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Wnt/β-catenin signaling controls mouse eyelid growth by mediating epithelial-mesenchymal interactions

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Abstract

Purpose—To investigate the role of Wnt/β-catenin signaling in mouse eyelid development.

Methods—Wnt/β-catenin signaling was disrupted by deleting supraorbital mesenchymal β-catenin or epithelial *Wls*. *p63* was removed to determine whether the expression of Wnts is affected. The eyelid morphology was examined at different stages. Proliferation, apoptosis, and expression of Wnt ligands and their target genes were analyzed via immunofluorescence staining, TUNEL assay, and in situ hybridization.

Results—Deletion of β-catenin in supraorbital mesenchyme abolishes eyelid growth by causing decreased proliferation in supraorbital epithelium and underlying mesenchyme. Inhibition of Wnt secretion by deleting *Wls* in supraorbital epithelium results in failure of eyelid development,

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Author contributions

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similar to the effects of deleting mesenchymal β-catenin. Knockout of *p63* results in formation of hypoplastic eyelids and reduced expression of several Wnt ligands in eyelid epithelium.

Conclusions—Epithelial Wnt ligands activate mesenchymal Wnt/β-catenin signaling to control eyelid growth and their expression is partially regulated by p63.

Keywords

Eyelid development; epithelial-mesenchymal interactions; signaling pathway; Wnt; β-catenin; *Wls* ; *Fgf10* ; *p63* ; proliferation

Introduction

Eyelid colobomas, characterized by partial or complete loss of eyelid tissue, occur in more than twenty human syndromes [1]. However, the mechanisms controlling eyelid development, and how these are perturbed to cause defective eyelid development, are incompletely understood [2].

The mammalian eyelid originates from periocular epithelium and underlying mesenchyme, and its development can be categorized into four main stages comprising specification, growth, fusion, and separation [3, 4]. Our current knowledge of the mechanisms of eyelid development is largely based on murine genetic studies, as a diverse collection of genetically modified mouse mutants displays an eye-open at birth (EOB) phenotype [4]. The course of mouse eyelid formation closely resembles that of human eyelid, making it an ideal model to study eyelid development.

Murine eyelid development initiates at approximately embryonic day 9 (E9) when the presumptive eyelid region can be identified by *Pax6* expression [5]. By E11.5, invagination of both dorsal and ventral periocular tissue outlines morphologically discernable eyelids, which continue to grow toward each other across the ocular globe. Starting at E15.5, the peridermal cell layers of both eyelids extend from the leading edges and fuse across the cornea by E16.5. Thereafter, the palpebral epidermis that bridges the eyelids begins to stratify, and other epidermal appendages such as hair follicles and Meibomian glands begin to develop. At birth, the eyelids are fused and subsequently start to separate. Separation of eyelids, and opening of eyes, is complete by postnatal day 12 (P12) [6].

EOB phenotypes in most of the mouse mutants that have been characterized to date are caused by impaired eyelid closure and are associated with altered expression and/or activity of a diverse set of transcription factors and signaling pathways. For example, Activin βB regulates eyelid closure by triggering a signaling cascade that integrates MAP3K1, JNK and c-JUN activities [7, 8]. C-JUN may also induce EGFR expression, which mediates the effect of EGF in promoting periderm migration via activation of the ERK pathway [9, 10]. The FGF10-FGFR2 pathway is required for eyelid epithelial proliferation and promotes periderm migration by inducing expression of Activin βB and TGF-α [11]. Additionally, FGF signaling acts upstream of the SMAD4-mediated BMP pathway in maintaining FOXC1 and FOXC2 expression, which regulate eyelid closure in a dosage-dependent manner [12,

13]. While the regulation of eyelid fusion has been intensively investigated, the mechanisms regulating eyelid growth are less well characterized.

Wnt proteins are secreted paracrine factors that trigger both β-catenin-dependent canonical and β-catenin-independent non-canonical Wnt pathways. The cargo protein WNTLESS (WLS) is required for secretion of Wnt ligands [14, 15]. Multiple Wnts are expressed in the periocular tissue during eyelid development [16], and Wnt/β-catenin signaling is reported to regulate eyelid closure by repressing MAP3K1 expression and JNK activity [17]. Deletion of DKK2, a secreted Wnt inhibitor, also causes abnormal eyelid development, indicating that excessive Wnt activity affects eyelid closure [18]. Therefore, the Wnt signaling pathway must be precisely controlled for normal eyelid development.

In the current study, we investigated the functions of Wnt/β-catenin signaling in eyelid growth by genetically manipulating the expression of mesenchymal β-catenin and epithelial *Wls*. We found that deletion of either mesenchymal β-catenin or epithelial *Wls* disrupts eyelid growth. In addition, we observed that expression of Wnt ligands in eyelid epithelium is decreased in *p63* loss of function mutants, which have hypoplastic eyelids, indicating that epithelial Wnt expression is at least partially regulated by p63 during eyelid development.

Materials and methods

Mice

The following mouse lines were used: *Prx1-Cre* (Jackson Laboratories, strain #005584); *Msx2-Cre* (Jackson Laboratories, strain #027892); $ROSA^{mT/mG}$ (Jackson Laboratories, strain #007676); $ROSA26^{LacZ}$ (Jackson Laboratories, strain #002073); $Wls^{tm1.1La/Jn}/J$ (Wls^{fl}) (Jackson Laboratories, strain #012888); $\beta\text{-}catenin^{fl/fl}$ (Jackson Laboratories, strain #004152); and $p63^{+/-}$ [19]. All mice were maintained on a mixed strain background. Mice were allocated to experimental or control groups according to their genotypes, with control mice included in each experiment. Male mice carrying *Prx1-Cre* or *Msx2-Cre* were crossed with $ROSA^{mT/mG}$, $ROSA26^{LacZ}$, $\beta\text{-}catenin^{fl/fl}$, or $Wls^{fl/fl}$ females to avoid potential deletion in the female germline. $p63^{+/-}$ male and female mice were intercrossed to yield $p63^{-/-}$ embryos. Investigators were not blinded during allocation and animal handling as information about genotype was required for appropriate allocation to experimental groups. Immunostaining and RNA in situ hybridization studies were carried out and results were recorded in a blinded fashion. Up to five mice were maintained per cage in a specific pathogen-free barrier facility on standard rodent laboratory chow (Purina, catalog #5001). All animal experiments were performed under approved animal protocols according to institutional guidelines established by the respective IACUC committees at the Icahn School of Medicine at Mount Sinai and Shanghai Jiaotong University. At least three embryos of each genotype and experimental condition were assayed for all the experiments in this study.

RNA in situ Hybridization (ISH)

Embryos at the indicated stages were harvested, fixed with 4% paraformaldehyde (PFA) (Affymetrix/USB) in PBS overnight at 4°C, and processed for whole-mount RNA ISH, or frozen or paraffin sectioning. Whole-mount RNA ISH procedures and the *Fgf10* ISH

probe were previously described [20]. RNAscope was performed on fixed frozen sections or paraffin sections following the user's guide provided by Advanced Cell Diagnostic (ACD) using the RNAscope Multiplex Fluorescent Reagent Kit v2 (ACD #323100) and probes for *Lef1* (ACD #441861), *Wnt4* (ACD #401101), *Wnt3* (ACD #312241), and *Twist1* (ACD #414701). The whole-mount ISH staining was photographed using a Leica MZ16F dissection microscope (Leica Microsystems).

β-Galactosidase staining

Embryos at the indicated stages were harvested and fixed with 4% paraformaldehyde (PFA) (Affymetrix/USB) in PBS for 30 minutes at room temperature. Subsequent whole mount staining for β-Galactosidase was carried out as previously described [21]. Embryo whole mounts were photographed using a Leica MZ16F dissection microscope (Leica Microsystems).

