REITERATIVE FGF SIGNALING DETERMINES THE IDENTITY AND MORPHOLOGY OF THE LACRIMAL GLAND

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The lacrimal gland plays an essential role in protection of the ocular surface by secreting the aqueous component of the tear film. Deficiency in the lacrimal gland is the main cause of dry eye disease, but existing treatments only alleviate the symptoms without curing the underlying disease. To develop curative measures, a thorough understanding of lacrimal gland development is needed. Lacrimal gland is formed as a result of interaction between the neural crest-derived mesenchyme and the conjunctival epithelium. The mesenchyme secretes the chemo-attractive signal of Fgf10, which binds to epithelial Fgfr2b and co-receptor heparan sulphate proteoglycans, to promote budding and branching morphogenesis of the lacrimal gland. However, the mechanism by which Fgf10 expression is regulated within the neural crest and the direct downstream targets of Fgf signaling in the epithelium are currently unknown. In this study, we show that FGF signaling mediated by protein phosphatase Shp2 is required for the proper patterning and differentiation of the neural crest-derived mesenchyme to produce Fgf10. Genetic evidence further demonstrates that Shp2 is recruited by Frs2α to activate Ras-MAPK signaling downstream to Fgfr1 and Fgfr2 but not to Pdgfrα in the neural crest. By differential gene expression analysis, we identified homeodomain transcription factor Alx4 as the key effector of Shp2 signaling to control expression of Fgf10 in the periocular mesenchyme. Loss of function *ALX4/Alx4* mutation disrupted lacrimal gland development in both human and mouse. Our results reveal a FGF-Shp2-Alx4-Fgf10 axis in regulating neural crests during lacrimal gland development. In addition, we also show that Fgf signaling cascade mediated by Pea3 family of transcription factors are critical for lacrimal gland duct elongation and branching. High-throughput gene expression analysis revealed that *Pea3* genes were important for establishing the tissue identity of the lacrimal gland. Loss of Pea3 resulted in upregulation of Notch signaling with the concomitant loss in the expression of the members of Six family of transcription factors and a switch of cell fate to the epidermal skin-like cells. These findings show that Fgf

signaling is used reiteratively to establish the identity of both the epithelium and mesenchyme of the lacrimal gland.

Weinian Shou, Ph.D., Chair

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List of Abbreviations

ALSG	Aplasia of lacrimal gland and salivary gland
Alx/ALX	Aristaless-like homeobox
BMP	Bone Morphogenetic Protein
Col2a1	Collagen, Type II, Alpha 1
DMEM	Dulbecco's Modified Eagle Medium
Dusp	Dual Specificity Phosphatase
E	Embryonic day
ERK	Extracellular Signal-Regulated Kinase
ECM	Extracellular matrix
En1	Engrailed Homeobox 1
ETS	E-26 transformation-specific
ETV	ETS variant
FBS	Fetal Bovine Serum
Fgf/FGF	Fibroblast growth factor
Fgfr/FGFR	Fibroblast growth factor receptor
GAGs	Glycosaminoglycans
GSEA	Geneset enrichment analysis
HS	Heparan Sulfate
HSSE	Heparan sulphate synthesizing enzymes
Hs2st	2-O-sulfotransferases
Hs6st	6-O-sulfotransferases
LADD	Lacrimo-auriculo-dento-digital
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix metalloproteinases
Ndst1/2	N-deacetylase/N-sulfotransferase
NES	Normalized enrichment score
NICD	Notch Intracellular domain
PBS	Phosphate buffer saline
PCA	Principal Component Analysis
Pdgfr	Platelet-derived growth factor receptor

PEA	Polyomavirus Enhancer Activator 3 Homolog
RIPA	Radio-Immunoprecipitation Assay
R26R	Rosa-26 reporter
Shp2	Src- homology protein 2
Six	Sine Oculis Homeobox Homolog
Sox	SRY-Related HMG-Box
Spry	Sprouty
Ugdh	UDP-Glucose 6-Dehydrogenase
α-SMA	α-smooth muscle actin

1 Introduction

1.1 Lacrimal gland development in human and mouse

1.1.1 Tear film and role of lacrimal gland

The lacrimal gland is a tubulo-acinar exocrine gland which produces the aqueous component of the tear, including water, electrolytes and proteins [1]. Critical for the ocular health and quality vision, the tear forms a smooth refractive film over the cornea, lubricating the cornea and conjunctiva, supporting the ocular surface metabolism, flushing away dirt and noxious stimuli. The tear film is composed of three layers- 1) the outermost lipid layer secreted by Meibomian glands prevents evaporation of tears, 2) the middle aqueous layer produced by the lacrimal gland and the accessory glands of Krause and Wolfring accounts for over 90% of the tear volume, and 3) the innermost mucous layer produced by the cornea and conjunctiva anchors the tear film to the ocular surface [2]. By releasing immunoglobulins into the tear, the lacrimal gland also functions as the secretary immune system of the eye to protect the ocular surface against infection arising from constant environmental exposure [3].

1.1.2 Structure of Lacrimal gland

In humans, the secretory component of the lacrimal system is the lacrimal gland located within the bony upper orbit of the eye, emptying its secretions into an anastomosed duct system that delivers the fluid to the ocular surface. The excretory part of the lacrimal system lies at the nasal side of the eye, draining the excessive fluid through the tear duct connected to the lacrimal sac and nasal passages (Fig. 1) [4]. In rodents, however, the lacrimal gland is comprised of two lobular structures: the intra-orbital and the ex-orbital. The primary lacrimal gland is the ex-orbital lobe, located just beneath the ear and connected to the eye via a long duct that joins the intra-orbital lobe just prior to reaching the eye (Fig. 2) [5]. The lacrimal gland epithelium is composed of three major cell types: acinar, duct and myoepithelial cells. The primary secretory apparatus are acinar cells which make up to 80% of the gland. The luminal side of the acinar cells are connected to the ducts lined by cuboidal duct cells, which constitute 10-12% of the lacrimal gland cell population and contribute to 30% of the lacrimal gland fluid secretions [6]. Myoepithelial cells are the third major component of the lacrimal gland, surrounding the basal side of

both acinar and ductal cells. Their function is to squeeze the secretory cells to expel the fluid into the duct [5]. Besides these three main cell types, the lacrimal gland stroma also contains fibroblast cells which produce collagens and mast cells secreting histamines and other matrix proteins in the interstitial spaces [4]. The vasculature of the lacrimal gland also brings in plasma cells, lymphocytes, dendritic cells and macrophages, which provide immune protection to the ocular surface [5, 7]. The function of the lacrimal system is controlled by sensory afferent nerves from the cornea and conjunctiva and motor efferent parasympathetic and sympathetic nerves innervating the lacrimal gland. Together, they ensures the optimum volume and quality of tear secretion in response to environmental stress [5].

1.1.3 Dry eye disease

Dry eye disease affects more than 3.2 million middle-aged or older women and 1 million males aged 50 or more within the United States and the prevalence is increasing rapidly [8, 9]. Dry eye occurs when the quality and amount of tears is not sufficient to maintain the ocular surface homeostasis and the risks of this incidence occurring increase with age. There are two forms of dry eye- 1) Evaporative: caused by deficiency in the lipid layer which is often due to the blockage of the Meibomian gland located in the eyelid. Poor quality or insufficient oil layer can result in faster vaporization of the tears 2) Aqueous-deficient: occurs due to deficiency in the lacrimal gland functional unit responsible for secreting the aqueous layer, the major component of the tear film. These clinical manifestations of these two etiologies could occur separately or in combination.

1.1.3.1 Current and proposed treatment strategies

Common forms of treatment include over-the-counter topical medications such as artificial tears, gels and ointments, prescription-based anti-inflammatory drugs such as cyclosporine and lifitegrast, recommendations for changing lifestyle and environment, devices which help stimulate tear production as well as surgical intervention to plug the tear drainage system. However, these treatments address the symptoms but do not tackle the underlying glandular deficiencies and hence, do not provide a permanent cure. To improve the treatment strategy for dry eye, several lines of research have been taking

place with the overarching goal of better understanding of the underlying cause of the disease which will help develop better treatments. One line of research is to study the mechanisms of development and function of Meibomian gland with the goal of understanding its anatomy and physiology, lipid composition of tears and ways by which lipid production can be stimulated and stabilized. Since the aqueous layer is the principal component of the tears, lack of which causes dryness in the eye, many laboratories are searching the ways to treat the lacrimal gland-associated deficiencies. In this direction, one area of research is development of topical nerve stimulators in cases of deficiency in the corneal nerves and their excitatory capacity and another line of focus is to understand the lacrimal gland development and ways by which it can be rebuilt via patient's own stem cells. Scientists in the latter field have proposed two strategies, one is to promote intrinsic regeneration by identifying the factors which can stimulate this process or develop artificial lacrimal gland implants. There have been significant advancements in this area which have pushed forward the task of rebuilding lacrimal gland and brought it closer to reality, however, clinical transition still remains elusive.

1.1.3.2 Why study lacrimal gland development?

Impairment of the lacrimal gland can result in the debilitating condition known as the aqueous-deficient dry eye disease, which may progress to corneal abrasion and vision loss. The most significant risk factor for the dry eye disease is aging, causing structural and functional changes in the lacrimal gland characterized by atrophied acini, duct obstruction, increasing lymphocytic infiltration and decrease in stimulated protein secretion [10]. Lacrimal gland dysfunction can also arise from inflammation triggered by the dry environment, auto-immune attack on exocrine glands as in Sjögren's syndrome and rheumatoid arthritis, side effects of chemo and radiation therapies and congenital defects [11-13]. However, all the current clinical interventions are palliative, not curing the underlying lacrimal gland deficiency. To this end, regeneration of the damaged lacrimal gland or replacement by bioengineered implants can potentially provide permanent cures for the dry eye disease. This would require a thorough understanding of the molecular mechanism of lacrimal gland development and regeneration.

1.1.4 The molecular mechanism of lacrimal gland development

1.1.4.1 Signaling interactions: the epithelium - mesenchyme interaction

Lacrimal gland forms as a result of interactions between the conjunctival epithelium and the periocular mesenchyme. In human, it begins as a thickening of the epithelium at the superior conjunctival fornix, which subsequently invades the underlying mesenchyme to form a highly branched gland [14]. This is recapitulated in mouse as the budding of the conjunctival epithelium at the temporal side of the eye at the E13.5 stage (Fig. 2) [15]. This tubular bud elongates posteriorly toward the ear, accompanied by condensation of the surrounding mesenchyme [16]. This process apparently can occur independently of retina and lens development, as the lacrimal gland bud develops even in mouse mutants lacking the eyeball [17]. Starting at E15.5, the lacrimal gland bud branches out to form a complex intra-orbital and ex-orbital multi-lobular structure, eventually composed of a system of acini, ducts, myoepithelial cells, nerves, plasma cells and connective tissue.

1.1.4.2 Role of FGF10 signaling

The inductive signals to initiate lacrimal gland budding and branching morphogenesis are Fibroblast growth factors (FGFs). *Fgf10* is expressed in a distinctive domain in the mesenchyme surrounding the epithelial bud and its expression persists throughout lacrimal gland development. By contrast, *Fgf7* expression in the mesenchyme is more diffusive [15, 18].

Both recombinant human FGF10 and FGF7 were able to induce ectopic budding of the lacrimal gland epithelium in explant culture of embryonic eye [15]. Remarkably, ectopic glands can even be induced in the cornea by transgenic expression of rat *Fgf10* or human *FGF7* in the lens, but not by other FGFs, underlying the potency and specificity of the FGF7 subfamily of FGFs for ocular gland development [18, 19]. *Fgf10* null mice exhibit a complete loss of the epithelial component of the lacrimal gland despite an intact mesenchyme, while *Fgf7* knockout mice have normal lacrimal glands, indicating that *Fgf10* is the primary driver of lacrimal gland development [15, 18, 19].

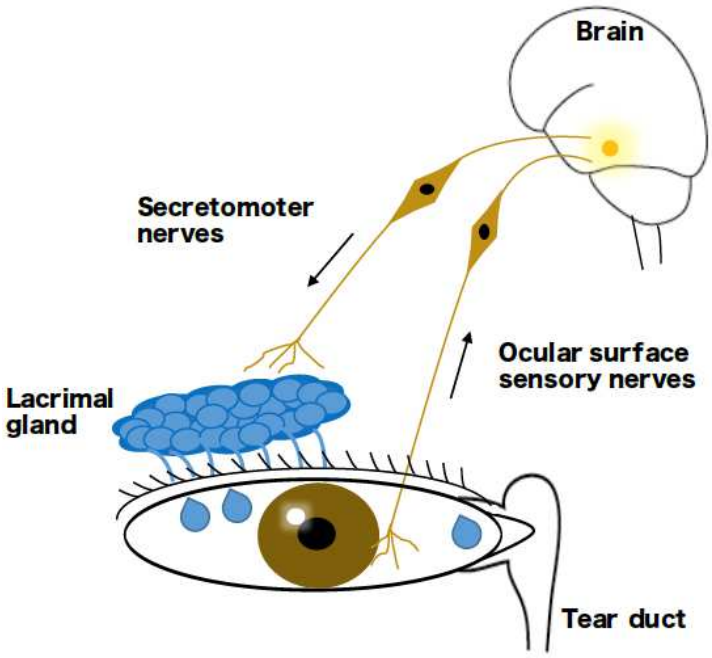


Figure 1. Schematic of Lacrimal gland functional unit. The lacrimal gland functional unit is comprised of (a) the lacrimal gland, (b) Sensory afferent nerves from the cornea and conjunctiva, (c) motor efferent nerves originating from the central nervous system which innervate lacrimal gland, (d) the excretory tear duct for drainage of the excess fluid. Impairment in any components of lacrimal gland function unit can destabilize the tear film and cause the dry eye disease.

In fact, *Fgf10* is haploinsufficient for the lacrimal gland development in mouse, whereas heterozygous *FGF10* mutations in humans can lead to aplasia of lacrimal gland and salivary gland (ALSG), a rare disorder characterized by dryness in the eye and mouth (OMIM #180920) [20]. A more severe congenital disorder called Lacrimo-auriculo-dento-digital (LADD) syndrome affecting lacrimal and salivary glands, ears, teeth and distal limbs has been associated with missense mutations in *FGF10* (OMIM #149730) [21]. The majority of *FGF10* mutations in LADD patients disrupt protein stability or receptor interaction, but missense mutation affecting secretion and nuclear localization of FGF10 has also been identified [22, 23]. The LADD mutations are thought to have dominant-negative instead of simple loss-of-function effect, which may explain why more organs are affected in LADD syndrome than ALSG syndrome [21]. These observations highlight the pivotal role of FGF10 /Fgf10 in multi-organ development, but also raise the interesting question why lacrimal gland development is particularly sensitive to the *FGF10/Fgf10* dosage.

In addition to the precise control of *Fgf10* at the gene dosage level, the concentration of Fgf10 protein in the periocular mesenchyme is also under exquisite regulation by proteoglycans within the extracellular matrix (ECM) (Fig. 3) [24]. Previous work from our lab have shown that glycosaminoglycans (GAGs) attached to proteoglycans in the periocular mesenchyme restricts diffusion of Fgf10 during lacrimal gland development [25]. Mesenchyme-specific knockouts of proteoglycan biosynthetic enzymes Ugdh (UDP-Glucose 6-Dehydrogenase) cause excessive diffusion of Fgf10 and disrupt lacrimal gland budding. Interestingly, the lacrimal gland defect can be reproduced by mesenchymal deletion of heparan sulfate (proteoglycan) modification enzymes Ndst1/2 (*N*-deacetylase/*N*-sulfotransferase), but not by 2-*O*-sulfotransferases (Hs2st) and 6-*O*-sulfotransferases (Hs6st1/2), suggesting that *N*-sulfation of heparan sulfates is essential for regulating Fgf10 dissemination [25].

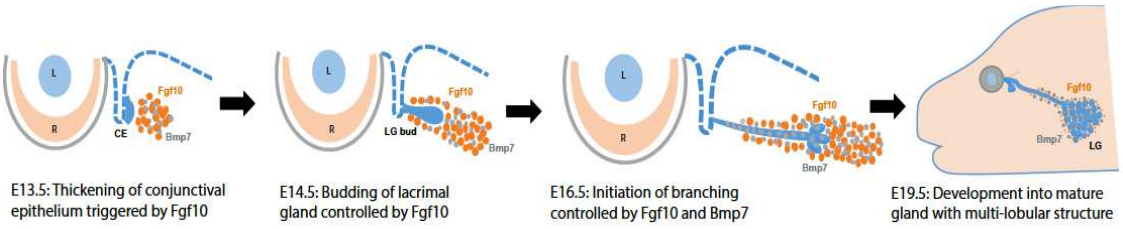


Figure 2. Lacrimal gland development in mouse. Transverse sections of mouse embryos at different stages are shown. Lacrimal gland development begins with thickening of the CE at E13.5 induced by Fgf10 from the surrounding mesenchyme. These epithelial cells further grow and elongate into a bud from E14.5 through E15.5. Branching of LG initiates at E16.5 under the additional influence of BMP7 signaling, eventually forming a multi-lobular tubulo-acinar structure at E19.5. Lacrimal gland continues to develop even during postnatal stages to become a mature gland capable of regulated tear secretion in adults. L, lens; R, Retina; CE, conjunctival epithelium; LG, lacrimal gland.

Consistent with this, mutating the key residues of FGF10 that interact with heparan sulfates also resulted in increased diffusion range of FGF10 in the ECM [26]. The resulting mutant FGF10 was found to behave like FGF7, which has lower affinity to heparan sulfates and preferentially promotes lacrimal gland branching instead of elongation. On the other hand, FGF signaling has been shown to cooperate with transcription factor Barx2 in the lacrimal gland epithelium to regulate the expression of ECM remodeling enzymes matrix metalloproteinases (MMP2 and MMP9), which are secreted into the mesenchyme to promote the release of Fgf10 from proteoglycans [27]. This presents a positive feedback mechanism to modulate the Fgf10 concentration ahead of the invading epithelial bud. One question that remains unresolved is whether the control of Fgf10 diffusion by proteoglycans generate a chemoattractive gradient to guide lacrimal gland development. This is partly due to the lack of a sensitive assay to determine the endogenous concentration of Fgf10 in the periocular mesenchyme. In this regard, it is worth noting that although endogenous *Fgf10* is also expressed in a localized fashion in the embryonic lung, a recent study showed that it can be functionally substituted by ubiquitous expression of *Fgf10* during branching morphogenesis [28]. This raises the possibility that Fgf10 gradient may even be dispensable for budding and branching of glandular organs.

The cognate receptor for Fgf10 is Fgf receptor 2(III)b (Fgfr2b) expressed in the lacrimal gland epithelium. Both epithelial ablation of *Fgfr2* in vivo and knock down of *Fgfr2b* ex vivo disrupts lacrimal gland development [15, 29]. Consistent with this, *FGFR2* mutations have been identified in LADD patients [21]. Nevertheless, a heterozygous mutation in the kinase domain of FGFR3 has also been reported in a LADD family. Because these affected patients do not exhibit congenital abnormalities typically associated with syndromes caused by activating *FGFR3* mutations, it was assumed that this LADD mutation is a loss-of-function allele. However, Fgfr3 is known to have a very low affinity to Fgf10 and *Fgfr3* knockout mouse has normal lacrimal gland (AG and XZ, unpublished results). Therefore, functional study is needed to resolve the nature of this LADD-associated *FGFR3* mutations. It is also interesting to note that heterozygous ablation of Fgfr2c, which is not the cognate receptor for Fgf10, results in secondary

branching defects in the lung, kidney and lacrimal gland [30]. Since *Fgfr2* heterozygous null mouse exhibits no overt phenotype, it is believed that *Fgfr2c* deletion creates a gain-of-function allele as a result of a splicing switch in *Fgfr2* locus, leading to ectopic expression and activation of Fgfr2b in the mesenchyme. *Fgfr2c* mutant lacrimal gland retains a mesenchymal sac without *Fgf10* expression. It remains to be determined how aberrant Fgfr2b signaling can be activated without Fgf10 in the mesenchyme.

The assembly of FGF signaling complex on the cell surface also requires heparan sulfates as co-receptors (Fig. 3). Interestingly, the lacrimal gland bud specifically expresses Ndst1 enzyme in the tip cells, but not in the follower cells that form the stalk of the lacrimal gland bud[29]. Epithelial ablation of Ndst1 not only disrupted *N*-sulfation of heparan sulfates, but also abrogated lacrimal gland bud formation. Similarly, both 2-*O* and 6-*O* sulfation of heparan sulfates contribute to Fgf10-induced signaling process since the deletion of *Hs6st* and *Hs2st* in the lacrimal gland epithelium resulted in the stunted growth or no bud formation [31]. Indeed, using a FGF ligand and carbohydrate engagement assay (LACE), we showed that recombinant Fgf10/Fgfr2b proteins were able to form a tight binding complex on the lacrimal gland bud in situ, which was disrupted in heparan sulfate *N*- or *O*-sulfation mutants [29, 31]. On the other hand, these modifications of heparan sulfates are also under the control of FGF signaling, as epithelial ablation of Fgfr2 abolished *N*-sulfation of heparan sulfates [29]. This positive feedback mechanism is mediated by Shp2, a non-receptor tyrosine phosphatase that transmits FGF signaling to Ras-MAPK pathway, partly by suppressing the negative Ras signaling regulator Sprouty2 [32]. The key targets of this signaling cascade are likely transcription factors Sox9 and Sox10, which have been shown to regulate the expression of heparan sulfate 3-O-sulfotransferases in an FGF-signaling-dependent manner [33].

