β* -null mice, the expression of DKK1 is reduced in corneal epithelium.¹⁹ DKK1 is an

inhibitor of Wnt signaling, implying that Wnt signaling is increased in the developing cornea in *Tfap2 β* -null mice.¹⁹ Based on our expression data, we would predict that *Lef1* expression would be increased in the *Tfap2 β* -null animals and suggest that *Tfap2 β* may function in a negative feedback loop to reduce Wnt signaling in corneal development.

Importantly, we observed overlapping expression domains of *Zic1* and *Zic3* with *Gli1* and *Ptch1* in optic cups and OS at E10.5, while the expression of *Zic1* and *Zic3* are not overlapped with *Gli1* and *Ptch1* at E12.5. *Zic* proteins has been reported to interact with Gli proteins,²³ and acts upstream of Sonic Hh target genes²⁴ to regulate forebrain development. These observations imply that *Zic* proteins regulate the early eye development through Hh signal.

Taken together, we have shown that *Tfap2 β* , *Casq2*, *Penk*, *Zic1*, and *Zic3* make a panel of five genes that are expressed in distinct, complementary, and overlapping tissues that will facilitate the study of eye morphogenesis.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

YW performed the experiments, analyzed data, and drafted the manuscript. CW analyzed data and drafted the manuscript. NR analyzed data. MBR analyzed data. HLS-R conceived and managed the project, analyzed data, and wrote the paper. All authors have read and approved the final manuscript.

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