BrdU administration—20mg/ml BrdU (B5002, Sigma) in PBS was used for BrdU labeling. Pregnant female mice were intraperitoneally injected with 2mg BrdU per 30 g body weight. After 1 hour, the mice were euthanized to collect embryos at the indicated stages.

Immunostaining and TUNEL assays

Frozen sections were incubated with Ki-67 antibody (#14-5698-82, Invitrogen, 1:200), β-catenin antibody (#C7207, Sigma, 1:1000), BrdU antibody (#B2531, Sigma, 1:200), LEF1 antibody (#2230s, Cell Signaling Technology, 1:200), KRT5 antibody (#905501, BioLegend, 1:500) and KRT14 antibody (#MA5–11599, Invitrogen, 1:500) overnight at 4°C, followed by incubation with secondary antibodies including goat anti-rabbit Alexa Fluor™ 488 or 555 (#A-11008 or # A-21428, Thermo Fisher Scientific, 1:1000), goat anti-mouse Alexa Fluor™ 488 or 555 (#A-11001 or #A-21422, Thermo Fisher Scientific, 1:1000), and Streptavidin-Texas Red™ (#SA-5006–1, Vector Laboratories, 1:500). For quantification of proliferating cells, at least 30 epithelial and 100 underlying mesenchymal cells in the presumptive upper eyelid from each embryo were analyzed. Unpaired two-tailed Student's *t*-test was used to calculate statistical significance for quantification of Ki-67+ or BrdU+ cells, and $p<0.05$ was considered significant. For TUNEL assays, fixed frozen sections were incubated with TUNEL labeling mix (Roche) at 37°C for 1 hour and then washed with PBST and mounted in DAPI containing medium (Thermo Fisher Scientific). Histology and section immunofluorescence (IF) data were documented using a Leica Microsystems DM5500B fluorescent microscope (Leica Microsystems). Whole-mount fluorescence signals were documented using a Leica MZ16F dissection microscope (Leica Microsystems).

Results

Prx1-Cre is active in supraorbital and developing upper eyelid mesenchyme

To determine the role of canonical Wnt pathway during eyelid growth, we sought to delete β-catenin in eyelid mesenchyme which displays a high level of Wnt activity as evidenced by expression of the ubiquitous Wnt target gene, *Axin2* [22]. To identify a Cre line that is active in eyelid mesenchyme, we first examined the activity of *Prx1-Cre* in periocular tissue during

eyelid development because *Prx1-Cre* was reported to be active in a subset of cranial-facial mesenchymal cells [23]. *Prx1-Cre* mice were crossed with the *Rosa26R*$^{mT/mG}$ Cre reporter allele to monitor Cre activity. We found that at E11.5, reporter gene activity was present in a subset of supraorbital mesenchyme of the presumptive upper eyelid (Fig. S1B–D). By E12.5, Cre reporter expression in the supraorbital mesenchyme had expanded rostrally, and increased numbers of deleted cells were detected in the developing upper eyelid primordia (Fig. S1E–G). By E13.5, the domain of reporter expression continued to expand, and the entire upper eyelid and surrounding mesenchyme were positive for mGFP expression (Fig. S1H–J). Published data showed continued absence of Cre activity in the lower eyelid at E16.5 [23]. Furthermore, adult (P33) *Prx1-Cre ROSA26R*^{mTmG} mice displayed Cre reporter expression in upper but not lower eyelid mesenchyme (Fig. S1K and S1L). These data suggest that *Prx1-Cre* can be used to manipulate gene expression in supraorbital and upper eyelid mesenchyme.

Deletion of β-catenin in supraorbital mesenchyme abolishes upper eyelid growth

To test whether mesenchymal canonical Wnt signaling regulates eyelid development, we used *Prx1-Cre* to delete β-catenin. We found that eyelid development at E18.5 was unaffected by heterozygous deletion of β-catenin in *Prx1-Cre β-catenin*$^{fl/+}$ embryos compared with wild-type. However, homozygous deletion in *Prx1-Cre β-catenin*$^{fl/fl}$ embryos resulted in an open eye phenotype (Fig. 1A and 1B). H&E staining of sectioned tissue revealed that the upper eyelids of these mutants were almost completely absent except for some rudimentary epidermal thickening, while the lower eyelids still developed (Fig. 1C and 1D). This observation was consistent with differential *Prx1-Cre* activity between upper and lower eyelid mesenchyme (Fig. S1). Notably, although the lower eyelid was present in mutant embryos at E18.5, it failed to extend over the eyeball; given the lack of Cre activity in the lower eyelid, this phenotype was likely secondary to failure of upper eyelid growth. To determine whether the upper eyelid defect was caused by defective eyelid growth, we examined eyelids at E12.5 and E14.5 and found that the upper eyelids of *Prx1-Cre β-catenin*$^{fl/fl}$ embryos failed to develop at E12.5 and E14.5 (Fig. 1E–H). These data indicate that supraorbital mesenchymal β-catenin is required for upper eyelid growth.

Wnt/β-catenin signaling is necessary for cell proliferation in supraorbital mesenchyme

To delineate the mechanisms by which β-catenin regulates eyelid growth, we assessed activity of the canonical Wnt pathway in the supraorbital region when eyelid growth initiates at E11.5. We found that supraorbital mesenchymal β-catenin was efficiently deleted by *Prx1-Cre* at E11.5, although small patches of undeleted cells could still be identified (Fig. 2A and 2B). Expression of *Lef1* and *Twist1*, which are direct Wnt/β-catenin target genes [24], was significantly reduced in β-catenin-deficient supraorbital mesenchyme (Fig. 2C–F). By contrast, expression of epithelial Wnt ligands including *Wnt3* and *Wnt4* was not affected in these mutants (Fig. 2G–J). To determine whether increased apoptosis contributes to the eyelid defects in *Prx1-Cre β-catenin*$^{fl/fl}$ embryos, we performed TUNEL assays. The results indicated absence of abnormal apoptosis in the supraorbital region of the mutants at E11.5 (Fig. 2K and 2L). Since Wnt/β-catenin pathway regulates proliferation of mesenchymal cells in multiple contexts [20, 25], we asked whether proliferation was affected in the supraorbital region of *Prx1-Cre β-catenin*$^{fl/fl}$ mutants at E11.5. The data indicated that proliferation was

decreased in both supraorbital mesenchyme and overlying epithelium in the mutant eyelids compared with controls (Fig. 2M-O). Taken together, these data suggest that mesenchymal β-catenin is indispensable for cell proliferation in the presumptive upper eyelid.

Epithelial Wnt ligands are required to maintain eyelid growth

Since Wnt genes are predominantly expressed in eyelid epithelium [16], we investigated whether epithelial Wnt ligands are necessary for eyelid growth. To disrupt the function of epithelial Wnt ligands, we utilized a conditional allele of *Wls* (*Wls*$^{tm1.1La/Jn/J}$) (*Wls*^{fl}) [26], which is required for Wnt protein secretion. *Wls*^{fl} mice were bred to mice carrying *Msx2-Cre*, which is active in periocular epithelium, including the presumptive epithelium of the upper eyelid from E11.5 (Fig. S2B–D), the upper eyelid from E12.5 to E13.5 (Fig. S2E–J), and mosaically in the developing epithelium of the lower eyelid from E12.5 to E13.5 (Fig. S2E–J). *Msx2-Cre* also exhibited mosaic activity in the developing lens (Fig. S2A–J). *Msx2-Cre Wls*$^{fl/fl}$ embryos exhibited absence of normal eyelids at E17.5 (Fig. 3A and 3B). H&E staining data showed that the upper eyelids failed to form, and the lower eyelids were hypoplastic (Fig. 3C and 3D) in these mutants. Some rudimentary lower eyelid epithelium was still present at E17.5, by contrast with the more severe lower eyelid phenotype seen in *Prx1-Cre Ctnnb1*$^{fl/fl}$ embryos at E18.5 (Figure 1A–D). Several factors may contribute to this apparent inconsistency. Firstly, *Msx2-Cre* has mosaic activity in the lower eyelid; secondly, deletion of *Wls* is likely to have less severe consequences in the upper eyelid than β-catenin deletion, and thus secondary effects on lower eyelid growth may be reduced in the *Wls* versus β-catenin mutant; thirdly, secondary consequences on the lower eyelid may be less obvious at E17.5 than at E18.5. Examination of eye tissue at E12.5 and E14.5 confirmed that growth of upper eyelid was completely disrupted in the mutants (Fig. 3E–H). *Msx2-Cre Wls*$^{fl/fl}$ mutants also displayed defects in development of the iris and retina (Fig. 3A–D). Partial abrogation of Wnt ligand secretion from developing lens cells due to mosaic *Msx2-Cre* activity in the lens (Fig. S2A–J) can potentially explain the abnormal development of adjacent retinal and iris tissues. Taken together, these data demonstrate that epithelial Wnt ligands are necessary for eyelid growth and reveal likely additional roles for *Wls* in the developing lens.