1.1.4.3 Role of BMP signaling

While Fgf10 is expressed exclusively in the lacrimal gland mesenchyme to guide budding and branching of the epithelium, another growth factor Bmp7 displays a more complex and dynamic expression pattern during lacrimal gland development. Initially expressed in the periocular mesenchyme surrounding the epithelial bud, Bmp7 is later present in both

the epithelial and mesenchymal compartments of the lacrimal gland [16]. The direct target of Bmp7 signaling, however, appears primarily to be the lacrimal gland mesenchyme. Exposing isolated lacrimal gland epithelium to Bmp7 did not affect budding induced by Fgf10, but in the mesenchymal culture, Bmp7 resulted in increasing cellular proliferation and aggregation marked by expressions of connexin43, cadherins and α-smooth muscle actin (α-SMA) [16]. Defective condensation of the periocular mesenchyme was found in *Bmp7* null mice, which also exhibited smaller glands with misplaced buds and reduced branching. It is thought that the condensation and proliferation of mesenchymal cells induced by BMP signaling is critical for proper branching morphogenesis of the lacrimal gland epithelium. In support of this model, it was shown that although transcription factor Foxc1 was dispensable in the lacrimal gland epithelium, its loss in the mesenchyme prevented BMP signaling from inducing cellular condensation [34]. As a result, *Foxc1* null mice exhibited reduced lacrimal gland size with fewer terminal buds, reminiscent of *Bmp7* null phenotype. It should be noted that, in contrast to Bmp7, Bmp4 is found to suppress Fgf10-induced growth and elongation of the lacrimal gland bud in isolated epithelial culture, suggesting that BMP signaling may also play a direct role in the lacrimal gland epithelium [16]. BMP signaling is mediated by phosphorylated Smad1/5/8 proteins, which form a complex with Smad4 to activate the downstream transcriptional events. Indeed, a recent study showed that epithelial deletion of Smad4 resulted in smaller lacrimal glands with fewer branches and acini [35]. Interestingly, *Smad4* mutant lacrimal gland accumulated pigments after birth and was eventually replaced by adipose tissue. These studies suggest that BMP signaling in both the epithelium and the mesenchyme is critical for lacrimal gland development.

1.1.4.4 Role of Wnt signaling

Canonical Wnt signaling has been shown to interact with both FGF and BMP signaling to modulate lacrimal gland branching morphogenesis. Activation of the canonical Wnt signaling pathway prevents degradation of β-catenin in the cytoplasm, which is translocated into the nucleus to bind Tcf/Lef transcription factors to induce gene expression. Transcripts of several Wnts (both canonical and non-canonical) are present in the lacrimal gland during development [36]. Inhibition of Wnt signaling by knocking

down β-catenin using morpholinos in lacrimal gland explants led to increasing branching and cell proliferation and an up-regulation of Fgf10 in the mesenchyme. Activating Wnt signaling by Wnt3a or LiCl treatment, on the other hand, reduced proliferation of both the epithelial and mesenchymal components of lacrimal gland with concurrent reduction in the number of branches. Wnt signaling also suppressed Bmp7-induced increase of cell proliferation in the lacrimal gland mesenchyme. Thus, Wnt signaling regulates branching morphogenesis by counterbalancing the effect of Fgf10 and Bmp7 [36].

1.1.4.5 Role of Notch signaling

Maturation and homeostasis of the lacrimal gland also require Notch signaling, which is transmitted by nuclear translocation of the Notch Intracellular domain (NICD) that subsequently interacts with recombination signal binding protein for immunoglobin J_k region (RBP-J_k), Histone acyl transferases and Mastermind-like transcriptional co-activator (Maml) to activate the transcription of target genes. After postnatal knockout of *Notch1* in the ocular surface, lacrimal gland degenerates with infiltrated monocytic cells, resulting in marked reduction in tear volume [37]. It has also been shown that Maml-mediated Notch signaling is responsible for maintaining the conjunctival epithelial identity and goblet cell differentiation. This is achieved by augmenting the expression of Klf4/5 transcription factors which control Muc5a expression. Indeed, lacrimal gland in mice with epithelial deletion of *Klf5* exhibited excessive inflammation and disorganization of the acini [38]. More recently, Notch signaling has also been proposed to regulate branching morphogenesis by suppressing cleft-formation [39]. These studies suggest that Notch signaling contributes to both development and function of the lacrimal gland.

In summary, after the critical role of Fgf10 in lacrimal gland development was discovered less than two decades ago, it is now appreciated that FGF signaling must interact with other pathways, including BMP, Wnt and Notch signaling, to regulate lacrimal gland budding and branching morphogenesis. The recent high throughput gene expression analysis has further implicated IGF, TGFβ and Hippo signaling in human

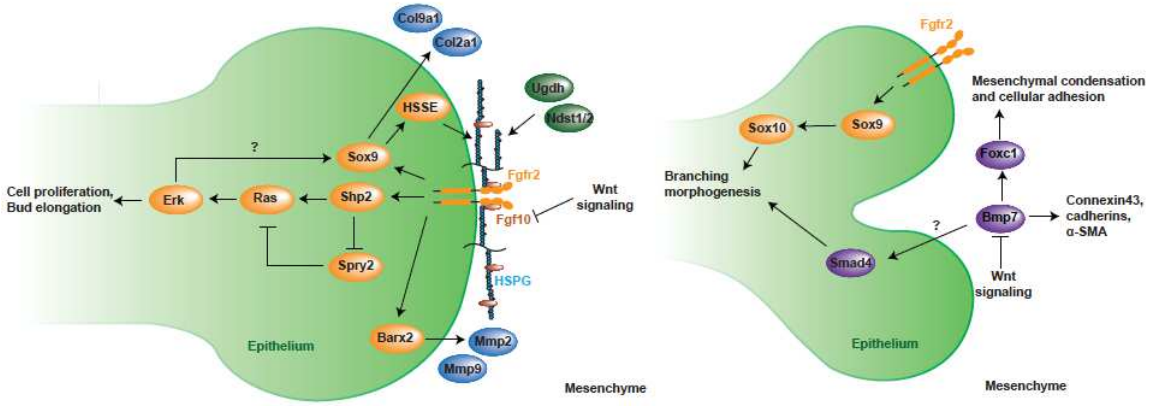


Figure 3. Summary of signaling interactions during lacrimal gland morphogenesis. (Left) Fgf10 forms a heparan sulfates (HS)-dependent gradient in the periocular mesenchyme, inducing lacrimal gland budding by binding to both Fgfr2b and HS in the epithelium. This activates Shp2, which inhibits the Ras signaling repressor Spry2 and promotes Ras-Erk cascade to stimulate cell proliferation, survival and bud elongation. With transcription factors Sox9 and Barx2, FGF signaling also stimulates expressions of HS synthesizing enzymes (HSSE) and metalloproteinases to remodel the ECM, forming a positive feedback loop to enhance FGF signaling activity. (Right) Bmp7 signaling mediated by Foxc1 is important for mesenchymal condensation during branching morphogenesis. Both FGF and BMP signaling are counterbalanced by canonical Wnt signaling in the mesenchyme. In addition, Smad4-mediated BMP signaling and Sox9-Sox10 cascade also directly regulates the epithelial elongation.

lacrimal gland, presenting a fertile research field for investigating additional pathways in lacrimal gland development [40]. On the other hand, our understanding of these known signaling in lacrimal gland remains rudimentary, lacking essential molecular details. For example, the lacrimal gland epithelum requires the inductive signal from the periocular mesenchyme, but how the mesenchyme itself is specified and whether there is reciprocal signals from the epithelium to the mesenchyme is not clear. There are also many unanswered questions regarding the mechanism of FGF signaling itself. For example, why Fgf10 is particularly potent in inducing ectopic ocular gland, while other Fgfs, such as Fgf1, lack such activity [18, 19, 41]. This can not be entirely explained by the specificity of Fgf10 to interact with Fgfr2b, because Fgf1 is also capable of activating the same receptor. Downstream to Fgf receptor, there are multiple intracelullar pathways, including Ras-MAPK, PI3K-AKT and PLC-PKC signaling. The specific roles of these pathways and their downstream targets are also important questions for future investigation.

1.1.4.6 Transcriptional network in lacrimal gland development

While signaling pathways provide the overall guidance of lacrimal gland morphogenesis, transcription factors are the ultimate interpreters and executors of the developmental program. The paired-domain transcription factor Pax6 is considered the master regulator of eye development [42]. Its expression precedes the budding of the lacrimal gland in the fornix of the conjunctival epithelium and continues in the lacrimal gland epithelium throughout development. Loss of function mutation in even a single allele of *Pax6* results in severe impairment in mouse lacrimal gland development, suggesting that Pax6 serves as a competence factor in the epithelium [15]. In fact, detailed characterizations of *Pax6* enhancers have led to the development of the *Le-Cre* transgene, which can act as both a Cre deletor and reporter in the lacrimal gland epithelium, greatly facilitating the genetic analysis of lacrimal gland development [29, 43]. Surprisingly, lacrimal gland defects have not been reported in human aniridia (OMIM 106210), a congenital disorder caused by heterozygous mutation in *PAX6*. Instead, patients with otofaciocervical syndrome-2 carrying homozygous mutation in *PAX1* (OMIM 615560) display lacrimal duct abnormalities, a phenotype shared with the closely related otofaciocervical syndrome-1

(OMIM 601653) that harbors mutations in *EYA1* gene [44]. On the other hand, branchiootorenal syndrome-1 (BOR1, OMIM #113650) caused by heterozygous *EYA1* mutation and branchiootic syndrome-3 (OMIM #608389) cause by *SIX1* heterodeficiency display many overlapping phenotype including lacrimal gland stenosis, suggesting that these two genes also act in the same genetic cascade. In mouse, Six1 is widely expressed in the head mesenchyme during early development and it is also found in the duct and acini of mouse lacrimal gland [45]. *Six1* knockout embryos exhibit small lacrimal glands with poor duct elongation and reduced branching, confirming the functional importance of *Six1* in lacrimal gland development. From *Drosophila* to mammals, *Pax*, *Six* and *Eya* genes have been shown to form a conserved transcriptional network in organogenesis. It would be interesting to investigate whether these interactions still occur in lacrimal gland development.

Whereas Pax/Six/Eya genes serve as competence factor for lacrimal gland development, additional transcription factors are required to specify the identity of the epithelium. TP63 is a transcription factor important for a variety of epithelial structures [46]. Mutations in this gene abolish lacrimal gland in mice and cause Limb-mammary syndrome (OMIM 603543) in humans, which presents lacrimal-duct atresia and obstructed lacrimal puncta [47]. Otx1 is a homeodomain transcription factor expressed in the conjunctival epithelium and *Otx1*$^{-/-}$ mice also fail to develop lacrimal gland [48]. In addition, loss of the epithelial gene, *Runx1,* resulted in a delay in embryonic lacrimal gland development as shown by reduced branching and smaller bud at E16.5. It is likely that *Runx1* is compensated by *Runx2* and *Runx3*, which are also expressed during lacrimal gland development [49].

There are transcription factors that may regulate development of both epithelial and mesenchymal compartments of lacrimal gland. The majority of patients carrying heterozygous mutations in *SOX10* (Waardenburg syndrome, OMIM 611584 and 613266) have hypoplastic or no lacrimal gland, underscoring the requirement of *SOX10* for lacrimal gland genesis [50]. This finding corroborates with lacrimal gland defects in mice with conditional deletion of *Sox10* in the epithelium [33]. However, Sox10 is also

expressed by migratory neural crest cells, which eventually form the periocular mesenchyme. It is possible that human systemic *SOX10* mutation may also indirectly affect lacrimal gland induction by disrupting neural crest migration and differentiation. Similarly, *TFAP2a* mutations carried by Branchiooculofacial syndrome patients cause lacrimal duct obstruction phenotype (OMIM #113620). Because Ap2a is expressed in both neural crest and the surface ectoderm, we predict that TFAP2a function may be required in both the mesenchyme and epithelium of the lacrimal gland. These findings have been summarized in Table 1.

1.2 Fgf signaling pathway

1.2.1 Fibroblast growth factors and Fibroblast growth factor receptors

Fibroblast growth factor family consists of 22 growth factors, dividing into seven subfamilies. Among them, six subfamilies are categorized as secreted Fgfs and one subfamily is intracellular Fgfs. Of these secreted Fgfs, five subfamilies (Fgf1, Fgf4, Fgf7, Fgf9, Fgf8) bind to their cognate Fgf receptors, with heparin/heparan sulphates in the extracellular matrix as co-receptors to limit the diffusion of Fgfs, and hence regulate the signal transduction. Another subfamily (Fgf15/19/21) comprises of endocrine Fgfs which have reduced binding affinity for heparan sulphates and they have been evolved to bind to cognate receptors with α/ß-Klotho, KLPH as co-receptors [51]. On the other hand, Fgf11 subfamily comprises of intracellular Fgfs (iFgfs) which are non-signaling proteins and they have been known to interact with intracellular proteins including voltage gated Na-channels and tubulins [52, 53].

Fgf receptors are ~800 aa proteins containing three extracellular immunoglobulin domains (I, II, III), one transmembrane domain and two intracellular tyrosine kinase domains. There are four such types of Fibroblast growth factor receptors. FGFR1, FGFR2, FGFR3, FGFR4. FGFR1-3 each have two splice variants via alternative splicing from the immunoglobulin domain III (IIIa, IIIb). A fifth FGF receptor, FGFRL1, have been discovered, which lacks the intracellular tyrosine kinase domain and hence, acts as a decoy receptor or modulator of Fgf signaling. These receptors have varying affinities for

Table 1: Transcription factors implicated during lacrimal gland development and maintenance.

	Functional relevance during Lacrimal gland development	
Genes	**Mice**	**Phenotype in humans**
Pax6	Budding, competence factor	Not reported
Pax1	Not studied	Lacrimal duct stenosis
Eya1	Not studied	Lacrimal gland aplasia, duct stenosis
Six1	Branching morphogenesis	Lacrimal duct stenosis
Otx1	Budding	Not reported
p63	Budding	Lacrimal-duct atresia, obstructed lacrimal puncta
Runx1-3	Branching morphogenesis	Not reported
Klf5	Preservation of glandular function during post-natal stages	Not reported
Sox10	Growth and branching morphogenesis	Hypo-plastic or no lacrimal gland
Ap2a	Not studied	Lacrimal duct obstruction

Fgfs for conferring specificity and regulation of Fgf signaling in different tissue types [51].

1.2.2 Downstream signaling molecules and transcription factors

Upon binding of Fgf ligand to Fgfrs, the receptors dimerized to trigger trans-autophosphorylation of the kinase domain. Once Fgf signaling is activated, it has been shown to induce Ras/MAPK pathway involved in cellular proliferation and differentiation; PI3K/AKT pathway in cell survival and cell fate determination; PLCγ associated with cell morphology, migration and adhesion and signal transducer and activator of transcription (STAT) pathway implicated in different processes depending upon the cell context [54].

For Ras-MAPK activation, relay of downstream signaling proteins is summarized in Fig. 4. Fgf signaling is mediated by phosphorylation of FGFR substrate 2α (Frs2α). Frs2α is an adaptor protein which is normally anchored to the plasma membrane through myristoylation and is constitutively docked to the highly conserved FGFR binding site close to the membrane [55, 56]. Phosphorylated Frs2α recruits another adaptor protein Growth factor receptor bound-2 (Grb2) and tyrosine phosphatase, Src- homology protein 2 (Shp2) [57].

The exact mechanism of how Shp2 regulates Fgf signaling is still debated in the field. In addition to its SH-2 domains, the catalytic activity of tyrosine phosphatase is found to be critical for Shp2 to mediate Fgf signaling, but the direct substrates of Shp2 are not clear. Several mechanisms have been proposed by which Shp2 can function, such as dephosphorylating RasGap and preventing its recruitment to receptor kinases, regulating Src-family kinases, or modulating the inhibitory function of Sproutys by dephosphorylating these proteins [58]. It is likely that Shp2 acts through one or more of these mechanisms in a context-dependent manner.

Grb2 recruits Ras-GTPase to the plasma membrane through guanine nucleotide exchange factors (GEF), son of sevenless (SOS). Ras-GTPase activates Raf-kinases

phosphorylating mitogen activated protein kinase kinase (MAPKK/MEK) which phosphorylates mitogen activated protein kinase (MAPK/ERK). Activated Erk translocates to the nucleus to phosphorylate E26 transformation-specific (ETS) factors [59]. More specifically, Pea3 subfamily of transcription factors, Etv1/Er81, Etv4/Pea3 and Etv5/Erm are often considered as downstream effectors of Fgf signaling [60, {Firnberg, 2002 #108, 61, 62].

Fgf signaling is regulated by inhibitory signaling molecules such as Sprouty, Dusps, Sef and CBL as negative feedbacks. Sprouty proteins bind to Grb2 and inhibit the recruitment of Grb2-Sos complex required for Ras-MAPK activation [63]. Dusp6 (Dual specificity phosphatase 6) directly dephosphorylates MEK when MEK is bound to its substrate Erk2 [64]. Sef (similar expression to Fgfs) which belongs to the fibroblast growth factor syn-expression group was found to inhibit Ras-MAPK pathway during zebrafish embryogenesis and in mammalian cultures [65, 66]. Later, it was demonstrated that Sef acted by preventing the dissociation of ERK from MEK [67] or preventing phosphorylation of FGFR [68]. CBL, an E3 ubiquitin ligase, forms a complex with Frs2α and Grb2 resulting in the ubiquitination and degradation of Fgfr and Frs2α, thus inhibiting Ras-MAPK pathway [69].

1.2.3 Pea3 family of transcription factors

Fgf signaling cascade stimulates the phosphorylation of Erk, resulting in its translocation to the nucleus to induce the expression of Pea3 family of transcription factors. Similar to Fgf signaling, these factors have been shown to be active at the sites of the epithelial to mesenchymal interaction during organogenesis, with Pea3 and Erm specifically exhibiting overlapping expression pattern in various tissues [70]. Interestingly, these *Pea3* genes also act as oncogenes as they have been implicated in many cancers mimicking oncogenic RAS-MAPK pathways in melanoma, breast, lung and prostate cancer [71]. Conditional inactivation of Pea3/Erm in the lung epithelium caused increased mesenchymal Fgf10 expression and decrease in epithelial Shh expression, resulting in a smaller lung size and branching defects, however, mice were grossly healthy and exhibited normal life-span [72, 73]. Similarly, in the limb buds, Pea3/Erm

can mediate Fgf signaling in the proximal-distal (P-D) limb patterning as well as promoting and inhibiting Shh expression in the posterior and anterior limb bud respectively, as evident by growth defect along the P-D axis and mild preaxial polydactyly along the A-P axis with the mesenchymal loss of Pea3/Erm [74, 75]. In contrast to mild developmental defects in the lung, limb and mammary gland, Pea3/Erm are found to be downstream targets of Ret signaling, and they are absolutely necessary for kidney development, lack of which caused renal agenesis and hypoplasia [76]. Notably, among Pea3 factors, Er81 doesn't add to the severity of the phenotype and cannot compensate for the loss of Pea3/Erm, highlighting its redundancy. These data show that Fgf signaling induces the transcription of Pea3 members and the extent of their functional relevance varies between the tissues. Although Pea3 transcription factors are expressed during development of lacrimal gland as reported previously [25, 32], the functional relevance of downstream Pea3 genes are yet to be investigated.

1.2.4 Physiological role of Fgf signaling

1.2.4.1 Embryogenesis

FGF signaling pathway plays an important role in early embryogenesis during gastrulation. Fgfr1 knockout mice exhibit reduced proliferation and mesodermal cell fate specification defects [77, 78]. Similarly, Fgf8 knockout mice are embryonically lethal as they fail to form the mesodermal-derived tissues [79]. In later studies, Fgfr1 has also been shown to regulate migration and specification of the mesodermal progenitor cells at the primitive streak [80]. In Xenopus, studies have shown that FGF2, 4, 8 play roles in the mesoderm specification downstream of Nodal and Activin signals [81]. Fgf signaling has also been implicated during neural induction in the dorsal ectoderm by inhibiting the expression of BMPs [82].