Epithelial Wnt ligands control supraorbital mesenchymal canonical Wnt signaling

Since *Msx2-Cre Wls*$^{fl/fl}$ mutants displayed similar defects in eyelid development to those observed in *Prx1-Cre β-catenin*$^{fl/fl}$ mutants, we asked whether epithelial Wnt ligand secretion is required for canonical Wnt signaling in the mesenchyme. We found that epithelial *Wls* was efficiently deleted in *Msx2-Cre Wls*$^{fl/fl}$ mutants (Fig. 4A and 4B), and expression of the Wnt/β-catenin target genes LEF1 and *Twist1* was downregulated in the underlying mesenchyme, indicating reduced canonical Wnt activity (Fig. 4C–F). By contrast, mRNA levels of *Wnt3* and *Wnt4* were comparable between control and *Wls*-deficient epithelium (Fig. 4G–J), indicating that *Wls* deletion blocked Wnt protein secretion but did not affect expression of Wnt genes. *Msx2-Cre Wls*$^{fl/fl}$ mutants displayed reduced proliferation in both supraorbital epithelium and mesenchyme, but increased apoptosis was not observed (Fig. 4K–O). These observations demonstrate that epithelial Wnts regulate eyelid growth by activating canonical Wnt signaling in supraorbital mesenchyme.

Expression of *Fgf10* in eyelid mesenchyme requires epithelial Wnt ligand secretion

We observed that loss of mesenchymal β-catenin in *Prx1-Cre β-catenin*$^{fl/fl}$ mutants caused reduced proliferation in the overlying epithelium as well as in supraorbital mesenchyme, suggesting that canonical Wnt signaling in the mesenchyme controls expression of secreted signals required for epithelial proliferation. Previous studies showed that FGF10, which is expressed in eyelid mesenchyme, controls epithelial proliferation by binding to its receptor FGFR2 in the overlying epithelium [11]. Since *Fgf10* is a direct target gene of canonical Wnt signaling in other developmental contexts, we asked whether *Fgf10* expression was affected in the eyelid of *Wls*-deficient mutants. Because significant *Fgf10* expression in the upper eyelid initiates from E12.5 [11], and the upper eyelid of *Msx2-Cre Wls*$^{fl/fl}$ mutants fail to form, we utilized *Msx2-Cre*^{low} mice that had been maintained for many generations and exhibited partial silencing of Cre expression. *Msx2-Cre*^{low} mice exhibited periocular Cre activity that was not as broad as that of typical *Msx2-Cre* in supraorbital epithelium at E11.5, being more restricted to the eyelid groove (Fig. S3B–D). At E13.5, Cre activity in *Msx2-Cre*^{low} mice was mainly present in the epithelium of the posterior part of the developing upper eyelid (Fig. S3E–G). *Msx2-Cre*^{low} *Wls*$^{fl/fl}$ mutants displayed an EOB phenotype (Fig. S4A and S4B), and H&E staining of sectioned tissue from independently derived samples showed that the upper eyelids of *Msx2-Cre*^{low} *Wls*$^{fl/fl}$ mutants formed but were hypoplastic at E18.5 (Fig. S4C and S4D). This result contrasts with lack of an obvious eyelid phenotype in a prior study of *Msx2-Cre*^{low} mice carrying an independent *Wls* allele (*Wls*$^{tm1.1Xzg}$) [20], which is distinct from the *Wls*$^{tm1.1La/Jn}$*/J* allele used in the current experiments. Use of a different conditional allele of *Wls* may explain this discrepancy. Analysis of upper eyelid development at E15.5 and E12.5 confirmed that the upper eyelids of *Msx2-Cre*^{low} *Wls*$^{fl/fl}$ mutants were significantly reduced in size compared to controls (Fig. S4E–H). Expression of LEF1 and proliferation were reduced in the hypoplastic upper eyelids of the mutants (Fig. 5A–D and 5G), similar to observations in *Prx1-Cre β-catenin*$^{fl/fl}$ and *Msx2-Cre Wls*$^{fl/fl}$ mutants. We found that *Fgf10* expression was lost in the upper eyelid of *Msx2-Cre*^{low} *Wls*$^{fl/fl}$ mutants (Fig. 5E and 5F). Together, these data demonstrate that epithelial Wnts are required for *Fgf10* expression in upper eyelid mesenchyme.

Expression of epithelial Wnts during eyelid development is disrupted by *p63* deletion

In developing dorsal skin, epithelial p63 regulates transcription of multiple Wnt genes [27]. Since *p63* knockout mice also show an EOB phenotype [28], we asked whether p63 is required for Wnt expression in eyelid epithelium. We first examined the eyelids of *p63*$^{+/-}$ and *p63*$^{-/-}$ embryos. We found that heterozygous deletion of *p63* did not affect eyelid development. By contrast, the eyelids of homozygous *p63* knockout mutants were significantly smaller than those of wild-type or heterozygous mutant littermates at E14.5 and E17.5 (Fig. S5A–D). Expression of the p63 target genes KRT5 and KRT14 was reduced in developing eyelid epithelium of *p63*$^{-/-}$ embryos at E14.5 (Fig. 6A–D). We found that expression of the Wnt ligands *Wnt3* and *Wnt4* was also down-regulated in *p63*-deleted eyelid epithelium (Fig. 6E–H). In line with this, expression of the Wnt target genes *Lef1* and *Twist1* was decreased in *p63*$^{-/-}$ mutant eyelids (Fig. 6I–L). Taken together, these data indicate that p63 regulates expression of epithelial Wnt genes during eyelid development.

Discussion

Reciprocal interactions between epithelium and underlying mesenchyme are critical for the development of multiple organs and appendages, including skin, lung, limb and eyelid [3, 25, 29, 30]. Eyelid development is a multi-stage process that is controlled by distinct networks involving diverse signaling pathways and transcription factors. Among these, FGF10 secreted from eyelid mesenchyme binds to its receptor FGFR2b, which is expressed in the overlying epithelium, to modulate proliferation and migration of epithelial cells [11]. This indicates the existence of mesenchyme-to-epithelium signaling during eyelid development. By comparison, the signaling relays from epithelium to mesenchyme required for eyelid formation are less well characterized.

Wnt ligands are secreted factors that mediate epithelial-mesenchymal interactions in various contexts. As examples, ectodermal Wnt ligands are required to maintain the outgrowth of mesenchyme during limb development [20]; and epithelial Wnt secretion regulates proliferation and differentiation of mesenchymal progenitors in the skin dermis [25] and in the developing lung [31]. During eyelid development, multiple Wnt genes including *Wnt3* and *Wnt4* show persistent expression in periocular epithelium and Wnt receptors such as FZD7 are expressed in the underlying mesenchyme [16]. These observations suggested that epithelial Wnt ligands might play key roles in mediating epithelial-mesenchymal interactions during eyelid development.