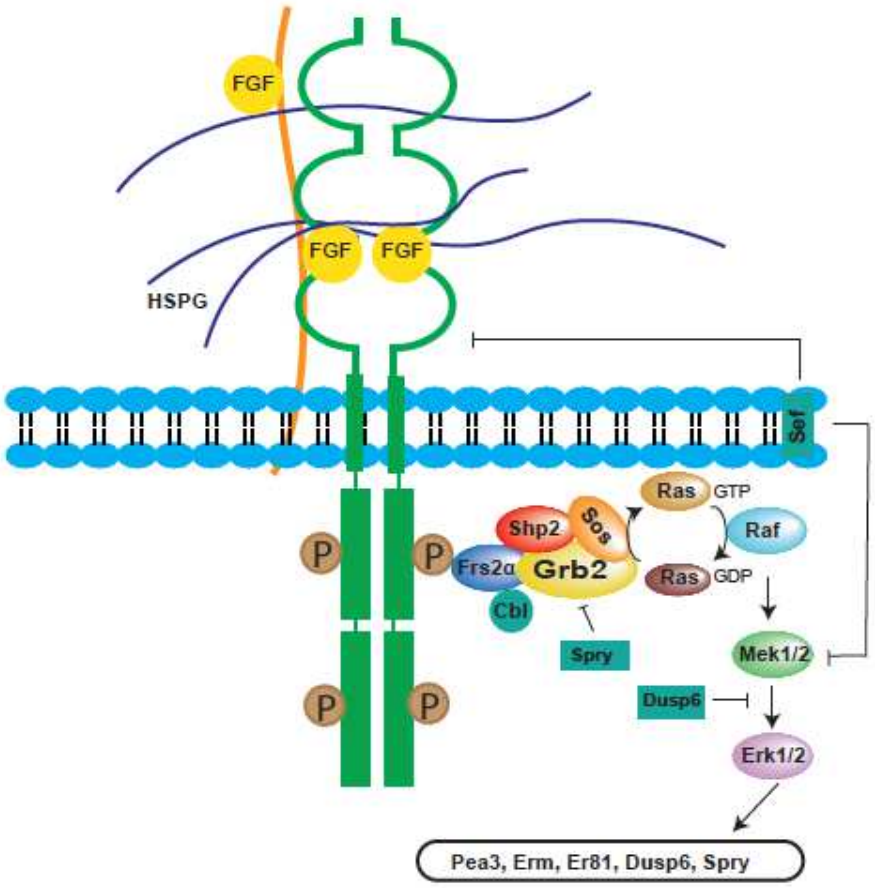


Figure 4. Schematic of Ras/MAPK-mediated FGF signaling pathway.

1.2.4.2 Epithelial-Mesenchymal interactions

In later stages, Fgf signaling has also been studied in the epithelial-mesenchymal interactions during limb development where the mesenchyme is derived from the mesoderm. Fgfr1 and 2 are expressed in the underlying mesenchyme which secretes Fgf10 to signal to the epithelium. As a result, Fgf8 is secreted by the epithelium to act on the mesenchyme to continue the expression of Fgf10 [83, 84]. During lung development, the mesodermal mesenchyme expresses Fgfr1 and Fgfr2 which respond to Fgf9 secreted by the lung epithelium to maintain the mesenchymal proliferation and Fgf10 expression, the latter signaling back to the epithelium to promote branching morphogenesis [85, 86]. Similarly, the mesoderm-derived pancreatic mesenchyme express the Fgfr2c isoform which binds to Fgf9 expressed by outer mesothelial lining of the mesenchyme [87]. Similar to lacrimal gland development as described in earlier section, the salivary gland is another example of the epithelial to mesenchymal interaction where the mesenchyme is derived from the cranial neural crest cells [88]. In this case, Fgf10 present in the mesenchyme is important for gland initiation and its expression is modulated by the ectodermal Fgf8 [89].

1.2.4.3 Fgf signaling in the neural crest

The neural crest is a multipotent stem cell population unique to vertebrates. It plays a critical role in the development of many different tissues including craniofacial structures, smooth muscle, ganglia of peripheral nervous system, adipose cells and melanocytes [90]. Neural crest is induced after gastrulation i.e. formation of three germs layers is completed. Upon induction at the neural plate border between the neural plate and non-neural ectoderm, the neural crest undergoes definitive specification as neural plate folds [91]. These cells then migrate to different regions of the embryo depending upon where they lie on anterior-posterior axis, guided by the signaling cues in the environment [92, 93]. These cells then differentiate into distinct cell types as they reach their destination, depending upon their origin. Signaling relay between neural crest cells and surrounding organ primordia not only influences the differentiation and patterning of the neural crest cells but also of the interacting tissues [94]. Because of its involvement in the formation of diverse array of tissues, neural crest cells are designated as the fourth

germ layer in the development biology field, in addition to the ectoderm, endoderm and mesoderm [95]. The research interest in studying signaling pathways in the neural crest stems from not only its extent of developmental contribution but also its implication in wide range of human diseases associated with defects in the heart, vision, facial structure, pigmentation, hearing, central nervous system and certain forms of cancer such as neuroblastoma [96].

It is reasonable to speculate that Fgf signaling is important for the neural crest-derived mesenchymal function during lacrimal gland development. Neural crest -specific deletion of Shp2 has revealed that it plays an important role in migration and differentiation of neural crest cells during heart and skull development, mediated by Erk phosphorylation [97]. In support of this finding, another study showed that Raf/Mek/Erk/Srf regulation is critical for development of the neural crest-derived craniofacial structures and cardiac outflow tract [98].

1.2.4.4 Fgf signaling as cell fate determinant for lacrimal gland epithelium

Fgf signaling is considered as important cell fate determinant mechanism during lacrimal gland development as ectopic expression of rat *Fgf10* or human *FGF7* in the cornea led to the switch in cell fate from the planar corneal epithelium to the secretory lacrimal gland epithelium [18, 19]. However, the key early response transcriptional factors which determine the lacrimal gland cell fate are still unclear. Previously, Sox transcription factors- Sox9 and Sox10, have been found to be downstream of Fgf signaling important for lacrimal gland development. Epithelial Sox9 is critical for induction of lacrimal gland and regulates Sox10 expression for acini formation at later stages [33]. However, it is not clear what are the direct transcriptional targets of Fgf signaling which regulate this process to establish Fgf signaling as an important cell fate determinant.

1.3 Hypotheses

1.3.1 Hypothesis #1

Fgf signaling plays an important role for lacrimal gland development. This process is primarily induced by growth factor Fgf10, present in the neural crest cells forming the

peri-ocular mesenchyme which bind to its cognate receptor Fgfr2 and co-receptor heparan sulphates in the conjunctival epithelium, triggering the activation of Fgf signaling cascade. However, there are several unanswered questions on how this process is accomplished. One of the questions which is still unaddressed is the mechanism by which Fgf10 is produced by the neural crest cells in the mesenchyme. Since Shp2 mediating Ras-MAPK signaling pathway is important for neural crest development and Fgf signaling pathway is frequently mediated by Shp2/Ras/MAPK, we hypothesized that Shp2- mediated Fgf signaling pathway is important for neural crest development to produce Fgf10 in the mesenchyme. To test this hypothesis, we used mouse models as lacrimal gland development in rodents which is very similar to what is present in humans. To specifically target the neural crest- derived mesenchyme, we used *Wnt-1 Cre* mouse line to conditionally delete genes within neural crest cells that are implicated in the canonical Fgf signaling pathway. To further investigate downstream targets of Shp2, we performed high throughput RNA-seq analysis of mesenchymal tissue in control and Shp2-ablated mouse embryos.

1.3.2 Hypothesis #2

It has been shown that Fgf signaling is critical for determining the fate of lacrimal gland cells as ectopic expression of rat *Fgf10* and human *FGF7* in the cornea, led to switching of the corneal fate to the glandular cell type. However, direct downstream targets of Fgf signaling which determine the fate of lacrimal gland epithelial cells are still unknown. Since Pea3 transcription factors are direct downstream effectors of Fgf signaling and are expressed in the lacrimal gland epithelium as shown previously, we hypothesize that Pea3 transcription factors are direct effectors of Fgf signaling which mediate the cell fate determination. To test this hypothesis, we used mouse models to genetically ablate *Pea3* genes *Erm* and *Er81* in the lacrimal gland epithelium using *Le-Cre* transgenic mouse line in *Pea3*-null background and determine their role during lacrimal gland development. In order to further determine the gene regulatory network downstream of Pea3 transcription factors, we performed RNA-seq analysis of lacrimal gland in control and *Pea3*-deficient mutant mouse embryos.

2 Materials and Methods

2.1 Mice and Genotyping

Mice carrying *Erk1*$^{-/-}$, *Erk2*^{flox}, *Frs2α*^{flox}, *Frs2α*^{2F}, *Mek1*^{flox}, *Mek2*^{KO}, *Shp2*^{flox} alleles were bred and genotyped as described [98-102]. We obtained *Etv1*^{flox} mice from Dr. Silvia Arber (University of Basel, Basel, Switzerland), *Etv4*$^{-/-}$ and *Etv5*^{flox} mice from Dr. Xin Sun (University of California at San Diego, San Diego, CA), *En1-Cre* and *R26-EtvEnR* from Dr. James Li (University of Connecticut Health Center, Farmington, CT), *Fgf8*^{flox} from Dr. Suzanne Monsour (University of Utah, Salt Lake city, UT), *Fgf15*$^{-/-}$ from Dr. Steven Kliewer (UT Southwestern Medical Center, Dallas, TX), *Fgfr1*$^{\Delta Frs}$ from Dr. Raj Ladher (RIKEN Kobe Institute-Center for Developmental Biology, Kobe, Japan), *Fgfr2*^{LR} from Dr. Jacob V.P. Eswarakumara (Yale University School of Medicine, New Haven, CT) and *Fgfr2*^{flox} from Dr. David Ornitz (Washington University Medical School, St Louis, MO) [74, 75, 103-109]. *LSL-Kras*^{G12D} mice was obtained from the Mouse Models of Human Cancers Consortium (MMHCC) Repository at National Cancer Institute [110]. *Alx4*$^{lst-J}$ (Stock No: 000221), *Fgfr1*^{flox} (Stock No: 007671), *p53*^{flox} (Stock No: 008462), *Pdgfrα*^{flox} (Stock No: 006492), *R26R* (Stock No: 003474), *R26RTdT* (*Ai14*, Stock No: 007914), *Sox10-Cre* (Stock No: 025807) and *Wnt1-Cre* (Stock No: 009107) mice were obtained from Jackson Laboratory [108, 111-116]. Le-Cre mice were kindly provided by Dr. Ruth Ashery-Padan (Tel Aviv University, Tel Aviv, Israel) and Dr. Richard Lang (Children's Hospital Research Foundation, Cincinnati, OH). Rosa-N1-ICD$^{flox/+}$ mice were obtained from Jackson lab (Stock # 008159). Animals were all maintained on mixed genetic background. *Wnt1-Cre, Le-Cre*, *Shp2*^{flox} and *Pea3*$^{+/-}$ */Erm*^{flox}*/Er81*^{flox} only mice did not display any lacrimal gland phenotypes and were used as controls. Mice were housed in virus-free facility in a 12 hour light-dark cycle and were given standard mouse feed.

2.1.1 Embryo collection

Females in breeding were checked for vaginal plugs which are considered 0.5 days pc. Embryos were harvested in 1XPBS at appropriate days based on the experiment and taken out of embryonic sac. Embryos were then fixed in 4%PFA or flash frozen in OCT

depending upon the experimental condition. For genotyping, embryonic tail samples were collected and stored in -20°C until processing.

2.1.2 Genotyping

For breeding strains and identifying embryos, tail samples were collected and heated in 300 µl of 50nM NaOH for 1 hour at 95ºC to release the DNA in solution. These samples were then neutralized with 17.1 µl of 1M Tris HCl, pH 8.0 and centrifuged at 13,000 rpm for 3 min. The supernatant was used as template for PCR reaction. PCR conditions for 10ul reaction are as follows. 10X PCR buffer mix- 1 µl, Primers, F and R – 0.1 µl each, DNA Template- 1 µl, Taq- 0.2 µl. PCR buffer mix was made in house with the addition of dNTPs and tracking dye.

2.2 Histology

Hematoxylin and Eosin staining (H&E) is performed as previously described [32]. Briefly, paraffin blocks were sections at 10 µm and were deparaffinized by heating and performing series of histosol washes (3 X 5 min), followed by rehydration by treating the slides through decreasing percentage of ethanol solutions (100% - 2X 3 min, 95% ethanol- 2 X 3 min, and 70% ethanol- 1 X 3 min). The slides were dipped into hematoxylin for 3 min followed by 10-15 min wash with tap water. The samples were decolorized with 1% acid alcohol for 15 sec, before dipping in Eosin solution for 1min. Samples were then dehydrated by passing through increasing concentration of ethanol, and transferred to histosol. The stained slides were mounted with cover-slips using Permount™ mounting medium.

2.3 Immunohistochemistry

For immunohistochemistry of paraffin samples, sections were deparaffinized and rehydrated by serial treatment with histosol followed by decreasing percentage of ethanol solutions (2X 100 %, 2 X 95%, 1X 70% for 5 min each). For cryosections, sections were briefly washed with PBS to remove OCT medium (Sakura). Antigen retrieval was performed with microwave boiling for 1-2 minutes followed by heating for 10 minutes at low power settings in citrate buffer (10 mM sodium citrate, pH 6.0). Sections were then

washed with PBS and blocked with 5% NGS/0.1% Triton in PBS. Primary antibody incubation was performed overnight at 4°C in humid chamber followed by incubation with florescent-conjugated secondary antibodies for 1 hour at room temperature in dark. For signal amplification, HRP-conjugated secondary antibodies are used, followed by washing and equilibration with TNT buffer. The slides were then incubated with Tyramide reagent for 10 min, washed with TNT buffer, stained with DAPI and mounted with anti-fade reagent, 0.2% NPG, 90% glycerol in 1X PBS. Following antibodies with respective dilutions were used as shown in Table 2.

2.4 Carmine staining

For carmine staining, post-natal P1-3 heads were decapitated and cheek skins were removed to expose lacrimal gland. Embryos were fixed in 4% PFA O/N at 4ºC, washed with PBS and transferred to 70% ethanol for O/N incubation at 4ºC. Embryo heads were stained in aceto-carmine for 5 minutes, 70% EtOH for 3 minutes, 1% Acid-EtOH for 2 minutes, 5% Acid-EtOH for 1 minute. To de-stain, 95% ethanol was used. Recipe for Aceto-carmine staining reagents: Aceto-carmine: 0.5gm carmine stain (SIGMA), 100 ml boiling 45% acetic acid; 1% Acid-EtOH: 1.0ml HCl, 100ml 70% EtOH; 5% Acid-EtOH: 5.0ml HCl, 100ml 70% EtOH.

2.5 X-gal staining

E13.5 embryos were incubated in 4% PFA for 1 hour at 4ºC and washed twice in PBS containing 0.02% NP-40, 0.01% sodium deoxycholate and 2 µg/ml $MgCl_2$ for 30 min each, followed by overnight incubation in X-gal staining solution (1 mg/ml X-gal, 10 mM Potassium Ferricyanide, 10nM Potassium Ferrocyanide, 2 µg/ml $MgCl_2$ in PBS) at 4ºC. The samples were then cryopreserved in OCT (Sakura Finetek), sectioned at 10 µm thickness and counter-staining with nuclear red.

Table 2: List of antibodies, catalog number and dilutions.

Antibodies	Catalog number	Dilution
Pax6	PRB-278P, Covance, Berkeley, CA, USA	1:250
RFP	600-401-379, Rockland	1:1000
Alx4	sc-33643, Santa Cruz Biotechnology	1:100
E-cadherin	U3254, Sigma, St Louis, Missouri	1:200
Cleaved-caspase 3	#9664, Cell signaling Technology	1:200
Col2a1	ab34712, Abcam	1:200
Connexin43	#3512, Cell signaling Technology	1:200
pHH-3	#06-570, Millipore	1:500
α-SMA	#C6198, Sigma-Aldrich	1:200
N1-ICD	Cell signaling	1:200
Jag1	sc-8303, H-114, Santa Cruz Biotechnology	1:200
Krt14	Covance, PRB-155P	1:1000
p-Erk	Cell signaling #4370	1:200

2.6 RNA in situ hybridization

2.6.1 Whole mount format

Whole mount in situ hybridization was performed as previously described [117]. Briefly, embryos were harvested in DEPC-PBS, fixed overnight in 4%PFA and dehydrated in graded solutions of 25%-100% methanol in PBS and stored in -20°C. On the day of experiment, embryos were rehydrated with decreasing concentrations of methanol in PBS. Embryos were then treated with Proteinase K at 10 μg /ml for 3-10 min at room temperature depending upon the stage, followed by quenching with 2mM glycine. Embryos were treated with RIPA buffer and additionally fixation in 2% glutaraldehyde, followed by washing in PBS and incubation in hybridization buffer at 65°C for 1 hour. Next, embryos were hybridized with diluted probe (1:1000) in hybridization buffer with

r-RNA (10 µg/ml) and incubated at 68°C overnight. The embryos were washed 8-10 times with 1x SSC and 50% formamide at 65°C, equilibrated with maliec acid buffer and blocked with anti-DIG antibody (Roche, 1:5000). Embryos were washed for 2-3 days with TBST and equilibrated with alkaline phosphatase buffer for 1 hour prior to incubation with AP-substrate BM purple (Roche) for several days at 4°C for color development.

2.6.2 Sectional format

RNA in situ hybridization was performed as previously described [118]. Briefly, the mouse embryos are harvested, fixed overnight in 4%PFA, equilibrated in 30% sucrose and cryo-frozen in OCT. On the day of the experiment, OCT blocks are sectioned at 10 µm and hybridized with diluted probe at 68°C overnight in a wet chamber, moistened with solution containing 50% Formamide and 1X Salt. Probe is diluted 1:200-500 in pre-warmed hybridization buffer and incubated at 70°C for at least 10 min. Next day, the slides were washed 3X in wash buffer (1X SSC, 50% Formamide) at 68°C. After cooling, slides were washed 2X with 1X MABT and incubated at room temperature for 30 min. Slides were blocked with 20% Sheep serum in 1XMABT for 1 hour, followed by incubation with anti-DIG antibody (1:1500) at 4°C for overnight. Next day, slides were washed 4-5X with 1X MABT and 2X with alkaline phosphatase buffer. For color development, slides were covered with BM purple substrate and incubated at room temperature for 4-24 hrs.

2.6.3 Probes

The following probes were used: *Alx1* (from Dr. Terence Capellini, Harvard University, Boston, MA), *Alx4* (from Dr. Yang Chai, University of Southern California, Los Angeles, CA), *Pea3/Etv4*, *Erm/Etv5* (from Dr. Bridget Hogan, Duke University Medical Center, Durham, NC, USA), *Foxc1*, *Sox10*, *Crabp1* (from Dr Anthony Firulli, Indiana University School of Medicine, Indianapolis, IN, USA), *Fgf10* (for whole mount) (from Dr. Suzanne Monsour, University of Utah, Salt Lake city, UT)), *Fgf10* and *Dusp6* (for sections) was generated from a full length cDNA clone (IMAGE: 6313081 and 3491528, Open Biosystems, Huntsville, AL, USA), *Pitx2* (from Dr. Valerie Dupé, CNRS, Strasbourg,

France), *Six1* (from Dr. Bernice Morrow, Albert Einstein College of Medicine), *Six2* (from Dr. Thomas Caroll lab, UT Southwestern Medical center), *Er81* (from Dr. Fischell, NYU Lagnone Medical Center).

2.7 Laser capture micro-dissection and Gene expression profiling

Freshly harvested embryos were frozen in OCT medium (Sakura Finetek), sectioned at 10 µm thickness and transferred to PEN slides (Ziess), which can be stored in -80°C under dry conditions (with silica beads in the tube) until further processing. For staining, slides were dipped in ice cold 95% ethanol for 2 min to fix the samples, stained with crystal violet stain (Stock- 3% in ethanol diluted in ethanol and Tris HCl pH 8-8.5 in 6:5:3.85) for 1 min on ice. The slides were then dipped 2X in ice cold 70% ethanol for 30-40 sec to remove the OCT and dehydrated in ice cold 100% ethanol for 2 min. For control and Pea3/Etv mutant embryos, lacrimal gland epithelial tissue was micro-dissected using Laser capture microscope (Zeiss AxioObserver.Z1 inverted microscope). For Pea3 conditional knockout experiment, RNA was extracted using Qiagen Micro Plus kit. Conversion to cDNA and amplification was performed using Clontech SMART-seq v4 Ultra low input RNA kit, as well as cDNA library construction was performed using Nextera XT DNA library preparation kit by core facility at Columbia university prior to RNA sequencing. On the other hand, for Shp2 conditional knockout experiment, 500 pg of RNA was isolated from each sample, converted to cDNA and amplified using Nugen Ovation kit v2 (Nugen) to obtain 2-3 µg cDNA, which was then converted to cDNA library for RNA-sequencing analysis at core facility in Columbia University.

2.8 Lacrimal gland mesenchyme culture

Lacrimal glands mesenchymal culture was performed as described previously [119]. Briefly, glands were isolated from P0-P2 pups and trypsinized (Gibco 1:250) at 4 ºC for 1 hr. After neutralizing trypsin, the mesenchyme was manually separated from the epithelium using fine needle and grown in the complete medium (DMEM+10% FBS with antibiotics) for 3 days before passage. The primary mesenchymal cells were transfected with siRNA using Lipofectamine RNAimax as previously described and harvested after

24-48 hrs [120]. For *Alx1* and *Alx4*, the results were confirmed using two different predesigned Silencer® Select siRNAs from Ambion (Life technologies).

2.9 Quantitative-PCR (qPCR)

Quantitative-PCR was performed as described [121]. Briefly, total RNA was extracted from MiniRNA Plus kit from Qiagen and converted to cDNA using High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. PCR SYBR green 2X master mix was used from Applied Biosystems and analyzed on StepOne plus Real time PCR instrument. Following primers were used. *Alx4*: 5'-ACACATGGGCAGCCTGTTTG3', 5-TGCTTGAGGTCTTGCGGTCT-3', *Alx1*: 5' GGAGGAAGTGAGCAGAGGTG-3', 5'- TTCAAATGCGTGTCCGTTGGT-3', *Fgf10*: 5' CAATGGCAGGCAAATGTATG-3', 5'- GGAGGAAGTGAGCAGAGGTG-3', *Gapdh*: 5'-AGGTCGGTGTGAACGGATTTG-3', 5'-TGTAGACCATGTAGTTGAGGTCA-3', *Shp2* (exon 4): 5'- CTGACGGAGAAGGGCAAGCA-3', 5'-CGCACGGAGAGAACGAAGTCT-3'

2.10 Chromatin Immunoprecipitation

The Chromatin Immunoprecipitation (ChIP) assays were performed in 3T3 fibroblasts cells and primary lacrimal gland mesenchymal cells as described [122]. Briefly, the cells grown in DMEM/10% FBS with antibiotics were crosslinked with 1% Formaldehyde for 8-10 min with gentle shaking. This was followed by quenching with 125 mM glycine or 5 min, 3X washing with cold PBS and addition of 1 ml of cold CHIP lysis buffer. After incubation for 10 min at 4°C, the lysed cells were centrifuged at 3000 rpm for 3 min and the pellet were stored at -80°C until later use. The pellet was then re-suspended in 1.2 ml of RIPA buffer, sonicated on ice for 8 min using probe sonicator (1 sec "on", 2 sec "off", power 3.5) and centrifuged at 13000 rpm for 15 min at 4ºC. The supernatant was precleared by adding 45 µl Protein G agarose beads (50% slurry, Millipore) and incubated for 2 hours at 4°C on rotor. After centrifugation at 5000 rpm for 1 min, the supernatant was transferred to a fresh tube and the protein concentration was measured by Bradford assay. For pull down, 1 µg of antibodies were added per 1mg of protein for overnight incubation at 4°C, followed by addition of 20 µl agarose beads for another 1-2

hours incubation. After brief centrifugation, the beads washed 1X with RIPA buffer at room temperature, 3X with cold RIPA buffer, 2X with cold Wash buffer A and Wash buffer B, 1X with TE/150mM NaCl. Next, the samples were decrosslinked in Elution buffer containing RNAase (40µg/ml) and Proteinase K (20µg/ml) for 1 hour at room temperature and 50°C overnight. After brief centrifugation, the supernatant was treated with equal vol. of Phenol/Chloroform and the DNA was precipitated with 2.5 vol. of 100% ethanol and Glycoblue for 1 hour at -80°C and dissolved in 20 µl sterile water for qPCR analysis. The antibodies used were IgG as isotype control (sc-2028, Santa Cruz Biotechnology) and anti-Alx4 (sc-22066, Santa Cruz Biotechnology). *Buffer recipes*: CHIP lysis buffer- 10mM Tris-Cl, pH-8, 85mM KCl, 0.5% NP-40, 5nM EDTA, 0.25% Triton; RIPA- 1% Triton, 150mM NaCl, 0.1% SDS, 0.1% Na-Deoxycholate, 10mM Tris-Cl, pH-8, 5mM EDTA; Wash buffer A- 50mM HEPES, pH7.9, 500mM NaCl, 1mM EDTA, 1% Triton, 0.1% Na-deoxycholate, 0.1% SDS, Wash buffer B- 20mM Tris-Cl, pH-8, 1mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate; Elution Buffer- 1% SDS, 30 mM Tris-Cl (pH-8), 15mM EDTA, 200mM NaCl. Protease inhibitor cocktail is added prior to use in all the buffers until ready to elute. The primers used for CHIP in intron 1 of Fgf10- F- 5'-GGTTGGAGCTTGTTGTGTGT-3', R- 5'-GCTCTGCTAATAAAGGTCTCCC-3'.

2.11 TUNEL assay

TUNEL assays were performed on 10-µm paraffin sections following the manufacturer's instructions in the In Situ Cell Death Detection kit, Fluoroscein (Roche Applied Science, Indianapolis, IN). Cell proliferation and apoptosis rates were calculated as the ratio of phospho-histone H3 or TUNEL-positive cells to DAPI-positive cells in control and mutant samples, and results were analyzed by one-way non-parametric t-test.

2.12 Bioinformatics analysis

Scanning for Alx4 binding sites on Fgf10 gene- We retrieved 200 KB upstream and 100 KB downstream of *Fgf10* transcription start site from Mouse Genome assembly GRCm38/mm10 and analyzed this sequence for evolutionary conservation using UCSC genome browser. These sequences were also overlaid with the DNase-hypersensitivity

data from 3T3 cell line retrieved from ENCODE database and scanned for Alx4 consensus binding sites based on TRANSFAC (release 2013.1) database using MATCH algorithm, with minFP as parameter to identity sites with minimum false positives. Analysis of RNA-seq data from Pea3 conditional knockout lacrimal gland: Unsupervised clustering analysis was performed using MATLAB using Clustergram function. We determined interquartile ranges of the gene expression levels in all the samples and top 200 genes were plotted. Gene set enrichment analysis (GSEA) was performed using MATLAB implementation of the same method as described in reference [123]. KEGG pathway enrichment analysis and functional annotation was performed in DAVID. For the functional annotation of downregulated genes, list of 476 genes was used for the analysis based on cut off on normal expression level (> 50 units), Log2(fold change) (<-1) and p-value (<0.05). Similarly, for the pathway analysis of upregulated genes, list of 640 genes was used for the analysis (normal expression level >50, Log2FC >1, p-value <0.05). Volcano plots representing Log2 (p-value) vs Log2(Fold change) were plotted in MATLAB. Log2(p-value)> 0.05 were set to 0.05 to avoid the scaling issues in the plot. "VennDiagram" library in R were used to generate Venn diagrams. To identify Er81/Etv1 and Pea3/Etv4 binding sites in the promotor region of genes, we retrieved 1000 bp upstream from Mouse Genome with assembly GRCm38/mm10 using UCSC genome browser. These sequences were scanned for consensus Etv1/4 binding sites retrieved from TRANSFAC, release 2013.1 using MATCH algorithm, with minFP as parameters to identity sites with minimum false positives. Principal component analysis with the previously published skin gene expression data was performed using script written in MATLAB.

3 Results

3.1 Alx4 relays sequential FGF signaling to induce lacrimal gland morphogenesis

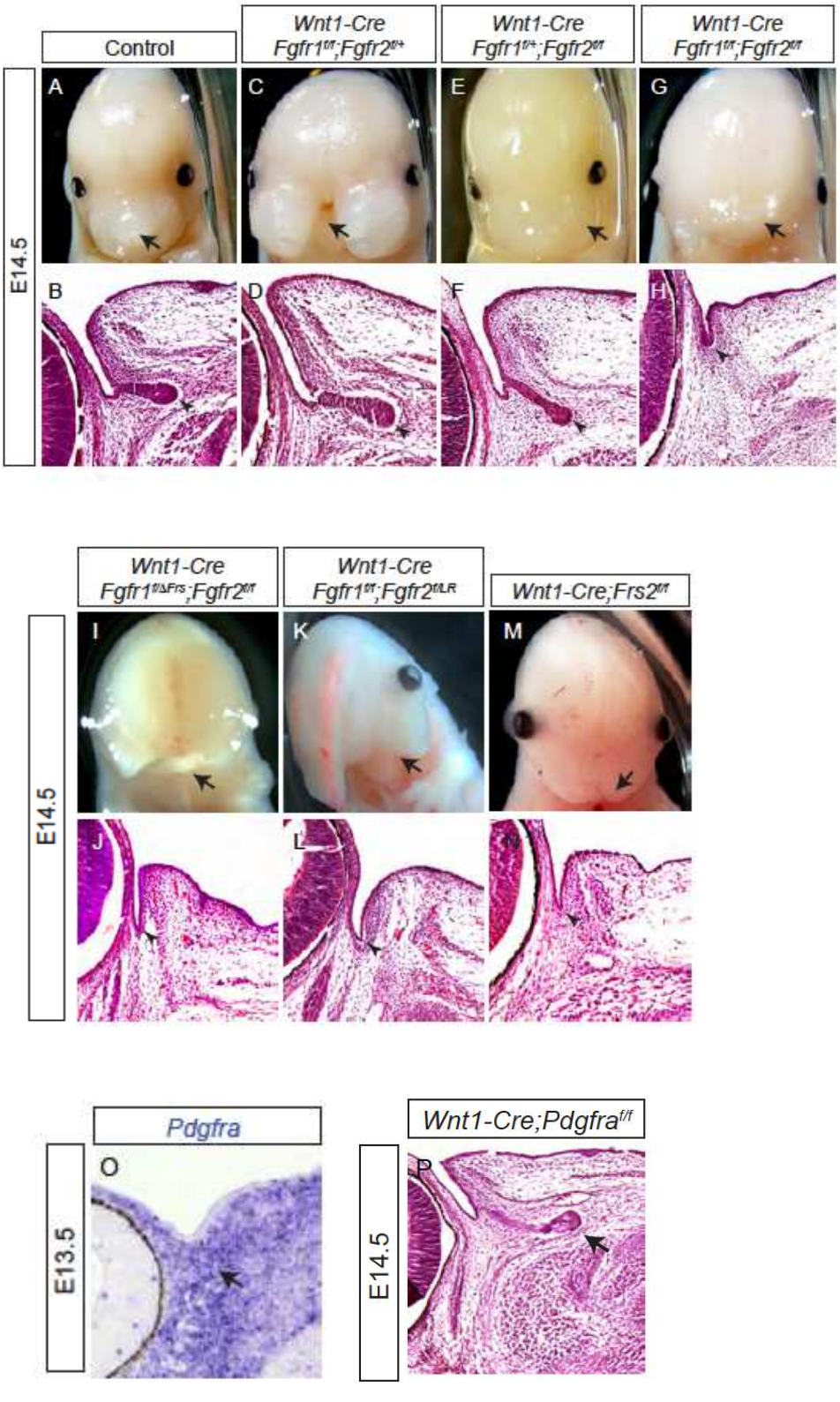
3.1.1 Lacrimal gland development requires FGF but not PDGF signaling in the neural crest

FGF signaling is important for development of the neural crest derived craniofacial structures [51, 108, 124-128]. Using the neural crest specific *Wnt1-Cre*, we observed that conditional knockout of *Fgfr1* resulted in significant craniofacial abnormalities, whereas deletion of *Fgfr2* did not exhibit obvious effect (Fig. 5A, C and E, arrows). Lacrimal gland development begins with the invasion of an epithelial bud from the conjunctiva into the periocular mesenchyme at embryonic day 14.5 (E14.5) (Fig. 5B, arrow). In *Fgfr1* and *Fgfr2* single mutants, lacrimal gland development was mostly unaffected (Fig. 5D and F, arrows). Combined deletion of both *Fgfr1* and *Fgfr2*, however, abrogated lacrimal gland budding (Fig. 5G and H, arrows), indicating that Fgfr1 and Fgfr2 can compensate for each other in the neural crest during lacrimal gland development. $Fgfr1^{\Delta Frs}$ and $Fgfr2^{LR}$ alleles encode the respective mutant Fgfr1 and Fgfr2 that lack the docking site for adaptor protein Frs2α [108, 109]. Although $Fgfr2^{L/R}$ homozygous mice were viable and fertile, the craniofacial and lacrimal gland phenotypes were observed in both *Wnt1-Cre;*$Fgfr1^{f/\Delta Frs}$;$Fgfr2^{f/f}$ and *Wnt1-Cre;*$Fgfr1^{f/f}$;$Fgfr2^{f/LR}$ mutants (Fig. 5I-L, arrows). The essential role of neural crest Frs2α for lacrimal gland development was further demonstrated in *Wnt1-Cre;*$Frs2\alpha^{f/f}$ mutants, which displayed less severe craniofacial phenotype than *Fgfr* mutants, but similar arrest of lacrimal gland budding (Fig. 5M and N, arrows). Finally, lacrimal gland development was also aborted in *Wnt1-Cre;*$Frs2\alpha^{f/2F}$ mutants, which carries mutation in two tyrosine residues in Frs2α ($Frs2\alpha^{2F}$) required for binding of Shp2 protein phosphatase (Fig. 5Q, n=6) [102]. In contrast, although *Pdgfrα* was expressed in the periocular mesenchyme and required for craniofacial development, its neural crest-specific knockout failed to impair lacrimal gland development (Fig. 5O-R, arrows). These results demonstrated that lacrimal gland development specifically requires FGF-Frs2-Shp2 signaling in the neural crest.

3.1.2 Neural crest Shp2 regulates *Fgf10* expression in the periocular mesenchyme for lacrimal gland development

To investigate the downstream target of the neural crest FGF signaling for lacrimal gland development, we next generated *Wnt1-Cre;Shp2*$^{f/f}$ mutants, which lacked lacrimal gland as expected (Fig. 6A and B, dotted lines, *n*=6). Consistent with the idea that the neural crest is the main contributor of the periocular mesenchyme, immunostaining confirmed that Shp2 protein was depleted in the periocular mesenchyme, but preserved in the ectoderm-derived conjunctival epithelium (Fig. 6C and D, arrows and dotted lines). Although the epithelial cells maintained Pax6 and E-cadherin staining, there was no increase in Col2a1 expression, a hallmark of the nascent lacrimal gland bud (Fig. 6E-H, dotted lines). By contrast, the periocular mesenchyme expression of Col2a1 was preserved, suggesting that the identity of these neural crest-derived cells was unchanged.

The budding of lacrimal gland requires the inductive signal of Fgf10 emanating from the periocular mesenchyme. In E13.5 control embryos, *Fgf10* was found in a ring pattern in the presumptive eyelid surrounding the eye (Fig. 6I, arrowheads), with the strongest signal in the mesenchyme adjacent to the future lacrimal gland bud (Fig. 6I and K, arrows). In both *Wnt1-Cre;Shp2*$^{f/f}$, however, *Fgf10* was absent in the entire periocular mesenchyme (Fig. 6J and L, arrows and arrowheads). As a result, ERK phosphorylation was maintained in the adjacent retina but abolished in the conjunctival epithelium (Fig. 6O and P, dotted lines), suggesting a specific loss of FGF signaling in the lacrimal gland primordia. This was further supported by downregulation of FGF signaling response genes, *Etv4* and *Etv5*, in the presumptive lacrimal gland epithelium (Fig. 6Q-T, dotted lines). Considering the essential role of Fgf10 signaling in inducing lacrimal gland bud, we concluded that the lack of *Fgf10* expression accounted for lacrimal gland aplasia in neural crest *Shp2* mutants.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Wnt1-Cre Fgfr1f/+;Fgfr2f/f
Wnt1-Cre Fgfr1f/f;Fgfr2f/f
E14.5
A
B
C
D
E
F
G
H
Wnt1-Cre Fgfr1f/ΔFrs;Fgfr2f/f
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Wnt1-Cre;Frs2f/f
E14.5
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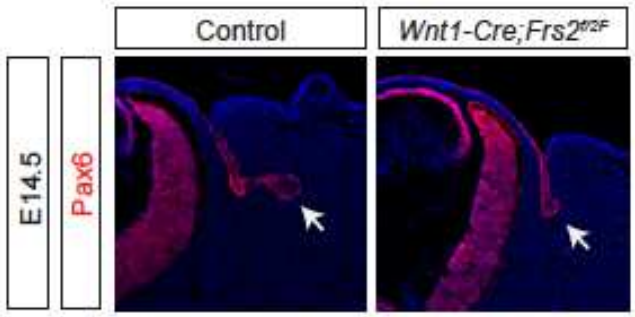
Pdgfra
E13.5
O
Wnt1-Cre;Pdgfraf/f
E14.5
P

Q



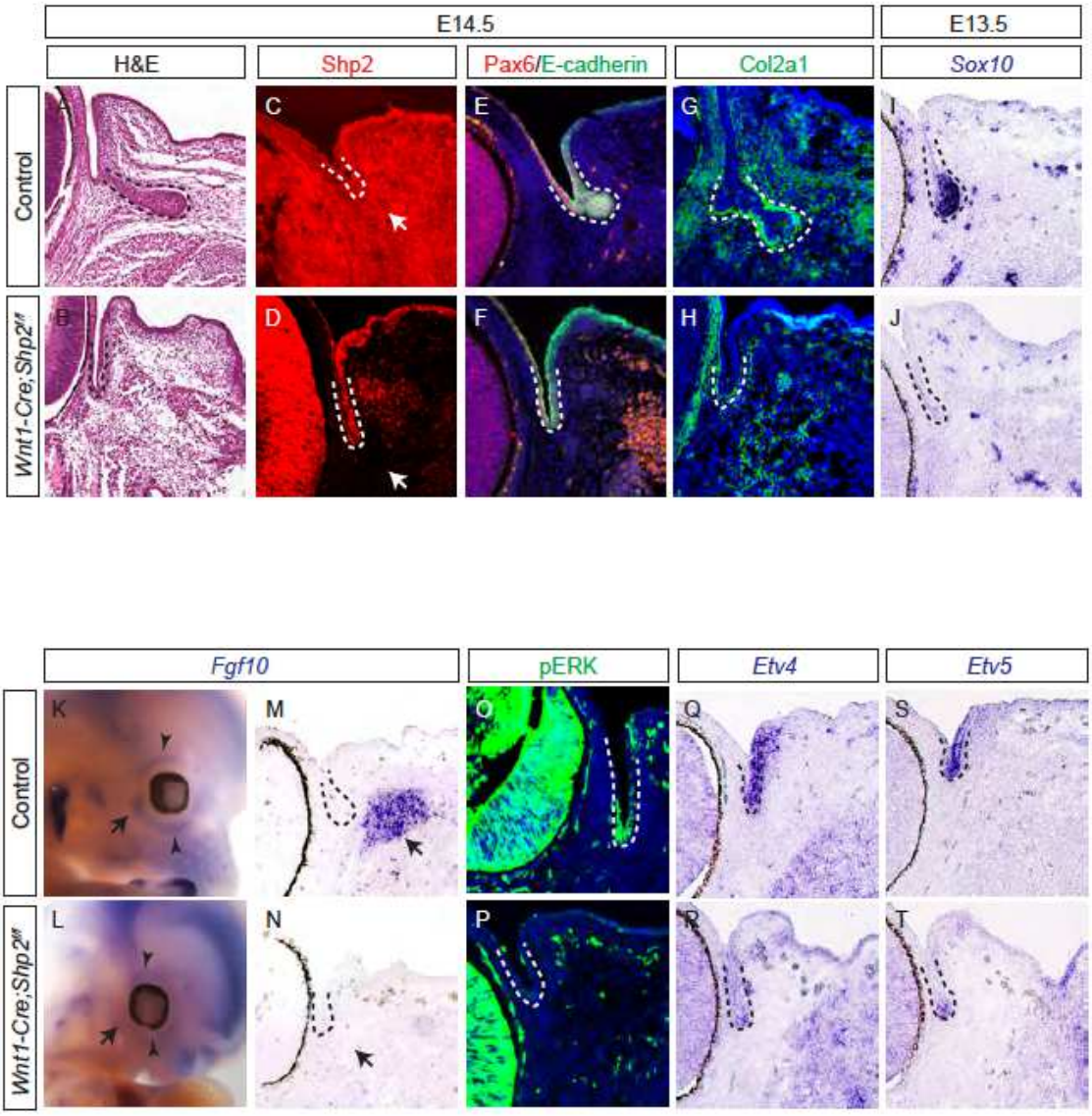
R

Figure 5. The neural crest specific ablation of Fgfr and Frs2α disrupted lacrimal gland. (A-N) Lacrimal gland budding occurred in Fgfr1 and Fgfr2 single, but not in double mutants (A-H, arrowheads). Mutation of Frs2α binding site on Fgfr1 ($Fgfr1^{\Delta Frs}$) or Fgfr2 ($Fgfr2^{L/R}$) or deletion of Frs2α itself also disrupted lacrimal gland development (I-N, arrowheads). Note the severity of the craniofacial phenotype does not always correlate with lacrimal gland defects (compare C, D, M and N). Arrow: craniofacial abnormalities. Arrowheads: lacrimal gland primordia. e: eye. (O-P) Pdgfrα was expressed in the periocular mesenchyme (O, arrow), but its deletion in the neural crest did not affect lacrimal gland budding (P, arrow). (Q) Frs2α-Shp2 interaction is required for lacrimal gland development. In Wnt1-Cre;$Frs2\alpha^{f/2F}$ mutant that disabled Shp2 binding to Frs2α, lacrimal gland development was aborted at E14.5 (n=6). (R) Quantification of lacrimal gland phenotype.