In support of this hypothesis, we found that deletion of mesenchymal β-catenin or prevention of epithelial Wnt ligand secretion resulted in failure of upper eyelid development, indicating that epithelial Wnts regulate eyelid development by activating canonical Wnt signaling in the mesenchyme. We further uncovered that deletion of β-catenin in developing eyelid mesenchyme abrogates *Fgf10* expression, consistent with prior data identifying *Fgf10* as a Wnt/β-catenin target gene [32]. Together with prior findings that *Fgf10* knockout mice display reduced proliferation of eyelid epithelium [11], our data suggest a model in which epithelial Wnt ligands signal to the mesenchyme to activate *Fgf10* expression, which then signals back to the epithelium to control its proliferation. Similar regulation of mesenchymal *Fgf10* by epithelial Wnt ligands is observed in other developing organs, such as limb and lung [20, 31], indicating that the Wnt-FGF10 axis is a common mechanism underlying epithelial-mesenchymal interactions. While *Fgf10* mutants display reduced proliferation only in the epithelium of the eyelid [11], we find that canonical Wnt signaling regulates cell proliferation in presumptive eyelid mesenchyme as well as in the epithelium. These observations indicate that the Wnt/β-catenin pathway controls proliferation of eyelid mesenchyme by mechanisms other than FGF10 signaling.

Overactivation of Wnt/β-catenin signaling is also known to disrupt eyelid development. For example, genetic deletion of the secreted Wnt inhibitor DKK2 or constitutive activation of epithelial β-catenin both cause EOB phenotypes [18, 33]. Therefore, it is crucial to precisely control activity of epithelial Wnt ligands during eyelid development. However, the mechanisms that regulate their expression have been unclear. To address this question, we examined a possible role for the epithelial transcription factor p63 in regulating epithelial Wnt gene expression.

p63 is a master regulator of epidermal development [34], and genetic deletion of *p63* results in the failed development of multiple organs and tissues including the epidermis, limb and eyelid [28]. p63 controls epidermal differentiation by orchestrating the expression and activity of multiple transcription factors and signaling pathways. In the developing skin epidermis, p63 directly activates transcription of epidermal Wnt genes to regulate epidermal differentiation [27]. Consistent with this observation, we find that homozygous deletion of *p63* impairs expression of several epithelial Wnt genes and causes reduced levels of canonical Wnt signaling in the developing eyelid, suggesting that the expression of epithelial Wnt genes during eyelid development is controlled at least in part by p63.

In summary, our data provide genetic evidence that Wnt/β-catenin signaling controls eyelid development by mediating epithelial-mesenchymal interactions (Fig. 7). These findings provide a mechanistic basis for investigating the etiology of eyelid colobomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data availability statement

The authors declare that the main data supporting the findings of this study are available within the article and its Supplemental Information files. All correspondence and material requests should be addressed to Sarah E. Millar or Gang Ma. This study includes no data deposited in external repositories.

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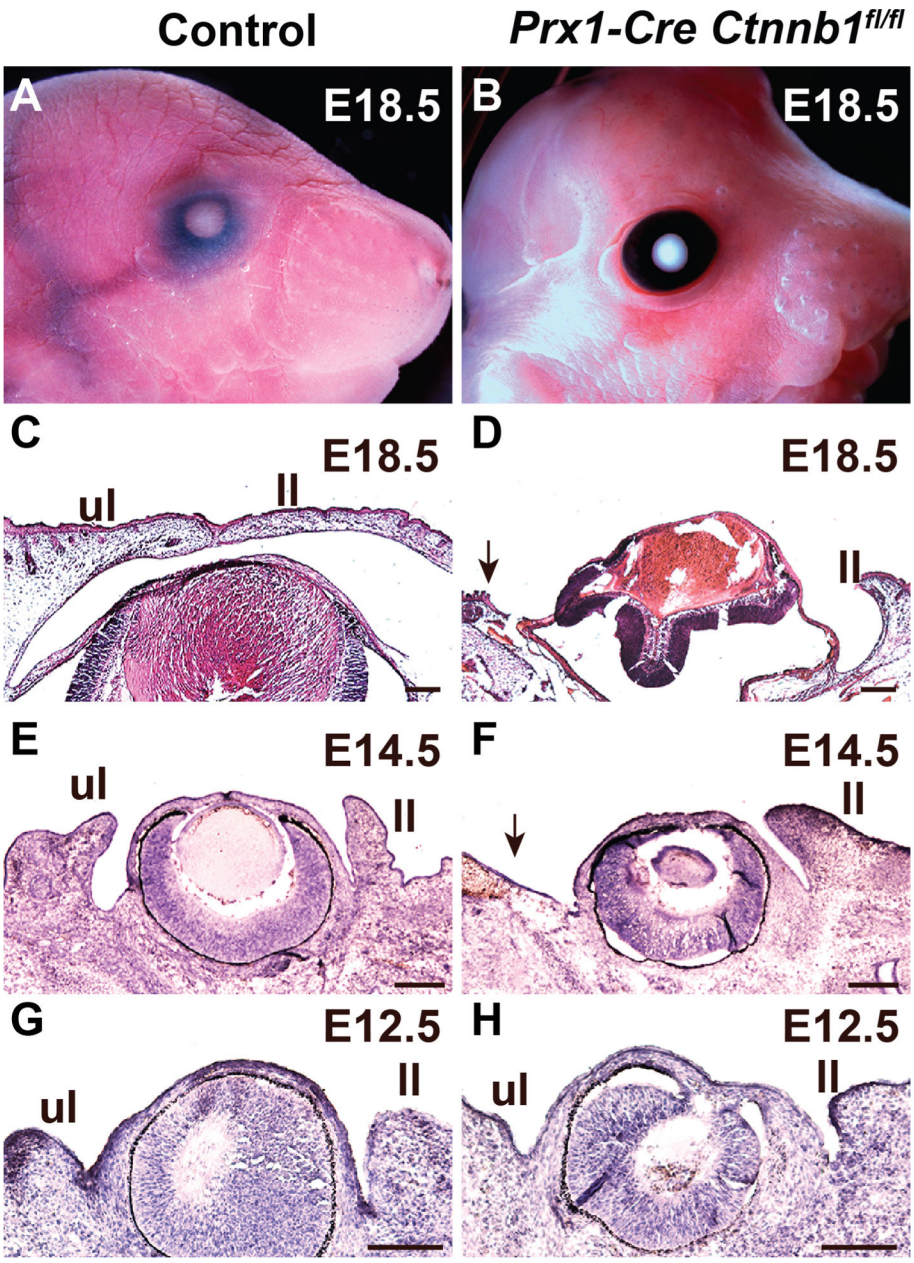

Figure 1. Deletion of β-catenin in supraorbital mesenchyme abolishes upper eyelid growth.

(A) The eyelids of control embryos are fused at E18.5. (B) The eyes of *Prx1-Cre β-catenin*$^{fl/fl}$ embryos are open at E18.5. (C) H&E staining of tissue sections shows that eyelids are fused in control embryos at E18.5. (D) H&E staining of tissue sections indicates that the upper eyelid is almost absent in *Prx1-Cre β-catenin*$^{fl/fl}$ embryos, except for some epidermal thickening (black arrow). The lower eyelid is still present in these mutants. (E) H&E staining of tissue sections shows that control eyelids are well-formed at E14.5. (F) H&E staining of tissue sections indicates that there is no discernable upper eyelid in *Prx1-Cre β-catenin*$^{fl/fl}$ embryos at E14.5 (black arrow). (G) H&E staining shows that at E12.5, control embryos have morphologically distinguishable eyelid primordia. (H) H&E staining shows that in *Prx1-Cre β-catenin*$^{fl/fl}$ embryos, no significant upper eyelid primordia

have formed at E12.5, but lower eyelid primordia are present. Samples in (A) and (B) were photographed at the same magnification. Scale bars: 150μm. Abbreviations: ul, upper eyelid; ll, lower eyelid.