The *Wnt1-Cre* transgene was recently reported to cause ectopic expression of *Wnt1* in the midbrain-hindbrain boundary [129]. To ensure that this complication did not compromise our results, we used another neural crest-specific deletor *Sox10-Cre* to ablate *Shp2*, which also abolished lacrimal gland development, likely caused by Fgf10 expression in the peri-ocular mesenchyme (Fig. 6U-X, arrows). Altogether, there results showed that Shp2 in the neural crest is required for lacrimal gland budding in a non-cell autonomous manner.

3.1.3 Ras-MAPK signaling and ETS transcription factors are downstream effectors of Shp2

FGF signaling is known to activate the Ras family of small GTP-binding proteins, which play important roles in cell proliferation, migration and differentiation. Previous studies have identified multiple downstream targets of Ras, including Raf kinases, type I phosphoinositide (PI) 3-kinases, Ral guanine nucleotide exchange factors, the Rac exchange factor Tiam1, and phospholipase C3 [130]. Among these molecules, Raf kinases activate the mitogen-associated protein kinase (MAPK) cascade that culminates at phosphorylation of Mek kinases (Mek1 and 2) and their direct targets Erk kinases (Erk1 and 2) [131]. At E10.5, ETS transcription factors *Etv1*, *4* and *5* were strongly expressed in tissues known to have active FGF signaling (Fig. 7A, arrows). In both *Wnt1-Cre;Shp2*$^{f/f}$ and *Wnt1-Cre; Mek1*$^{f/f}$*;Mek2*$^{-/-}$ embryos, these expressions were significantly down regulated in the cranial neural crest-derived mesenchyme in the midbrain, branchial arches and nose (Fig. 7A, arrowheads), supporting that Shp2 and Mek operate in the same signaling cascade leading to *Etv1, 4,* and *5 expression*. Furthermore, lacrimal gland development never initiated after genetic removal of *Mek1/2* in the neural crest (Fig, 7B, arrowhead, *n*=8). Interestingly, however, a small lacrimal gland protrusion was seen in *Wnt1-Cre; Erk1*$^{-/-}$*;Erk2*$^{f/f}$ embryos, suggesting that Mek may have additional key targets other than Erk (Fig. 7B, arrowhead, *n*=2) that participate in budding morphogenesis. Furthermore, by taking advantage of a conditional allele of oncogenic *Kras* (*LSL-Kras*^{G12D}*)*, we showed that constitutively active Ras signaling in the neural crest rescued *Shp2* deficiency during lacrimal gland budding (Fig. 7B, arrows, *n*=10), supporting the downstream role of Ras-MAPK activation in the FGF-Shp2 signaling cascade in the neural crest [132-135].


E14.5
E13.5
H&E
Shp2
Pax6/E-cadherin
Col2a1
Sox10
Control
Wnt1-Cre;Shp2f/f
A
B
C
D
E
F
G
H
I
J
Fgf10
pERK
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O
P
Q
R
S
T

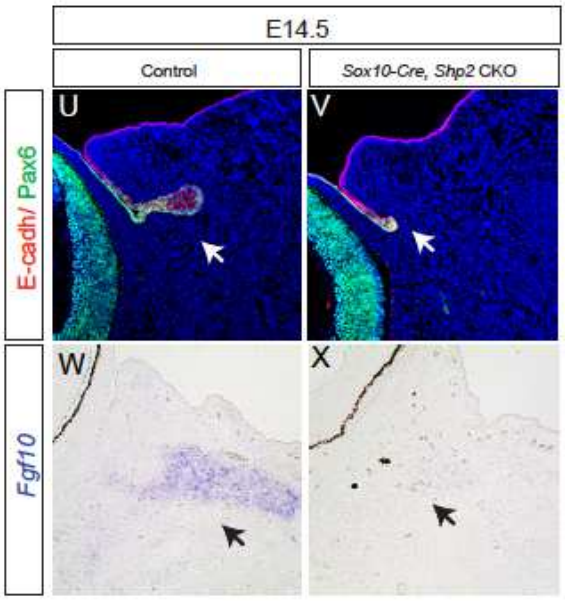


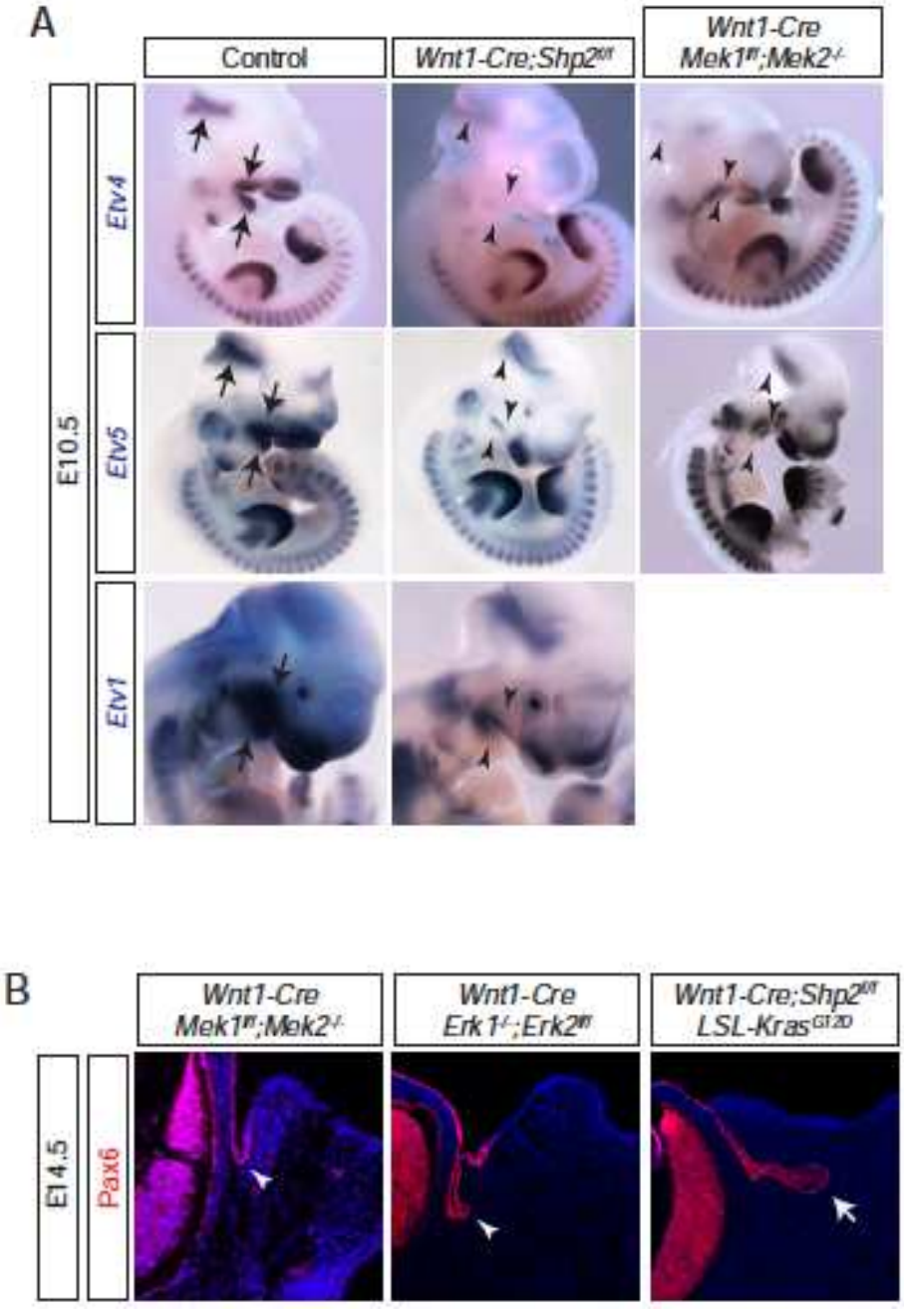
Figure 6. Lacrimal gland budding requires Shp2 in the neural crest. (A-J) *Wnt1-Cre* mediated ablation of Shp2 in the neural crest resulted in loss of Shp2 staining in the periocular mesenchyme (A and B, arrows) and abrogation lacrimal gland buds at E14.5 (C-D, dotted lines). The lacrimal gland primordia in *Shp2* mutants still expressed Pax6 and E-cadherin (E-F, dotted lines), but failed to upregulate Col2a1 and *Sox10* expressions (G-J, dotted lines). (K-T) *Fgf10* was normally expressed in the E13.5 periocular mesenchyme to induce downstream targets pERK, Etv4 and Etv5 in the lacrimal gland bud, but these were all down regulated in *Shp2* mutants. Arrow: *Fgf10* expression next to the future lacrimal gland bud. Arrowhead: *Fgf10* expression in the eyelid mesenchyme. The lacrimal gland primordia were outlined with dotted lines. *Shp2* deletion in the migratory neural crests disrupted lacrimal gland development. (U-V) *Sox10-Cre* mediated ablation of Shp2 in the migrating neural crest also abolished lacrimal gland budding at E14.5 (arrows). (W-X) *Fgf10* expression was lost in the periocular mesenchyme (arrowheads). Lacrimal gland primordia are outlined with dotted lines.

The faithful expression of *Etv1*, *4* and *5* in response to Ras-MAPK activity prompted us to investigate the functional significance of these three transcription factors. Surprisingly, even combined inaction of *Etv1/4/5* in the neural crest lineage failed to perturb lacrimal gland development (Fig. 7C, *n*=8), suggesting that these genes may be compensated by other ETS domain transcription factors that share similar binding specificity. To overcome this genetic redundancy, we used a Cre-inducible transgene (*R26-EtvEnR*) to express *Etv4* fused with the *Engrailed* repressor domain, which acts as a dominant negative ETS transcription factor [75]. *Wnt1-Cre; R26-EtvEnR* embryos not only exhibited craniofacial defect, but also showed reduced elongation of the lacrimal gland (Fig. 7D, *n*=8). This result suggests that ETS domain transcription factors are downstream effectors of FGF-Shp2-Ras-MAPK signaling in neural crest development.

3.1.4 Lacrimal gland aplasia is not due to aberrant neural crest induction, migration and death

FGF signaling has been implicated in the induction, proliferation, migration and differentiation of neural crest cells [125, 136-139]. The periocular mesenchyme originates from the neural tube in the midbrain, where active FGF signaling indicated by *Etv5* expression coincides with *Fgf8* expression (Fig. 8A, arrows), suggesting that Fgf8 may activate FGF signaling during induction of cranial neural crest progenitors. Considering that *Fgf15* is also expressed in the midbrain, we ablated *Fgf8* in the midbrain-hindbrain junction using *En1-Cre* in the *Fgf15* null background. As expected, both *Fgf8* and *Etv5* midbrain expressions were absent in *En1-Cre;Fgf8*$^{f/f}$*;Fgf15*$^{-/-}$ embryos (Fig. 8A, arrowheads), demonstrating a loss of FGF signaling. Nevertheless, the lacrimal gland bud still developed normally in these mutants (Fig. 8A, asterisks; *n*=3), showing that FGF signaling at the induction of the cranial neural crest is not required for lacrimal gland development.

After induction at the dorsal neural tube, the neural crest progenitors express *Sox10* as they begin to migrate toward their destination. At E10.5, although *Sox10*-positive neural crest cells were present in the cranial mesenchyme in *Wnt1-Cre;Shp2*$^{f/f}$ mutants, their number and the extent of migration were slightly reduced as compared to those in the


A
Control
Wnt1-Cre;Shp2fl/fl
Wnt1-Cre
Mek1fl/fl;Mek2-/-
E10.5
Etv4
Etv5
Etv1
B
Wnt1-Cre
Mek1fl/fl;Mek2-/-
Wnt1-Cre
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Wnt1-Cre;Shp2fl/fl
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E14.5
Pax6

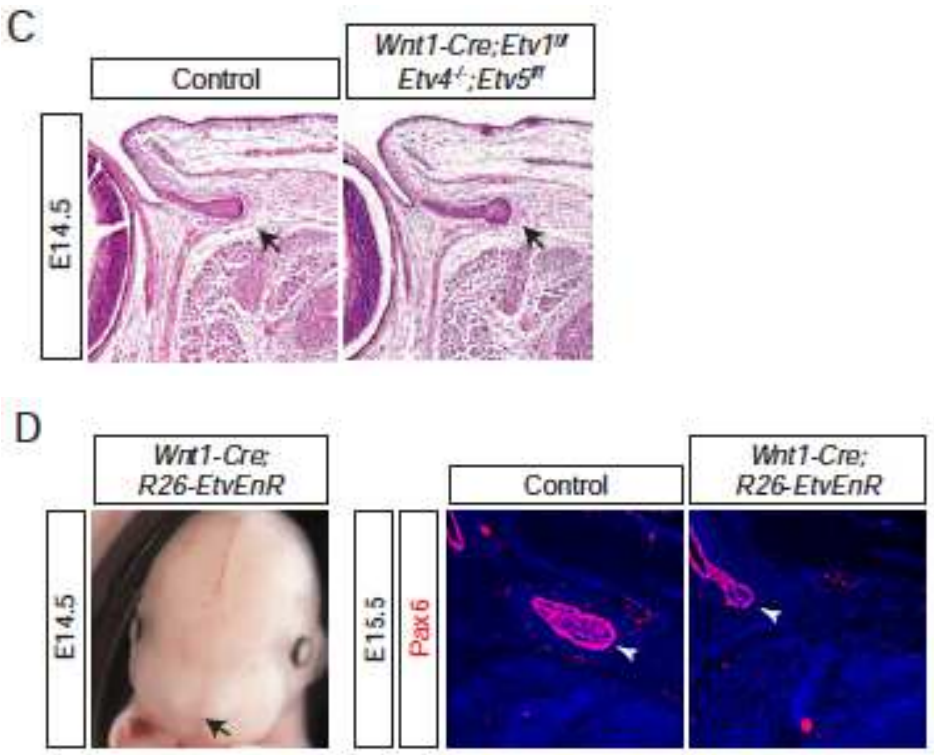
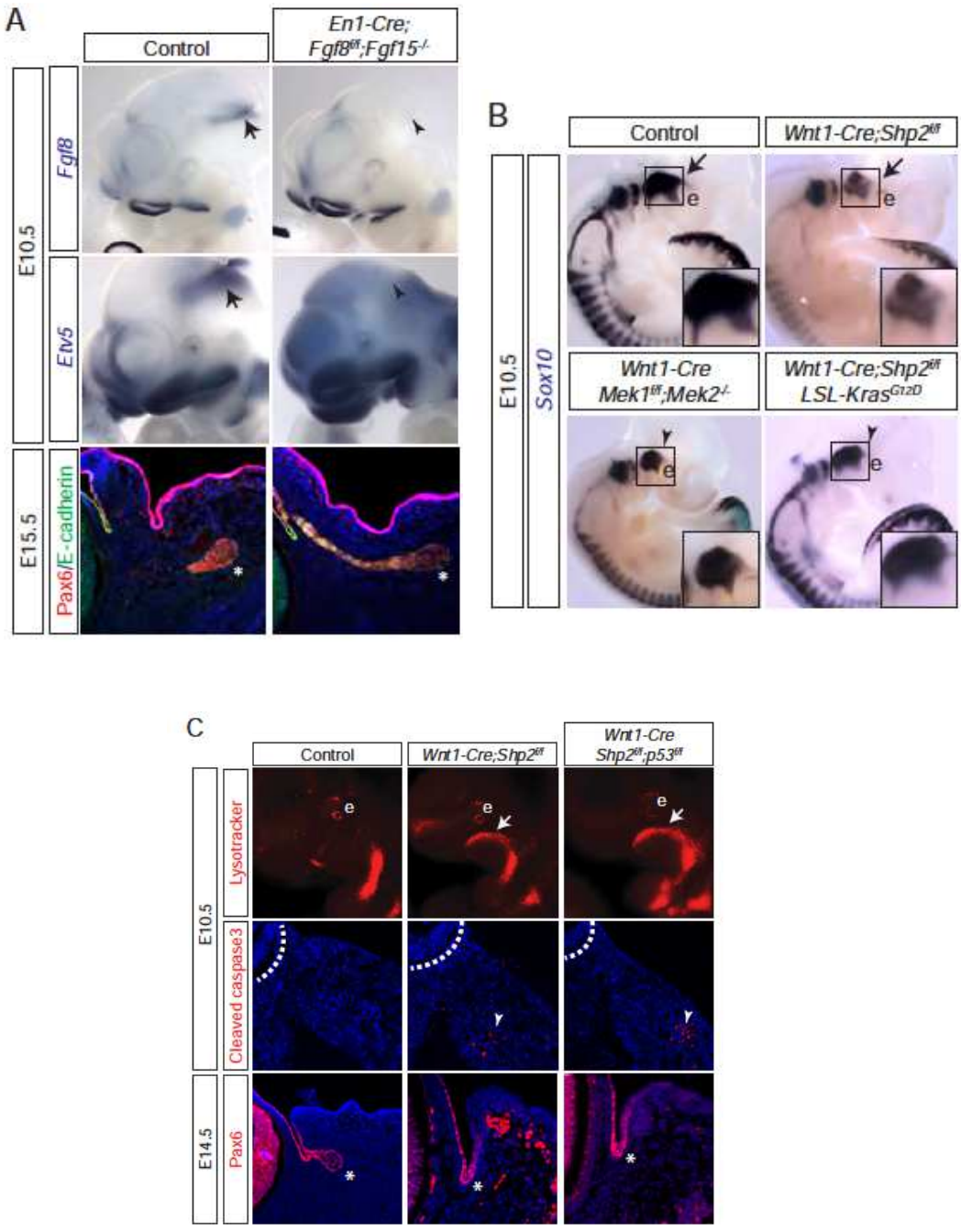


Figure 7. Shp2 regulates MAPK-Etv signaling in the neural crest. (A) FGF signaling target genes *Etv1*, *Etv4* and *Etv5* were expressed in the midbrain-hindbrain junction, branchial arches and nasal placode. These expressions were reduced by deletion of *Shp2* and *Mek1/2* in the neural crest. Arrows point to Etv-expressing regions in the brain. (B) Lacrimal gland budding was lost in *Wnt1-Cre; Mek1$^{f/f}$;Mek2$^{-/-}$* and *Wnt1-Cre; Erk1$^{-/-}$;Erk2$^{f/f}$* mutants, but rescued by constitutive activation of Ras signaling in *Wnt1-Cre; Shp2$^{f/f}$; LSL-Kras^{G12D}* embryos. Arrow: lacrimal gland primordia. (C) *Wnt1-Cre* mediated deletion of *Etv1*, *4* and *5* failed to disrupt lacrimal gland development. (D) Expression of Etv4-Engrailed repressor (EnR) fusion protein in the neural crest led to craniofacial defects (arrow) and reduced lacrimal gland budding (arrowheads), ameliorated in *Wnt1-Cre;Shp2$^{f/f}$;LSL-Kras^{G12D}* embryos (Fig. 7B, arrowheads), supporting a role for Shp2-Ras-MAPK signaling in the post-inductive neural crest cells.

control embryos (Fig. 8B, arrows), suggesting that the loss of Shp2 produces subtle defects in neural crest proliferation and migration. This phenotype was reproduced in *Wnt1-Cre; Mek1*$^{f/f}$*;Mek2*$^{-/-}$ embryos, but ameliorated in *Wnt1-Cre;Shp2*$^{f/f}$*;LSL-Kras*^{G12D} embryos (Fig. 8B, arrowheads), supporting a role for Shp2-Ras-MAPK signaling in the post-inductive neural crest cells. Previous studies in zebrafish suggested that Shp2 may have a MAPK-independent function in preventing p53-mediated apoptosis in the neural crest [134]. Using lysotracker dye to stain acidic lysosomes in cells undergoing apoptosis, we indeed observed extensive cell death in the first pharyngeal arch in E10.5 *Shp2* mutant embryos (Fig. 8C, arrows). In sections, cleaved-caspase 3 staining also detected abnormal cell apoptosis in the periocular mesenchyme, although the apoptotic regions were far removed from the conjunctiva (Fig. 8C, arrowheads). We reasoned that if the apoptosis induced by *Shp2* deletion was indeed dependent or partially dependent on p53, it may be reduced by removal of *p53*. However, ablation of *p53* in *Shp2* mutants failed to prevent cell death in the first pharyngeal arch or to rescue any craniofacial phenotype (Fig. 8C, arrows and arrowheads). In lacrimal gland development, budding morphogenesis was still aborted in *Wnt1-Cre; Shp2*$^{f/f}$*;p53*$^{f/f}$ embryos (Fig. 8C, asterisks, *n*=6). Therefore, p53 was not responsible for the neural crest cell death or lacrimal gland aplasia in *Shp2* mutants.

To determine whether these early neural crest defects affect the periocular mesenchyme development, we crossed *Wnt1-Cre* with the *R26R* Cre reporter to follow the fate of neural crest cells. Interestingly, by the time of lacrimal gland budding at E13.5, the periocular mesenchyme adjacent to the conjunctival epithelium was already occupied by the neural crest derived cells in *Shp2* mutants (Fig. 8D, arrows). Furthermore, the expression of *Pitx2* and *Foxc1*, two markers of the neural crest derived periocular mesenchyme, were similar in control and *Shp2* mutant eyes (Fig. 8E, arrows). Therefore, despite causing an initial delay in neural crest migration and abnormal apoptosis, *Shp2* ablation did not disrupt the occupancy of the periocular mesenchyme by the neural crest-derived cells at the time of lacrimal gland budding. We thus concluded that the subtle neural crest migration, survival and proliferation defects in *Shp2* mutant were unlikely to account for the failure of lacrimal gland development.


A
Control
En1-Cre;
Fgf8fl/fl;Fgf15-/-
E10.5
Fgf8
Etv5
E15.5
Pax6/E-cadherin
B
Control
Wnt1-Cre;Shp2fl/fl
E10.5
Sox10
Wnt1-Cre
Mek1fl/fl;Mek2-/-
Wnt1-Cre;Shp2fl/fl
LSL-KrasG12D
e
C
Control
Wnt1-Cre;Shp2fl/fl
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Cleaved caspase3
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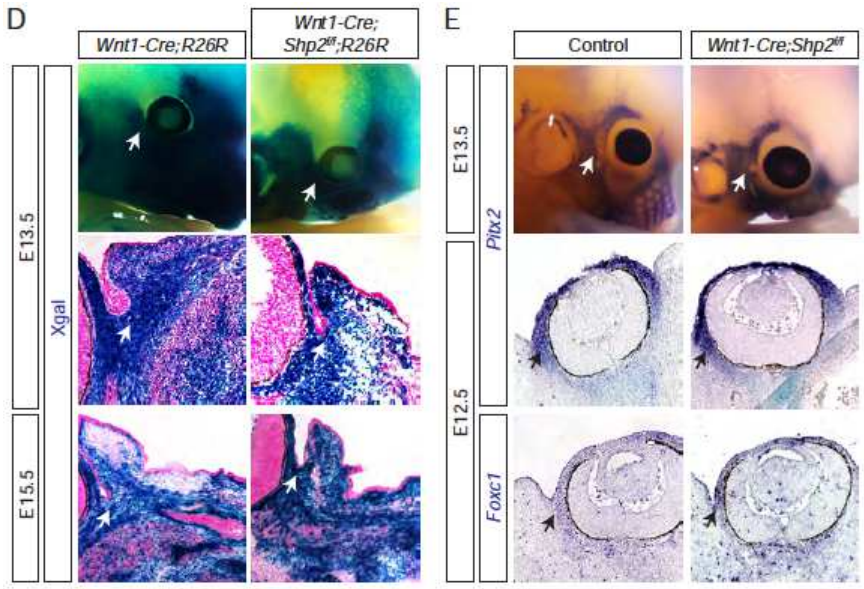


Figure 8. Shp2 deletion did not prevent the neural crest from giving rise to the periocular mesenchyme. (A) In E10.5 *En1-Cre;Fgf8*$^{f/f}$*;Fgf15*$^{-/-}$ embryos, *Fgf8* was ablated in the midbrain- hindbrain junction, where FGF signaling response gene *Etv5* was also down regulated, indicating a loss of FGF signaling. However, lacrimal gland budded at E15.5 was unaffected. Arrow and arrowhead: *Fgf8* and *Etv5* expressions in the midbrain-hindbrain junction. Asterisks: lacrimal gland bud. (B) The migrating neural crest marked by Sox10 expression was reduced in *Wnt1- Cre;Shp2*$^{f/f}$ and *Wnt1-Cre; Mek1*$^{f/f}$*;Mek2*$^{-/-}$ mutants, but rescued in *Wnt1-Cre;Shp2*$^{f/f}$*;LSL*^{KrasG12D} embryos. Arrow and arrowhead: Sox10 positive neural crest cells in the periocular mesenchyme. e: eye. (C) Deletion of *p53* in *Shp2* mutants failed to prevent aberrant apoptosis in the branchial arches shown by lysotracker (upper panel) and in periocular mesenchyme shown by cleaved caspase 3 staining (middle panel). Lacrimal gland budding was not rescued in *Shp2/p53* double mutant (bottom panel). Arrow: lysotracker staining in the branchial arch. Arrowhead: apoptotic cells in the periocular mesenchyme. Asterisk: developing lacrimal gland bud. (D) Lineage tracing by crossing *Wnt1-Cre* with R26R reporter showed that Shp2 ablation did not prevent neural crest cells from populating the periocular mesenchyme after E13.5. Arrow: X-gal stained neural crest cells. (E) Periocular mesenchyme markers *Fox1* and *Pitx2* were unperturbed in *Shp2* mutants.

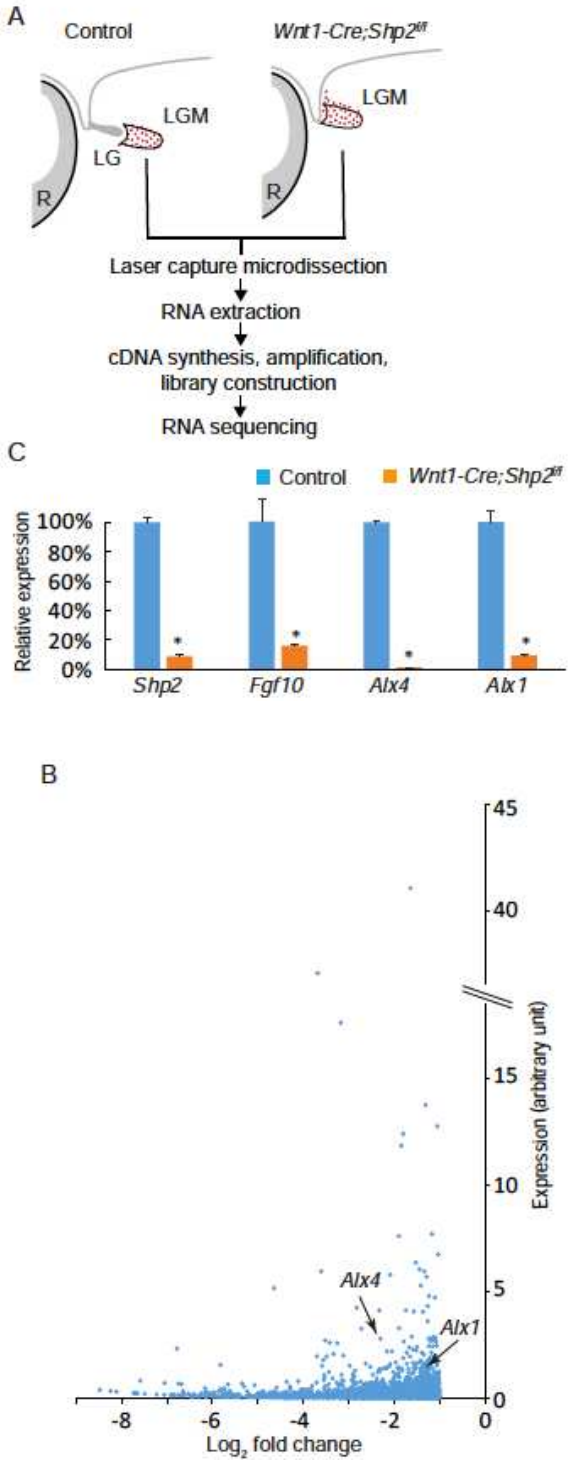
3.1.5 Shp2 signaling regulates Alx1 and Alx4 expression in the periocular mesenchyme

To determine the molecular basis of lacrimal gland defect in *Shp2* mutants, we isolated E14.5 periocular mesenchyme by laser capture micro-dissection and performed RNA-seq analysis (Fig. 9A). Among genes down regulated at least two folds in *Shp2* mutants, the third and eighth most highly expressed transcription factors were *Alx4* and *Alx1*, respectively (Fig. 9B). These results were confirmed by qPCR analysis of micro-dissected tissues, which also showed significant reductions in *Shp2* and *Fgf10* expressions as expected (Fig. 9C).

We next focused on *Alx4* and *Alx1* as downstream targets of Shp2 signaling. At E10.5 and E11.5, *Alx4* was widely expressed in the cranial mesenchyme surrounding the control eye, but the expression was moderately reduced in *Shp2* mutants (Fig. 9D, arrows). At E12.5, more pronounced reduction of *Alx4* expression was evident at the temporal side of mutant eye, where the lacrimal gland bud would have emerged. By E13.5, *Alx4* expression was absent in the periocular region except at the dorsal side, but recovered in *Wnt1-Cre;Shp2*$^{f/f}$*;LSL-Kras*^{G12D} embryos (Fig. 9D, arrowheads). Immunostaining on sections further confirmed that *Shp2* deletion led to progressive down regulation of Alx4 in the periocular mesenchyme, until it was entirely lost by E14.5 (Fig. 9E, arrows). Similarly, *Alx1* in control embryos was expressed just anterior to the elongating lacrimal gland bud at E14.5, but this domain of *Alx1* expression vanished in *Shp2* mutant embryos (Fig. 9E, arrowheads). These results demonstrate that the periocular expressions of both *Alx1* and *Alx4* are regulated by Shp2 signaling.

3.1.6 Alx4 binds a terrestrially conserved *Fgf10* genomic element to regulate its expression in the lacrimal gland mesenchyme

The results above revealed a close resemblance of *Alx1* and *Alx4* expressions in the periocular mesenchyme to that of *Fgf10* during embryonic development. To evaluate this further, we examined their expression patterns in the neonatal lacrimal gland. At postnatal day 0 (P0), *Fgf10* was detectable in the mesenchymal cells, whereas the FGF-


A
Control
Wnt1-Cre;Shp2f/f
LGM
LG
R
R
LGM
Laser capture microdissection
RNA extraction
cDNA synthesis, amplification, library construction
RNA sequencing
C
Control
Wnt1-Cre;Shp2f/f
Relative expression
100%
80%
60%
40%
20%
0%
Shp2
Fgf10
Alx4
Alx1
B
45
40
15
10
5
0
Expression (arbitrary unit)
Alx4
Alx1
-8
-6
-4
-2
0
Log2 fold change

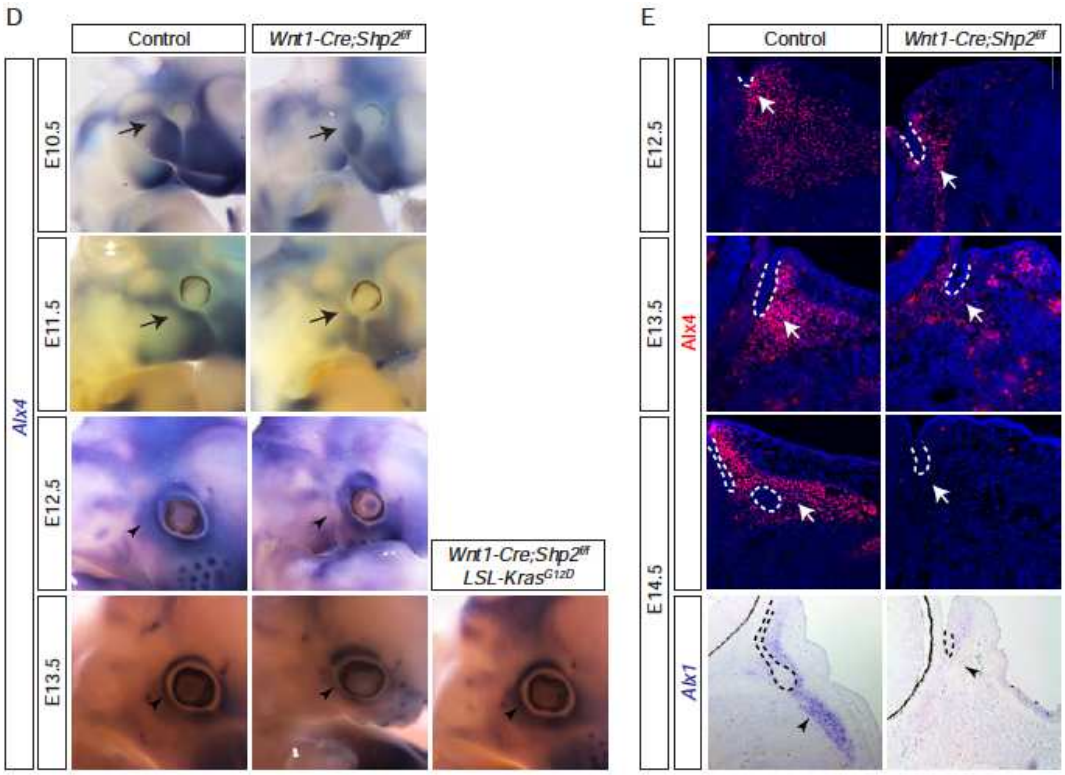
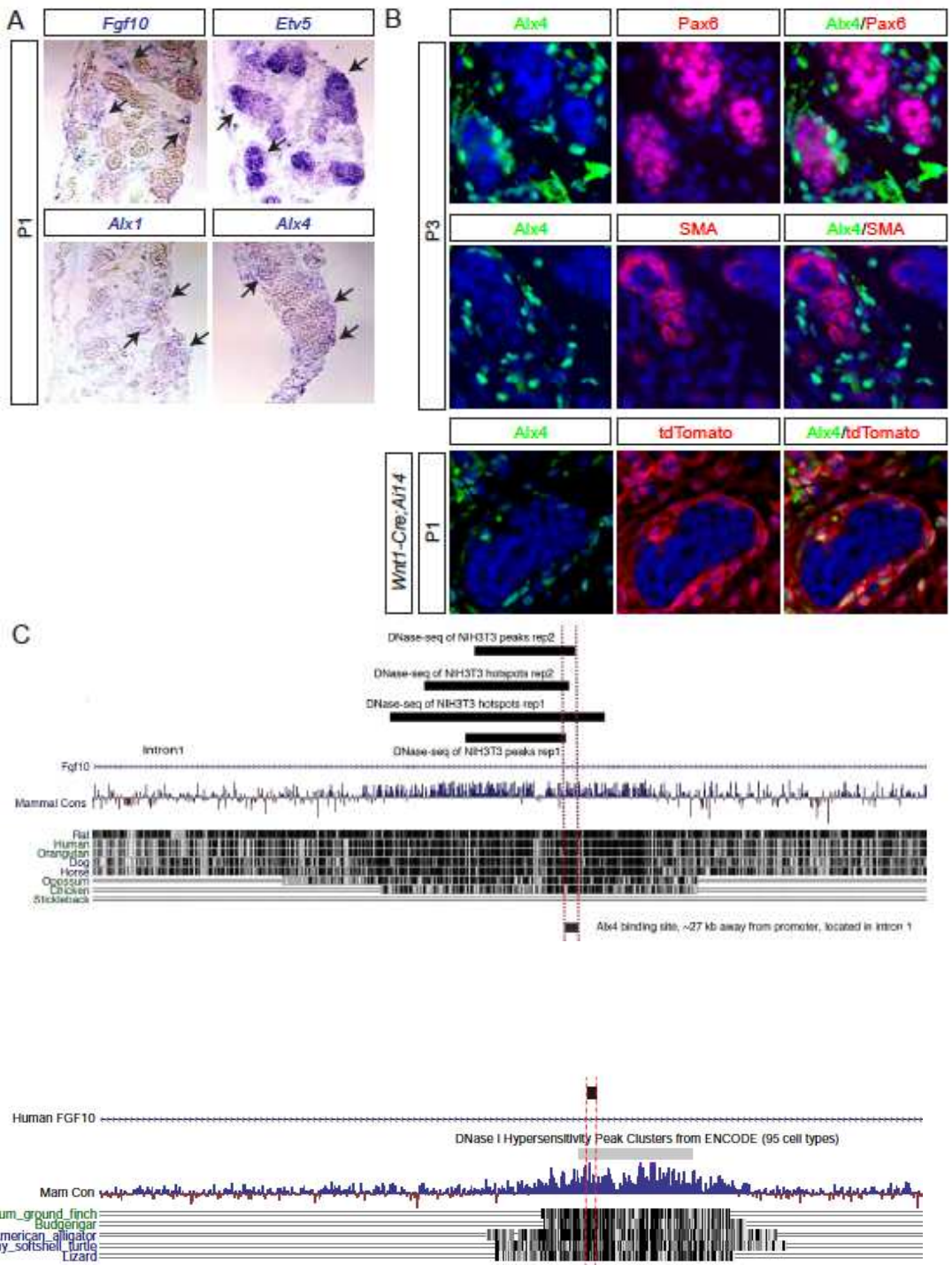


Figure 9. Identification of Alx genes downstream to Shp2 in lacrimal gland development. (A) Schematic diagram of laser capture microscopy to isolate the periocular mesenchyme for RNA-seq analysis. (B) Dot plot of genes down regulated at least two folds in *Shp2* mutant. *Alx4* and *Alx1* genes is marked by arrow. (C) qRT-PCR confirmed the deletion of *Shp2* and down regulation of Fgf10, Alx1 and Alx4 in the laser captured periocular mesenchyme from *Shp2* mutant. Student's t test: *P<0.001, n=3. (D) *Shp2* deletion reduced *Alx4* expression in the cranial mesenchyme, especially at the periocular region next to the future lacrimal gland at E13.5, which was ameliorated in *Wnt1-Cre;Shp2$^{f/f}$;LSL-Kras^{G12D}* embryos. Arrow: *Alx4* expression in the cranial mesenchyme at E10.5 and E11.5. Arrowhead: *Alx4* expression in the periocular mesenchyme at E12.5 and E13.5. (E) In *Shp2* mutants, Alx4 was progressively reduced in the periocular mesenchyme adjacent to the conjunctival epithelium from E12.5 to E13.5. By E14.5, both Alx1 and Alx4 were lost. Arrow: Alx4 immunostaining in the periocular mesenchyme. Arrowhead: *Alx1* expression surrounding the lacrimal gland bud. Lacrimal gland primordia are outlined in dotted lines.

inducible gene *Etv5* was expressed in the adjacent ducts and acini, suggesting that FGF signaling remained active at this stage (Fig. 10A, arrows). As expected, both *Alx1* and *Alx4* mRNA were also found in the lacrimal gland mesenchyme. By immunostaining, we further demonstrated that P3 lacrimal gland expressed Alx4 protein, which was separated from both epithelial marker Pax6 and myoepithelial marker SMA (Fig. 10B). Finally, to trace the origin of these Alx4-expressing cells in the lacrimal gland, we crossed *Wnt1-Cre* with an *R26TdT* (*Ai14*) reporter to indelibly label the neural crest-derived cells with tdTomato fluorescence. By double immunostaining, we confirmed that Alx4 resided exclusively in the tdTomato-positive cells, demonstrating that Alx4 persisted in the neural crest lineage during lacrimal gland development.