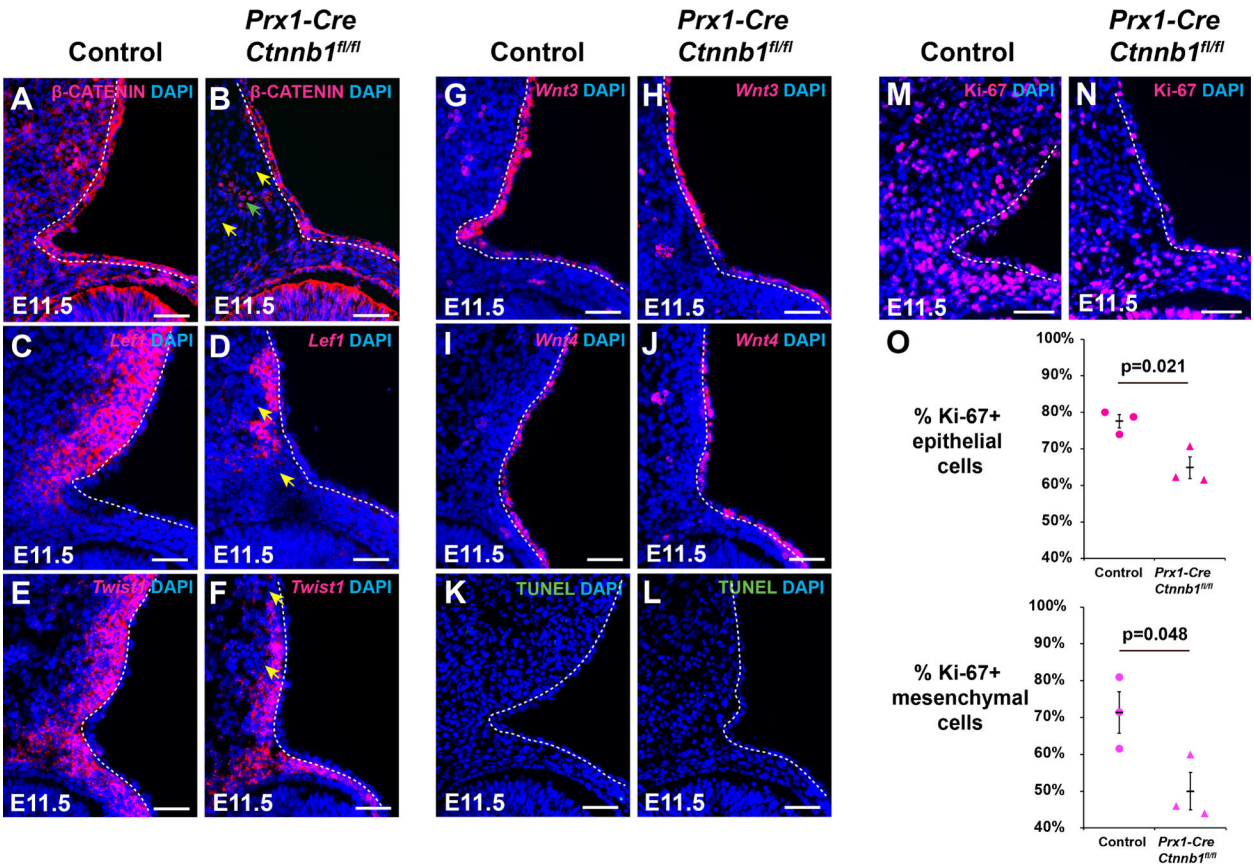

Figure 2. Deletion of β-catenin in supraorbital mesenchyme results in reduced canonical Wnt activity and decreased cell proliferation.

(A) IF shows that β-catenin is expressed in the supraorbital epithelium and mesenchyme of control embryos at E11.5. (B) IF shows that β-catenin is deleted in the supraorbital mesenchyme (yellow arrows), but patches of undeleted cells can also be identified (green arrow) in *Prx1-Cre β-catenin*$^{fl/fl}$ embryos at E11.5. (C) *Lef1* expression is detected in supraorbital mesenchyme in control embryos at E11.5. (D) *Lef1* expression is reduced or absent (yellow arrows) in the supraorbital mesenchyme of *Prx1-Cre β-catenin*$^{fl/fl}$ embryos at E11.5. (E) *Twist1* is expressed in the supraorbital mesenchyme of control embryos at E11.5. (F) Expression of *Twist1* is decreased in the supraorbital mesenchyme of *Prx1-Cre β-catenin*$^{fl/fl}$ embryos at E11.5 (yellow arrows). (G and H) Expression of epithelial *Wnt3* is similar between control and *Prx1-Cre β-catenin*$^{fl/fl}$ embryos at E11.5. (I and J) Epithelial *Wnt4* levels are comparable between control and *Prx1-Cre β-catenin*$^{fl/fl}$ embryos at E11.5. (K and L) TUNEL assay indicates that levels of apoptosis are similar in control and *Prx1-Cre β-catenin*$^{fl/fl}$ embryos at E11.5. (M and N) Ki-67 staining at E11.5 shows that proliferation is reduced upon deletion of mesenchymal β-catenin. (O) Statistical analysis of Ki-67+ cells in supraorbital epithelium and underlying mesenchyme. Three pairs of control and mutant embryos were analyzed. Unpaired student *t*-test was used to evaluate significance. $p<0.05$ was considered significant. Scale bars: 50μm. The white dashed line indicates the boundary between epithelium and mesenchyme.

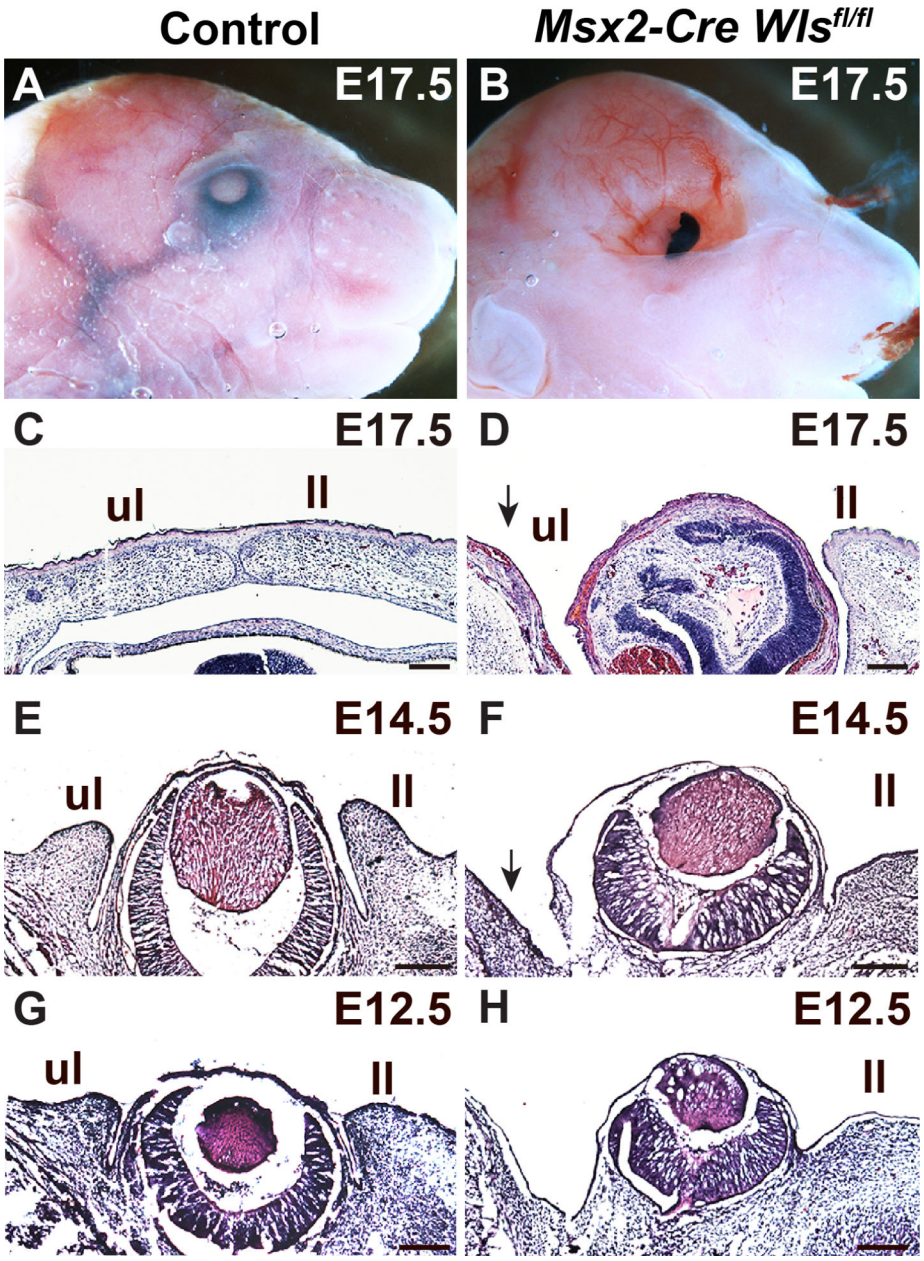

Figure 3. Epithelial Wnt ligands are required to maintain eyelid growth.

(A) The eyelids of control embryos are fused at E17.5. (B) The eyes of *Msx2-Cre Wls*$^{fl/fl}$ embryos exhibit defects in multiple ocular tissues including loss of supraorbital skin and eyelid, and malformed eye at E17.5. (C) H&E staining shows that eyelids are fused in control embryos at E17.5. (D) H&E staining indicates that the upper eyelid is lost in *Msx2-Cre Wls*$^{fl/fl}$ embryos (black arrow). The lower eyelid is still present in these mutants. (E) H&E staining shows that control eyelids are well-formed at E14.5. (F) H&E staining indicates that the upper eyelid is lost in *Msx2-Cre Wls*$^{fl/fl}$ embryos at E14.5 (black arrow). (G) H&E staining shows that at E12.5, control embryos have morphologically distinguishable eyelid primordia. (H) H&E staining shows that in *Msx2-Cre Wls*$^{fl/fl}$ embryos, no upper eyelid primordia have formed at E12.5. Samples in (A) and

(B) were photographed at the same magnification. Scale bars: 150μm. Abbreviations: ul, upper eyelid; ll, lower eyelid.

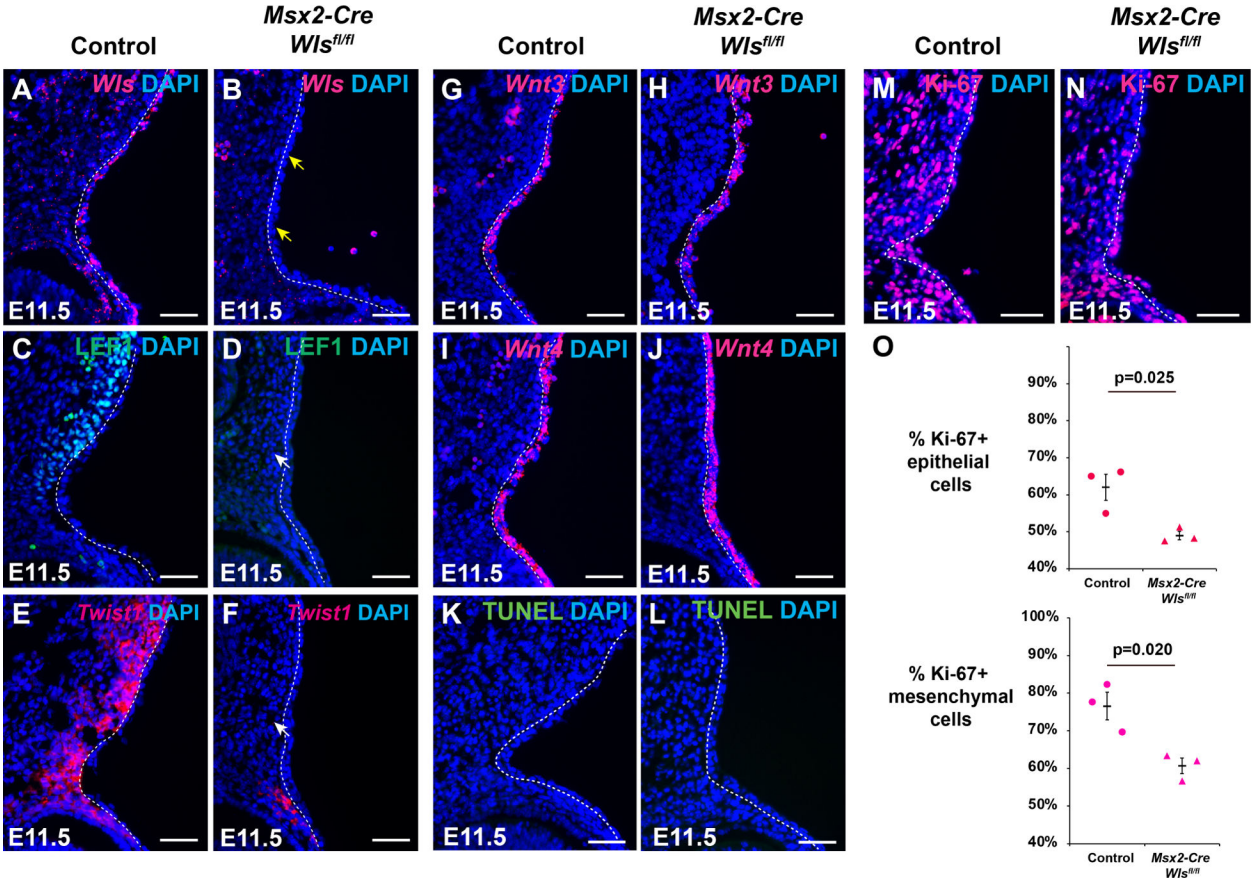

Figure 4. Epithelial Wnt ligands control mesenchymal canonical Wnt signaling in the eyelid.

(A) RNAscope ISH shows that *Wls* is expressed in the supraorbital epithelium and mesenchyme of control embryos at E11.5. (B) RNAscope ISH shows that *Wls* is deleted in the supraorbital epithelium (yellow arrows) of *Msx2-Cre Wls*$^{fl/fl}$ embryos at E11.5. (C) IF staining shows that LEF1 is expressed in the supraorbital mesenchyme of control embryos at E11.5. (D) IF staining shows that LEF1 expression is absent (yellow arrow) in the supraorbital mesenchyme of *Msx2-Cre Wls*$^{fl/fl}$ embryos at E11.5. (E) *Twist1* is expressed in the supraorbital mesenchyme of control embryos at E11.5. (F) Expression of *Twist1* is lost in the supraorbital mesenchyme of *Msx2-Cre Wls*$^{fl/fl}$ embryos at E11.5 (white arrow). (G and H) Expression of epithelial *Wnt3* is similar between control and *Msx2-Cre Wls*$^{fl/fl}$ embryos at E11.5. (I and J) Epithelial *Wnt4* levels are comparable between control and *Msx2-Cre Wls*$^{fl/fl}$ embryos at E11.5. (K and L) TUNEL assay indicates that levels of apoptosis are similar in control and *Msx2-Cre Wls*$^{fl/fl}$ embryos at E11.5. (M and N) Ki-67 staining at E11.5 shows that proliferation is reduced upon deletion of epithelial *Wls*. (O) Statistical analysis of Ki-67+ cells in supraorbital epithelium and underlying mesenchyme. Three pairs of control and mutant embryos were analyzed. Unpaired student *t*-test was used to evaluate significance. $p<0.05$ was considered significant. Scale bars: 50μm. The white dashed line indicates the boundary between epithelium and mesenchyme.