Based on the similarity between *Alx1/4* and *Fgf10* expression patterns in lacrimal gland development, we hypothesized that Alx1/4 were direct regulators of *Fgf10* transcription. Since the lacrimal gland system was an adaptation of terrestrial animals to the airy environment, we searched *Fgf10* locus for regions that were evolutionarily conserved from human to chicken but not to stickleback fish (Fig. 10C). We next overlaid these regions with DNase hypersensitive sites in 3T3 fibroblast cell line identified by the ENCODE project, because this cell line expressed both *Alx4* and *Fgf10* at high levels [140]. Finally, we screened these sequences using the Alx1/3/4 binding motif and identified a perfect match within the intron 1 of *Fgf10* (Fig. 10D). Interestingly, sequence alignment showed that this site was evolutionarily conserved in amphibians such as lizard which have the lacrimal gland, but not in Xenopus frog, which lacks the lacrimal gland [141]. Even among animals living both on land and in water, the lacrimal gland is only present in reptiles such as alligator, but not in amphibians such as frog (Fig. 10C). To ascertain whether this sequence was a bona fide Alx binding site, we performed chromatin immunoprecipitation in 3T3 cells followed by qPCR using specific primers. Compared to IgG control, there was a ~3 fold enrichment of this putative Alx binding element in chromatins pulled down by Alx4 antibody (Fig. 10E). This was further validated in vivo by Alx4 chromatin immunoprecipitation using the lacrimal gland mesenchyme isolated from neonatal pups, which resulted in a ~11 fold enrichment. We


A
Fgf10
Etv5
Alx1
Alx4
P1
B
Alx4
Pax6
Alx4/Pax6
P3
Alx4
SMA
Alx4/SMA
Alx4
tdTomato
Alx4/tdTomato
Wnt1-Cre;Ai14
P1
C
DNase-seq of NIH3T3 peaks rep2
DNase-seq of NIH3T3 hotspots rep2
DNase-seq of NIH3T3 hotspots rep1
DNase-seq of NIH3T3 peaks rep1
Intron1
Fgf10
Mammal Cons
Rat
Human
Orangutan
Dog
Horse
Opossum
Chicken
Stickleback
Alx4 binding site, ~27 kb away from promoter, located in intron 1
Human FGF10
DNase I Hypersensitivity Peak Clusters from ENCODE (95 cell types)
Mam Con
Medium_ground_finch
Budgerigar
American_alligator
Spiny_softshell_turtle
Lizard

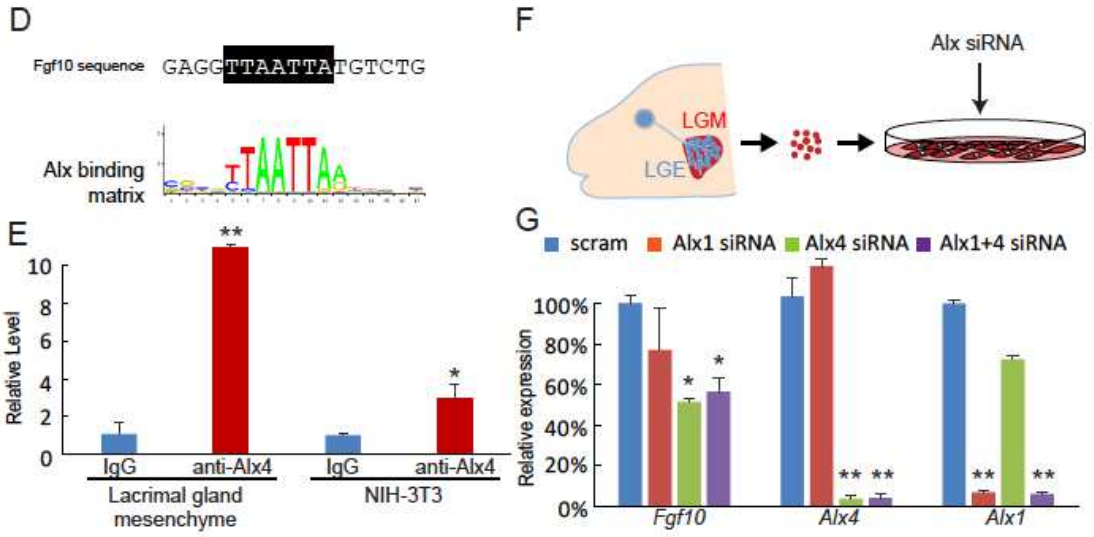


Figure 10. Alx4 binds a terrestrially conserved element in Fgf10 locus. (A) In new born pups, *Alx1*, *Alx4* and *Fgf10* were expressed in the lacrimal gland mesenchyme, whereas FGF response gene Etv5 was expressed in the epithelium. (B) Alx4 protein was excluded from Pax6-positive epithelial cells and SMA-positive myoepithelial cells, but it was expressed in the neural crest derived mesenchymal cells labeled by *Wnt1-Cre* induced tdTomato fluorescence. (C) Sequence alignment identified an Alx4 site within an intronic region of Fgf10, which was conserved from human to lizard, but not to Xenopus and fish. It resided next to DNase hypersensitivity peaks in NIH3T3 cells. (D) The Alx4 site in *Fgf10* locus matched with the Alx consensus sequence. (E) Chromatin immunoprecipitation showed that Alx4 directly bound the *Fgf10* intronic site in both lacrimal gland mesenchyme and NIH3T3 cells. Student's t test: *P<0.01, n=4; **P<0.001, n=3. (F) Schematic diagram of mesenchymal cell culture isolated from newborn pups and treatment with Alx siRNA. LGM: lacrimal gland mesenchyme. LGE: lacrimal gland epithelium. (G) Alx4 siRNA significantly down regulated Fgf10 expression in lacrimal mesenchymal cells, whereas additional application of Alx1 siRNA did not lead to further reduction. One Way ANOVA: *P<0.01, **P<0.001, n=3.

next knocked down *Alx1* and *Alx4* using siRNAs in cultured lacrimal gland mesenchymal cells (Fig. 10F). Interestingly, *Alx1* depletion led to a modest reduction in *Fgf10* mRNA level, but the effect was not statistically significant (Fig. 10G). In contrast, *Alx4* knockdown decreased Fgf10 expression by ~50%, which was not further reduced by combined treatment of both *Alx1* and *Alx4* siRNAs. This suggested that *Alx4* plays a more dominant role than *Alx1* in regulating *Fgf10* in the lacrimal gland mesenchyme.

3.1.7 Alx4 is required for lacrimal gland development in mouse and human

To determine the functional role of Alx4 in lacrimal gland development, we analyzed $Alx4^{lst\text{-}J}$ mice, which carried a frameshift mutation removing both the homeodomain and downstream CAR domain. Homozygous $Alx4^{lst\text{-}J}$ animals displayed craniofacial defects, dorsal alopecia and preaxial polydactyly at birth as previously reported in *Alx4* knockouts [142, 143]. At E14.5, $Alx4^{lst\text{-}J}$ homozygous embryos maintained Connexin43 and Col2a1 expressions in the periocular mesenchyme, but the domain of *Alx1* expression was more restricted (Fig. 11A, arrows). Importantly, there was a drastic reduction of *Fgf10* adjacent to the lacrimal gland bud (Fig. 11A, arrows), accompanied by down regulation of FGF-target genes *Etv4* and *Etv5* in the lacrimal gland epithelium (Fig. 11A, dotted lines). At E16.5, histology and immunostaining revealed a complete loss of Alx4 expression in the periocular mesenchyme and a much shorter Pax6-expressing lacrimal gland bud, characterized by reduced phospho-Histone H3 (pHH3) and increasing TUNEL signal (Fig. 11B, dotted lines). By P1, no lacrimal gland was detectable by Carmine staining in $Alx4^{lst\text{-}J}$ homozygous pups (Fig. 11B, black arrows). These results demonstrated that inactivation of *Alx4* markedly disrupted Fgf10 expression and downstream FGF signaling, affected cell proliferation and survival, and ultimately caused a failure of lacrimal gland development.

In human, *ALX4* loss-of-function mutations underlie autosomal recessive frontonasal dysplasia 2 syndrome, characterized by skull defects, wide nasal bridge, notched nares, depressed nasal tip, hypertelorism and alopecia (OMIM 613451). We reanalyzed one patient carrying a homozygous c.503delC mutation in exon 2 of *ALX4* gene, which resulted in truncation of the homeobox (HD) and C-terminal OAR domains [144]. MRI

imaging in that patient revealed an absence of lacrimal glands bilaterally (Fig. 11C, arrows). The patient lacked tearing and experienced irritable eyes and multiple episodes of eye infection since birth. This finding is consistent with the role of *ALX4* in human lacrimal gland formation.

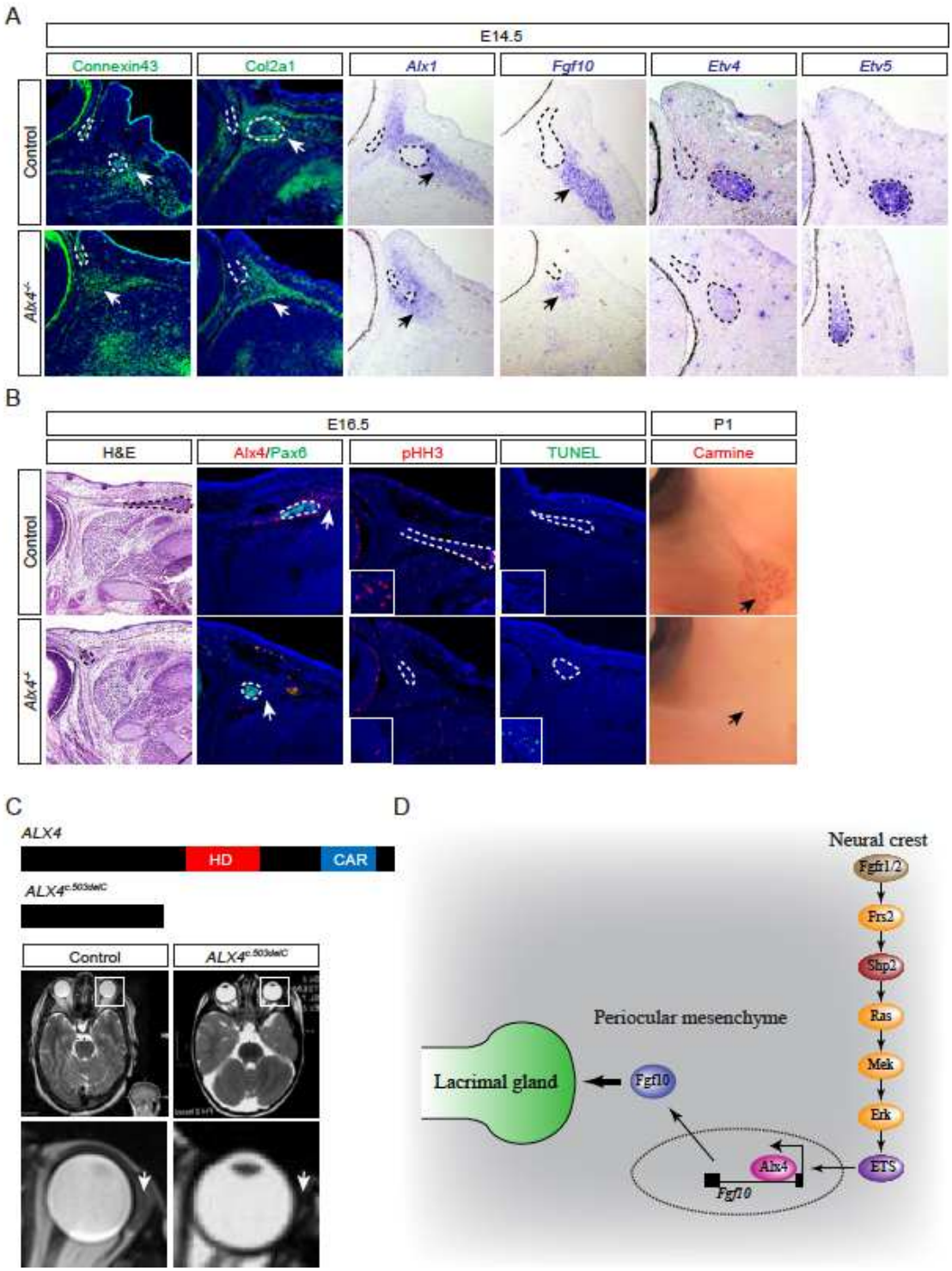


Figure 11. Alx4 inactivation led to lacrimal gland aplasia in human and mouse. (A) In E14.5 *Alx4* knockout embryos, Connexin43 and Col2a1 expression remained in the periocular mesenchyme, whereas Alx1 expression domain was reduced. Fgf10, Etv4 and

Etv5 were significantly down regulated. Arrows: staining in the periocular mesenchyme. Lacrimal gland buds are outlined in dotted lines. (B) E16.5 *Alx4* null mutant displayed only rudimentary lacrimal gland shown by histology and Pax6 staining, while Alx4 immunostaining was lost. There were reduction of pHH3 and increase in TUNEL staining in the residual bud (Inserts showed magnified region of lacrimal gland buds). At P1, carmine staining revealed an absence of lacrimal gland in Alx4 null pups. Lacrimal gland buds are outlined in dotted lines. (C) MRI revealed bilateral absence of lacrimal gland in a patient carrying c.503delC mutation that removed the functional domains of ALX4. Lower panel showed enlarged region of the eye and arrows point to the lacrimal gland. (D) Model of neural crest Shp2 signaling in lacrimal gland development. Shp2 mediates FGF signaling in the developing neural crest to activate Ras-MAPK signaling, which is required for Alx4 expression in the periocular mesenchyme. By binding to an intronic element of Fgf10, Alx4 activates Fgf10 expression to induce lacrimal gland budding.

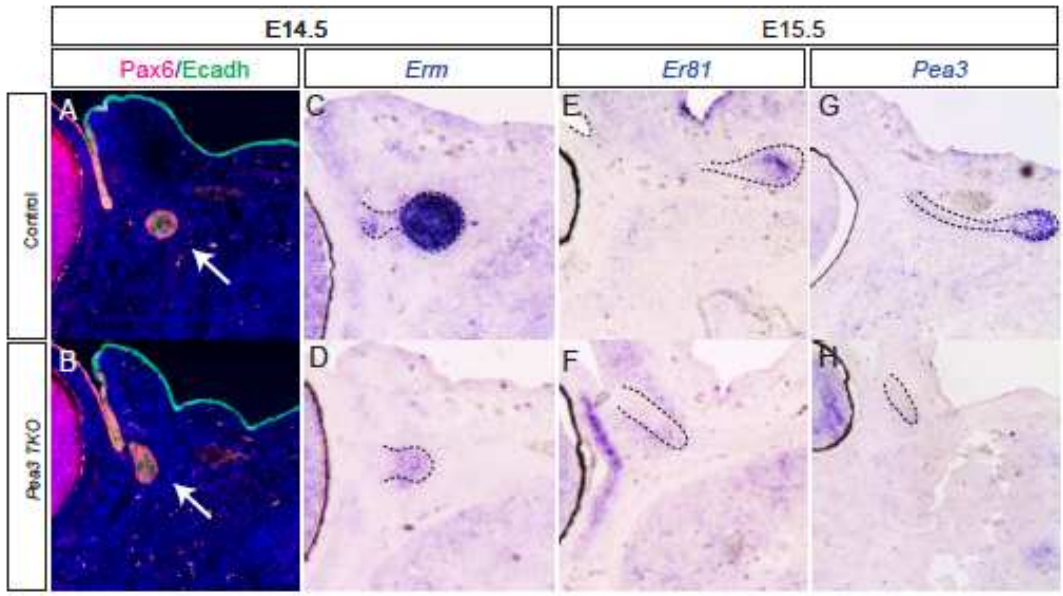
3.2 Etv/Pea3 transcription factors are required for establishing lacrimal gland cell fate, duct elongation and branching morphogenesis

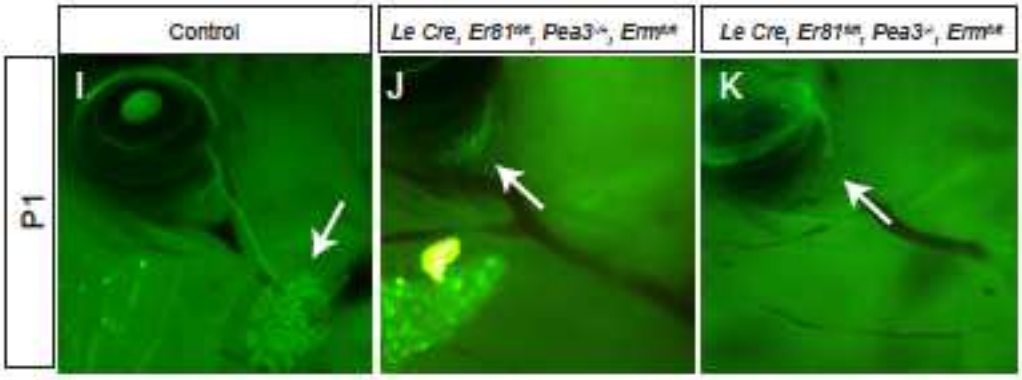
3.2.1 Pea3 transcription factors are critical for lacrimal gland development

Lacrimal gland development begins with the thickening of the conjunctival epithelium at E13.5 and forms a bud invaginating into the surrounding periocular mesenchyme by E14.5. This process is triggered by the mesenchymal Fgf10 which activates Fgf signaling in the epithelium. Pea3 family of Ets transcription factors: Pea3/Etv4, Erm/Etv5, Er81/Etv1, which are early response genes downstream of Fgf signaling are upregulated in the lacrimal gland epithelial bud (Fig. 12C, E and G, dotted lines). In order to study the function of these transcription factors, we conditionally deleted two members of the Pea3 family of transcription factors, Erm and Er81 in the *Pea3* KO background using *Le-Cre* transgenic mouse line, where Cre-recombinase is linked to an IRES-GFP reporter [43]. Deletion of *Pea3* genes was confirmed by in situ hybridization of *Etv4, Etv5* and *Er81* (Fig. 12D, F and H, dotted lines). In *Le-cre, Pea3*$^{-/-}$*, Erm*$^{fl/fl}$*, Er81*$^{fl/fl}$ (hereafter, referred to as *Pea3 TKO*), we found that the lacrimal gland bud is smaller in size compared to control at E14.5 as shown by the expression of lacrimal gland progenitor cell marker, Pax6 and the epithelial marker, E-cadh (Fig. 12A, B, arrows). Analysis at post-natal stages P1-3 showed that duct elongation and branching are severely affected as evident by malformed gland marked by GFP expression in *Pea3 TKO* (Fig. 12I-L). This phenotype in *Pea3 TKO* is more severe compared to the mice carrying one normal copy of *Pea3* gene, indicating that Pea3 is not redundant during lacrimal gland development. These results indicate that Pea3 transcription factors are important for lacrimal gland development.

3.2.2 Pea3 transcription factors mediate Fgf signaling during lacrimal gland development

In order to decipher the gene regulatory network of Pea3 transcription factors, mouse lacrimal gland epithelial tissue from control (*Le-Cre*) and mutant (*Pea3 TKO*) mice were micro-dissected using laser capture microscopy and subjected to RNA-seq at E14.5 stage (n=3 per condition, Fig. 13A). Unbiased clustering analysis of the normalized data


E14.5
E15.5
Pax6/Ecadh
Erm
Er81
Pea3
Control
Pea3 TKO
A
B
C
D
E
F
G
H


Control
Le Cre, Er81fx, Pea3-/-, Ermfx
Le Cre, Er81fx, Pea3-/-, Ermfx
P1
I
J
K

L

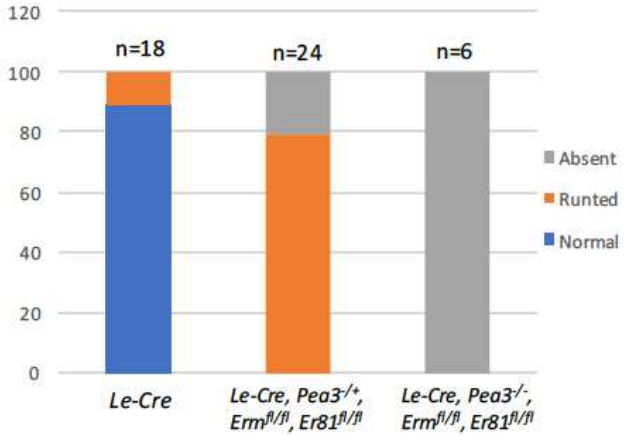


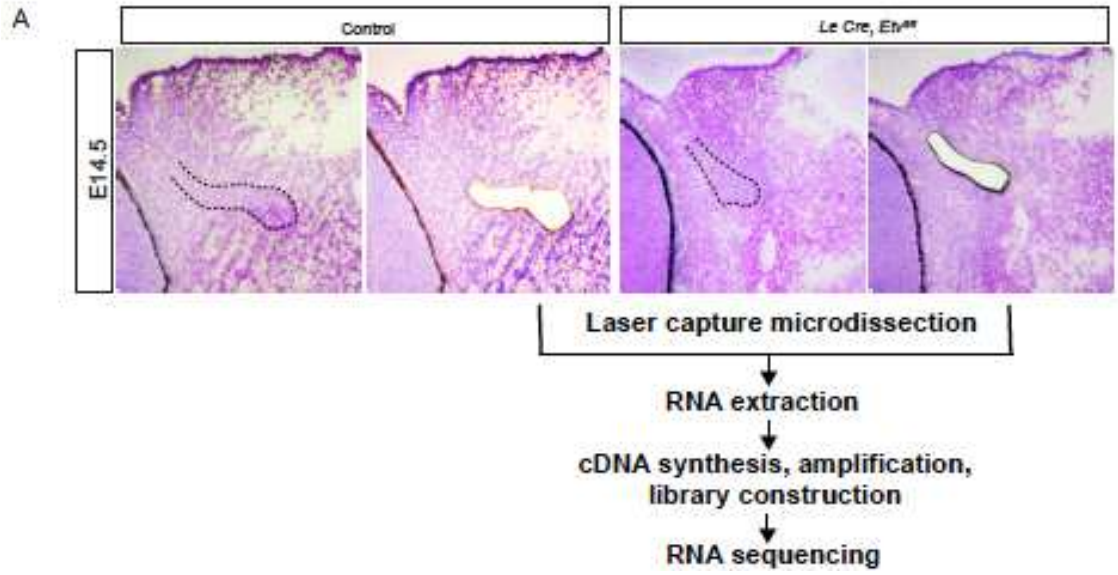
Figure 12. Pea3 transcription factors are important for lacrimal gland development. (A-H) *Le-Cre* mediated deletion of *Pea3* genes resulted in smaller lacrimal gland buds (A-B, arrows) and abrogation of expression of Pea3 transcription factors Erm, Er81 and Pea3 at E14.5-15.5 stage (C-H, dotted lines). (I-L) Analysis at post-natal stage, P1, demonstrated that complete lack of Pea3 transcription factors led to more severe phenotype as shown by GFP (I and K, arrows) in comparison to embryos which have one wild-type copy of *Pea3* still present (J and K, arrows).