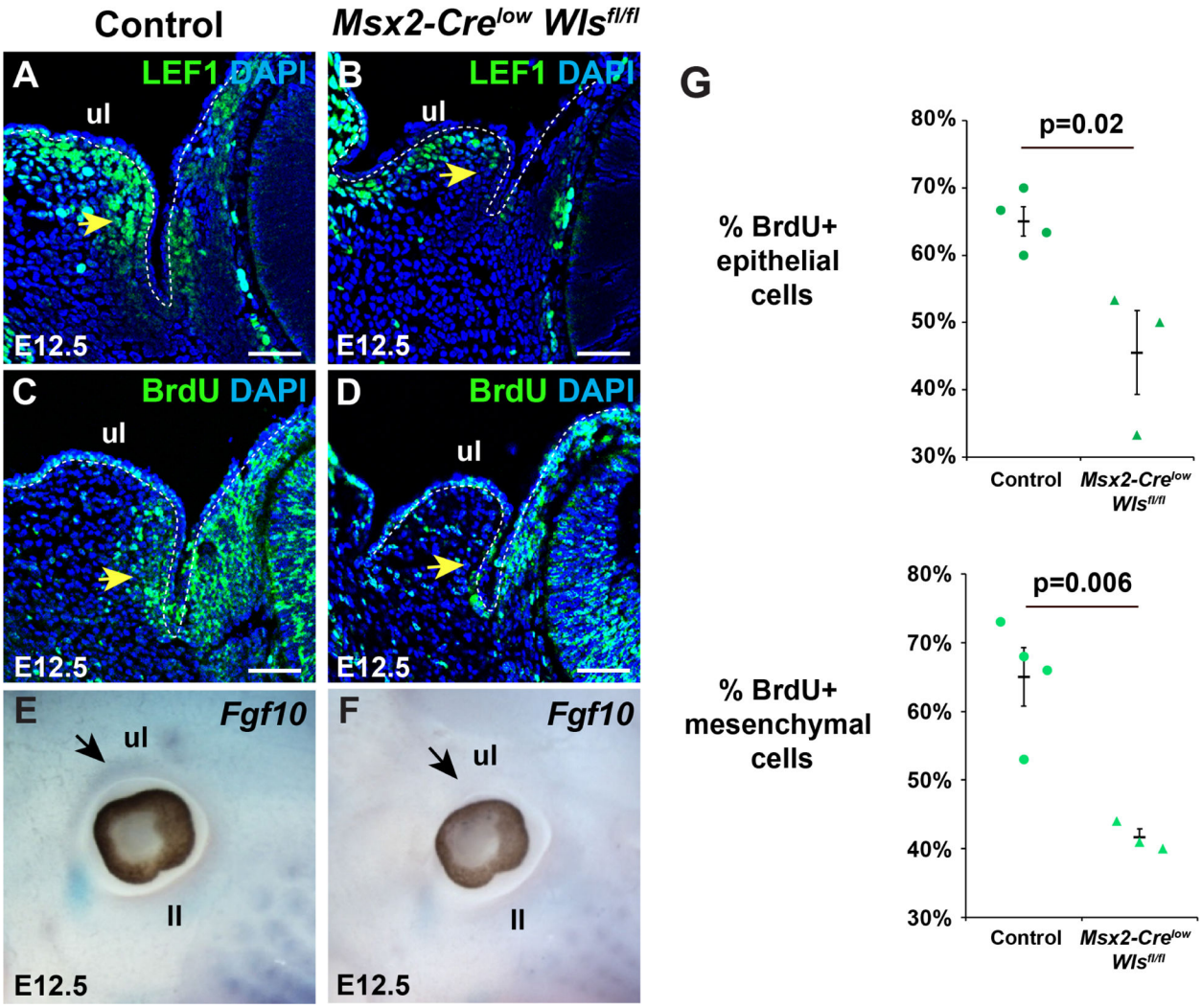

Figure 5. Expression of *Fgf10* in eyelid mesenchyme is dependent on secretion of epithelial Wnt ligands.

(A) IF staining shows that LEF1 is strongly expressed in the mesenchyme of developing upper eyelid primordia of control embryos at E12.5 (yellow arrow). (B) IF staining shows that LEF1 expression is significantly reduced in the supraorbital mesenchyme of the hypoplastic upper eyelid primordia of *Msx2-Cre*^{low} *Wls*$^{fl/fl}$ embryos at E12.5 (yellow arrow). (C) BrdU staining shows that cell proliferation is enriched in the epithelium and posterior mesenchyme of upper eyelid primordia in control embryos at E12.5 (yellow arrow). (D) BrdU staining shows that proliferation is reduced in the upper eyelid primordia of *Msx2-Cre*^{low} *Wls*$^{fl/fl}$ embryos at E12.5 (yellow arrow). (E) *Fgf10* is expressed in the developing upper eyelid primordia of control embryos at E12.5 (black arrow). (F) *Fgf10* expression is undetectable in the developing upper eyelid primordia of *Msx2-Cre*^{low} *Wls*$^{fl/fl}$ embryos at E12.5 (black arrow). (G) Statistical analysis of BrdU+ cells in supraorbital epithelium and underlying mesenchyme. Four control and three mutant embryos were analyzed. Unpaired student *t*-test was used to evaluate significance. $p<0.05$ was considered significant. Samples in (E) and (F) were photographed at the same magnification. Scale bars:

50μm. The white dashed line indicates the boundary between epithelium and mesenchyme. Abbreviations: ul, upper eyelid; ll, lower eyelid.

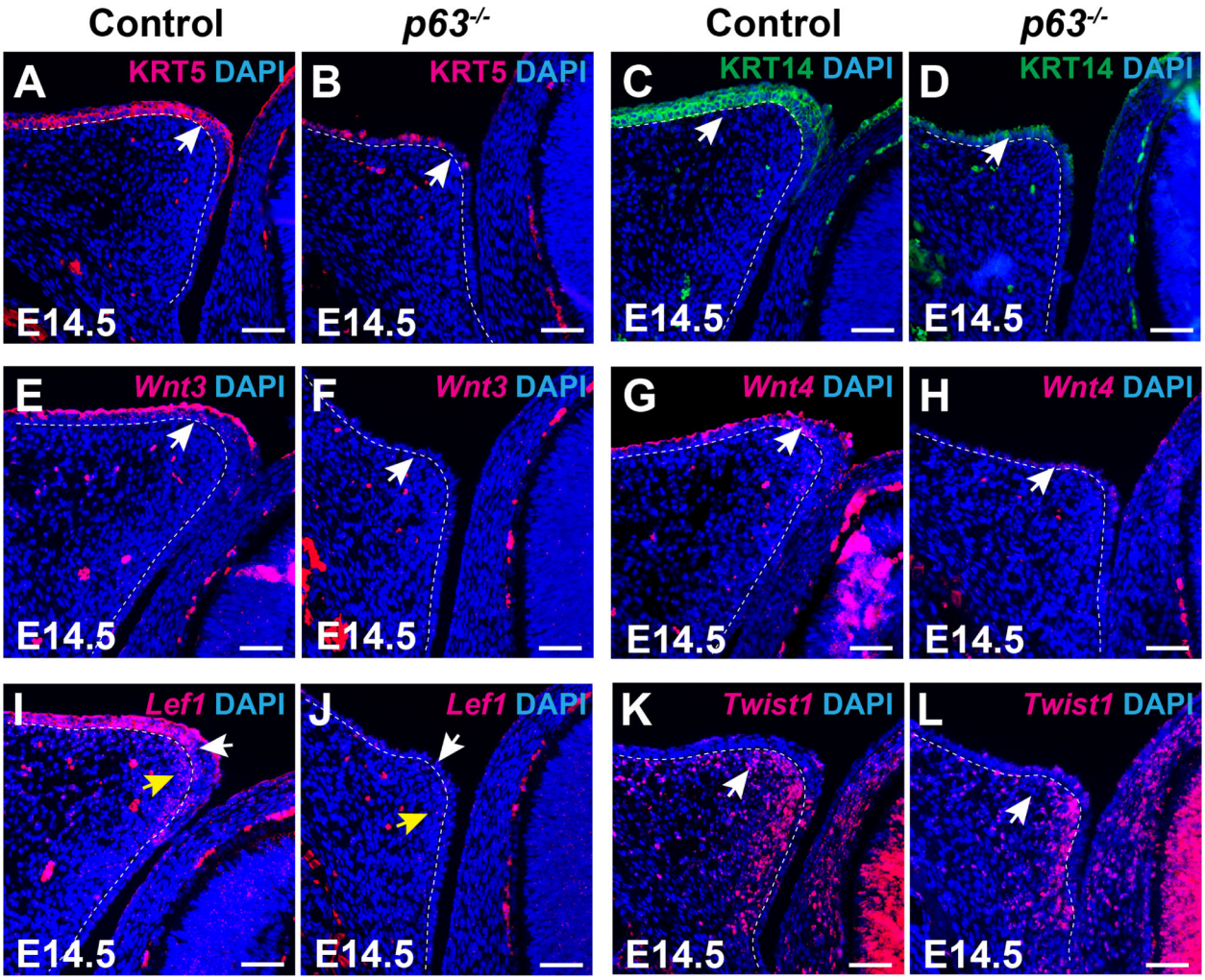

Figure 6. p63 regulates expression of Wnt genes in eyelid epithelium.

(A and C) KRT5 and KRT14 are expressed in the epithelium of the upper eyelid of control embryos at E14.5 (white arrows). (B and D) KRT5 and KRT14 expression is diminished in the epithelium of the upper eyelid of E14.5 *p63*$^{-/-}$ embryos (white arrows). (E and G) *Wnt3* and *Wnt4* are expressed in the epithelium of the upper eyelid of control embryos at E14.5 (white arrows). (F and H) Expression of *Wnt3* and *Wnt4* is significantly reduced in the epithelium of the upper eyelid of *p63*$^{-/-}$ embryos at E14.5 (white arrows). (I) *Lef1* is expressed in the epithelium (white arrow) and underlying mesenchyme (yellow arrow) of the upper eyelid of control embryos at E14.5. (J) Expression of *Lef1* is lost in both epithelium (white arrow) and underlying mesenchyme (yellow arrow) of the upper eyelid of E14.5 *p63*$^{-/-}$ embryos. (K) *Twist1* is expressed in the mesenchyme of the upper eyelid of control embryos at E14.5 (white arrow). (L) Expression of *Twist1* is reduced in the mesenchyme of the upper eyelid of E14.5 *p63*$^{-/-}$ embryos. Scale bars: 50μm. The white dashed line indicates the boundary between epithelium and mesenchyme.

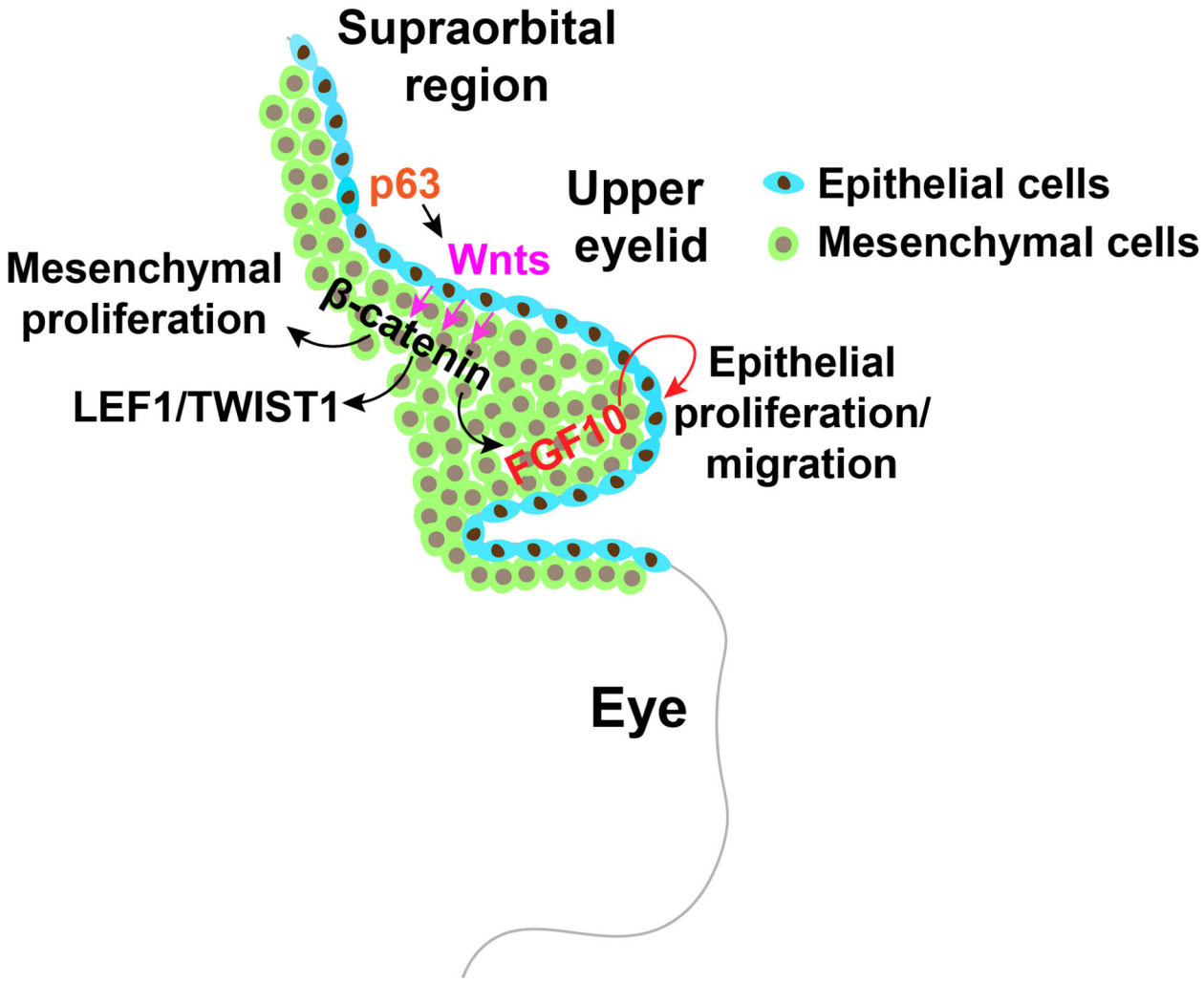

Figure 7. Schematic diagram of epithelial-mesenchymal interactions mediated by Wnt/β-catenin signaling in the developing eyelid.

Our data support a model in which p63 controls the expression of epithelial Wnt proteins, which in turn activate mesenchymal β-catenin-dependent Wnt signaling resulting in increased mesenchymal proliferation and activation of downstream target genes LEF1 and TWIST1. Epithelial Wnt ligands also control mesenchymal FGF10 expression, which is required for the proliferation and migration of epithelial cells in the developing upper eyelid [11].