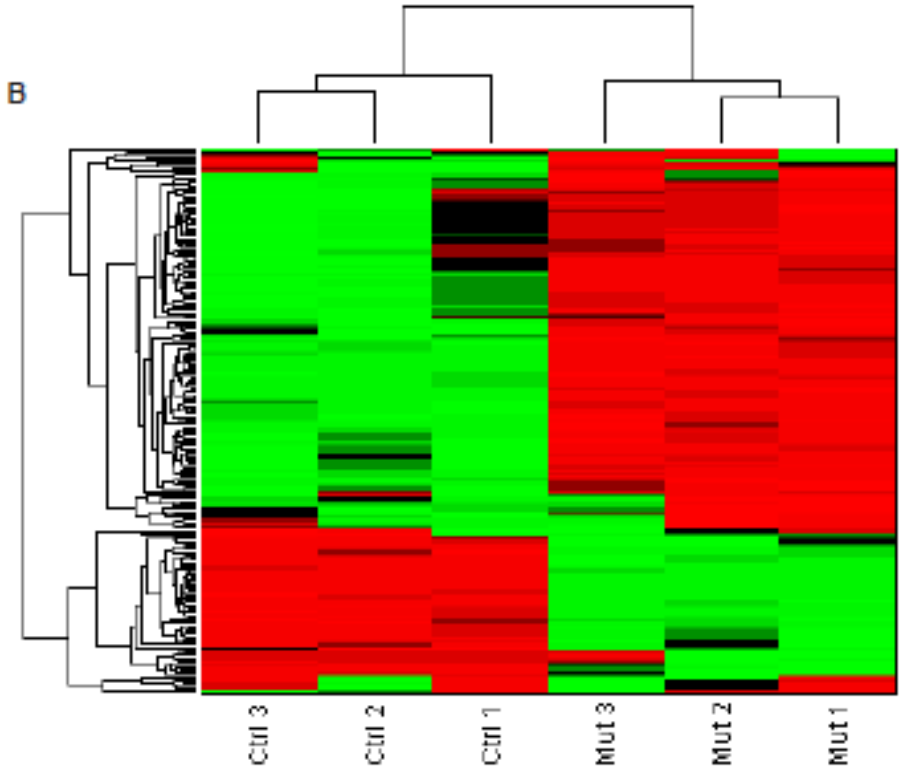
revealed that control and mutant samples were clustered in two separate groups and data from individual samples within each group were highly correlated (r= 0.8, Fig. 13B). These results indicated the robustness of our data. To analyze which signaling pathways or processes are downregulated in *Pea3 TKO* mutants, KEGG pathway analysis using DAVID was performed, which showed that several genes involved in PI3-Akt, Ras pathways and ECM receptor interaction processes are significantly downregulated (Fig. 13C).

These data were further validated by gene set enrichment analysis (GSEA) using a reference database previously published for lacrimal gland epithelium in Fgfr2 conditional knockout mice generated in a similar fashion as described above at E13.5 stage in mouse [33]. GSEA is a computational tool which evaluates whether a given set of genes shows a statistical significant difference between two biological states (phenotypes). The results of this analysis revealed that significantly downregulated genes in *Pea3 TKO* mutants were also downregulated in Fgfr2 conditional mutants, with normalized enrichment score (NES) as -6.8 and $p < 0.01$(Fig. 13D). NES is a statistic to compare the enrichment results and takes into account the differences in the size of the genesets as well as the different correlations between the genesets and expression datasets. Similarly, significantly upregulated genes in *Pea3 TKO* mouse mutants are downregulated in the Fgfr2 dataset (NES = -4.4, $p < 0.01$) (Fig. 13E). One of the reasons could be that since Fgfr2 is at the very top of the signaling cascade, its ablation can suppress the upregulation of compensatory genes in *Pea3* mutants. Taken together, these results are consistent with the earlier findings that Pea3 family of genes are downstream of the Fgf signaling cascade.

3.2.3 Pea3 genes are required for establishing the lacrimal gland fate

From RNA-seq data, another interesting observation was the upregulation of keratins-Keratin 5, 14, 17 in *Pea3 TKO* mutant mice, confirmed by immunostaining against Krt14

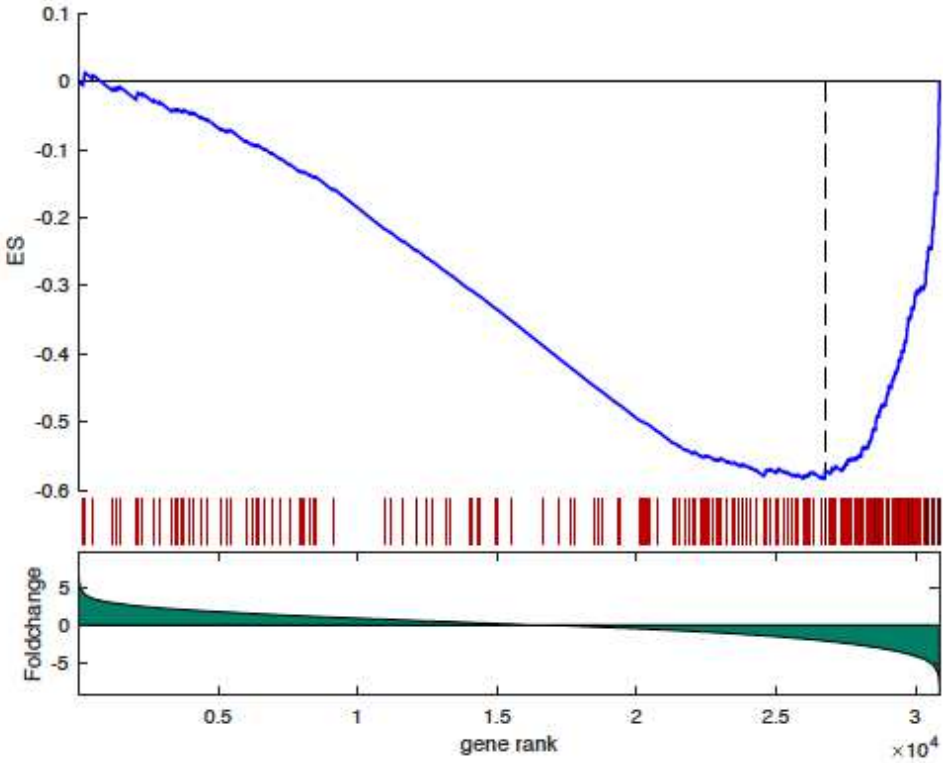

A
Control
Le Cre, Etv
E14.5
Laser capture microdissection
RNA extraction
cDNA synthesis, amplification,
library construction
RNA sequencing


B
Ctrl 3
Ctrl 2
Ctrl 1
Mut 3
Mut 2
Mut 1

C

DAVID- KEGG Pathway analysis | Downregulated genes	p-value
Protein digestion and absorption	7.20E-04
ECM-receptor interaction	7.30E-02
PI3K-Akt signaling pathway	5.10E-02
Pathways in cancer	6.30E-02
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	7.20E-02
Cell adhesion molecules (CAMs)	2.60E-01
Proteoglycans in cancer	3.00E-01
Hippo signaling pathway	3.30E-01
Ras signaling pathway	3.80E-01
Transcriptional misregulation in cancer	3.60E-01

D



E

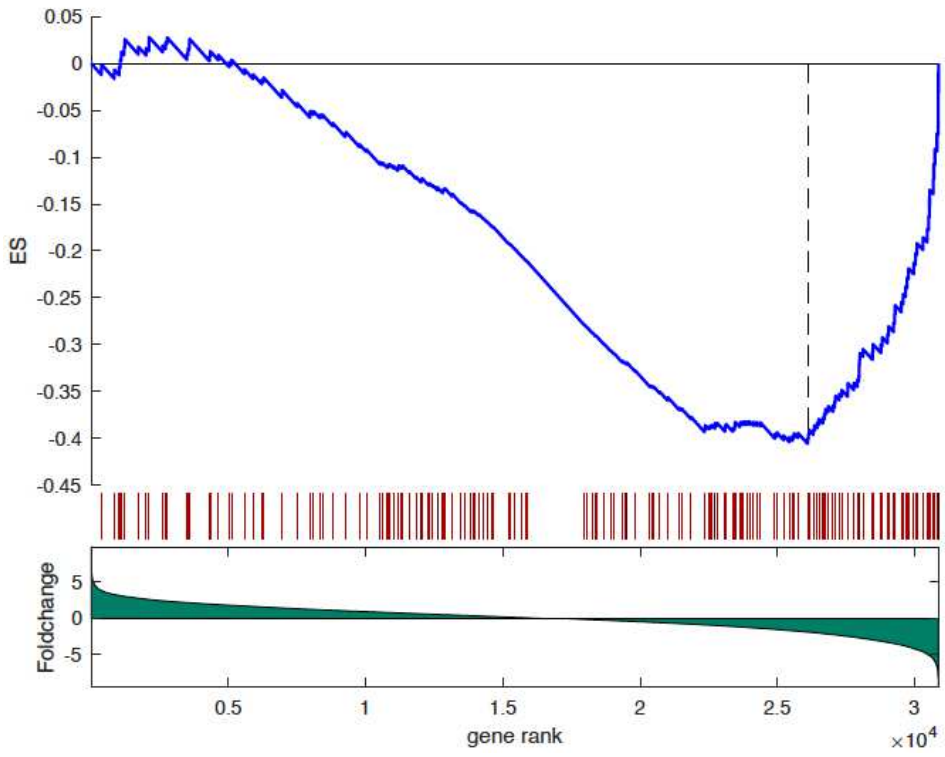
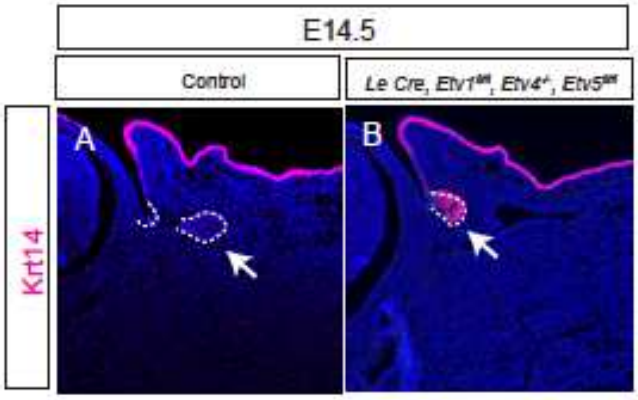


Figure 13. Bioinformatics analysis indicates Pea3 transcription factors are downstream of Fgf signaling during lacrimal gland development. (A) Schematic of RNA-seq following Laser capture microdissection of lacrimal gland bud in control and *Pea3 TKO* mutants. Lacrimal gland images of before and after the dissection process are shown. (B) Clustergram analysis of top 200 differentially expressed genes in RNA-seq data from 6 samples (Control, n=3, Mutant, n=3), showed that control and mutant samples form two separate clusters, indicating robustness of our samples (r = 0.8). (C) KEGG pathway analysis of downregulated genes in *Pea3* mutant using DAVID indicated several key pathways such as PI3-AKT, Receptor interaction, MAPK, Glycosaminoglycan synthesis pathways were significantly downregulated. (D-E) Gene set enrichment analysis of downregulated genes in *Pea3* mutants indicated that these genes were significantly enriched in reference database curated from published RNA-seq data from the *Fgfr2*-deleted conjunctival epithelium at the time of lacrimal gland development (D, $p<0.01$). Similar analysis with upregulated geneset also shows significant enrichment (E, $p<0.01$).

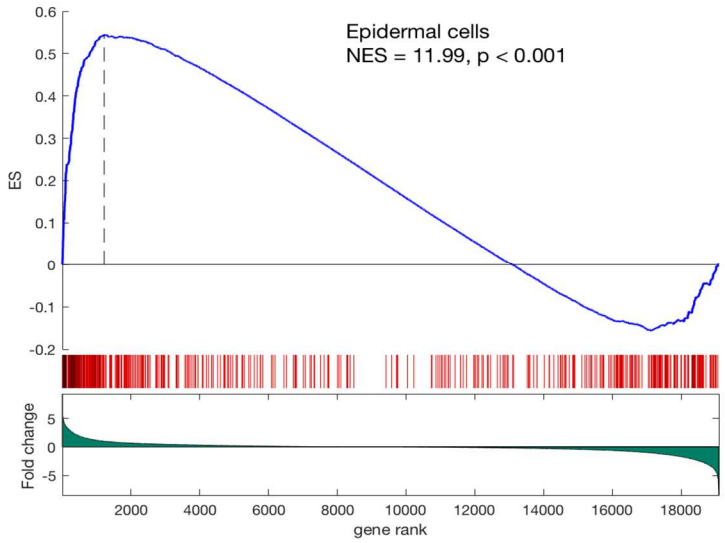
(Fig. 14A, B, arrows). These keratin genes are typically expressed in skin cells, more specifically in the epidermis, although the lacrimal gland progenitor cell marker Pax6 was still present in *Pea3 TKO* mutants (Fig. 12A, B). This led us to hypothesize that there was shift in cell identity from the lacrimal gland fate to the epidermal skin-like fate. To test this idea, we performed GSEA of differentially upregulated genes in *Pea3 TKO* mutants with reference to published gene expression datasets of different cell types present in mouse embryonic skin at E14.5 stage [145]. Upon analysis, we found that there is significant enrichment of upregulated genes in the epidermis and placodal datasets (epidermis- $p< 0.001$; placodal cells- $p<0.001$) (Fig. 14C, D). Notably, similar analysis with other skin cell types such as dermis, schwann cells, melanoctyes, and fibroblasts did not show any enrichment (Fig. 14E-I). Supporting this observation, we also performed principal component analysis (PCA) of our data with the published gene expression datasets of different cell types within the embryonic skin. PCA reduces the dimensionality of the data and linearly maps the data into lower-dimensional space in such a way that it captures the maximum variation in the data. This analysis revealed that the gene expression profile of *Pea3 TKO* mutant, but not that of the control lacrimal gland, clusters with gene expression pattern of epidermal cells along first and second principal component axes as well as first and third principal component axes (Fig. 14J, K). These data demonstrate that Pea3 transcription factors are required for maintaining the lacrimal gland cell fate during its development, absence of which pushes the lacrimal gland progenitor cells into the skin-like cell fate.

3.2.4 Pea3 genes control expression of Six1 and Six2 required for lacrimal gland development

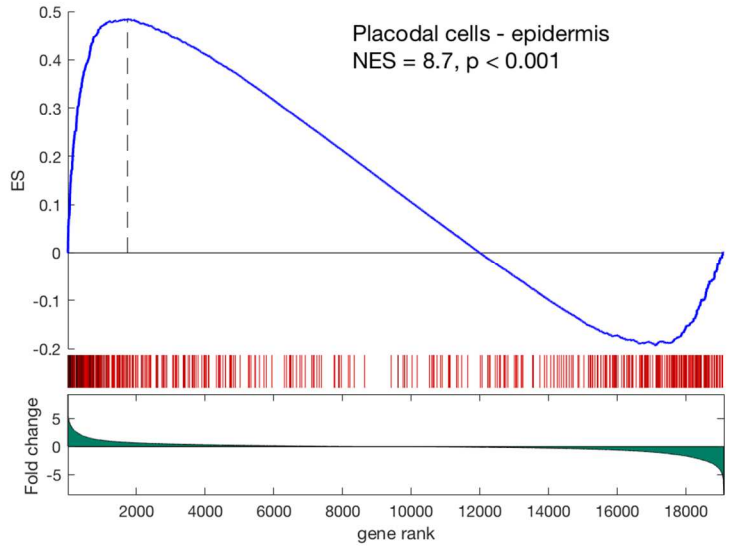
To understand the mechanism of this change in the cell fate, we sought to determine the most differentially regulated genes in our dataset. For this analysis, gene expression values, Log_2 (fold change), was plotted on x-axis against the corresponding Log_2 (p-value) on y-axis (Fig. 15A). Several known Fgf-responsive genes such as *Spry4*, *Dusp6*,


E14.5
Control
Le Cre, Etv1fl, Etv4+, Etv5fl
A
B
Krt14

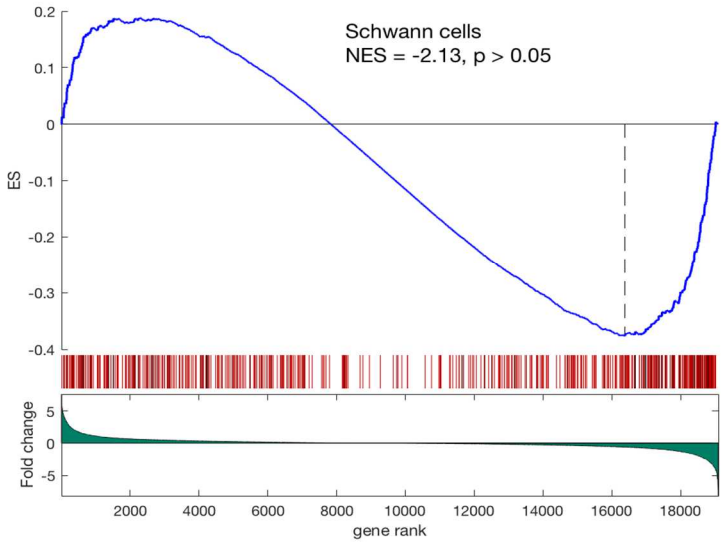
C


Epidermal cells
NES = 11.99, p < 0.001
ES
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
Fold change
5
0
-5
2000
4000
6000
8000
10000
12000
14000
16000
18000
gene rank

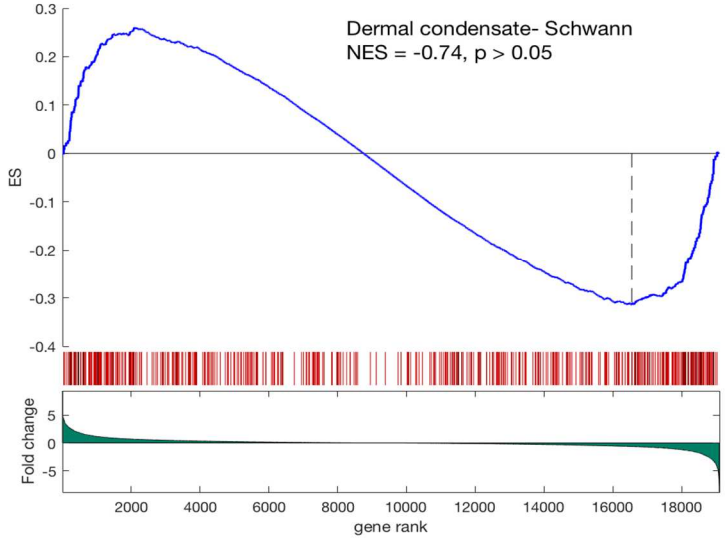
D


Placodal cells - epidermis
NES = 8.7, p < 0.001
ES
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
Fold change
5
0
-5
2000
4000
6000
8000
10000
12000
14000
16000
18000
gene rank

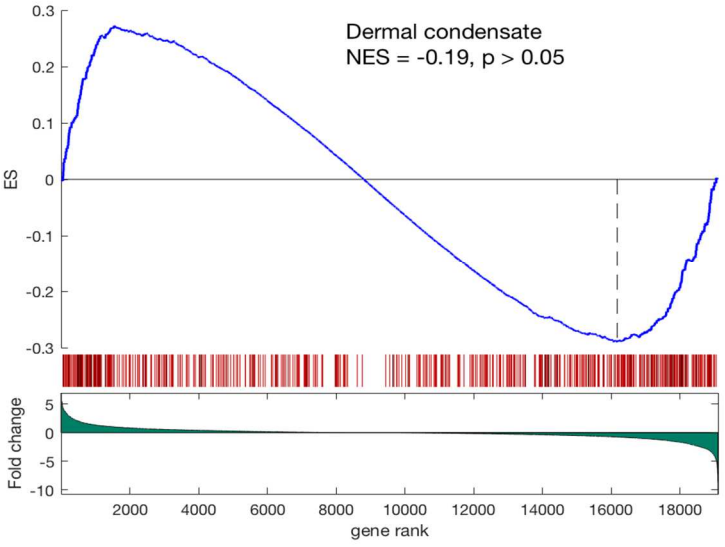
E


Schwann cells
NES = -2.13, p > 0.05
ES
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
Fold change
5
0
-5
2000
4000
6000
8000
10000
12000
14000
16000
18000
gene rank

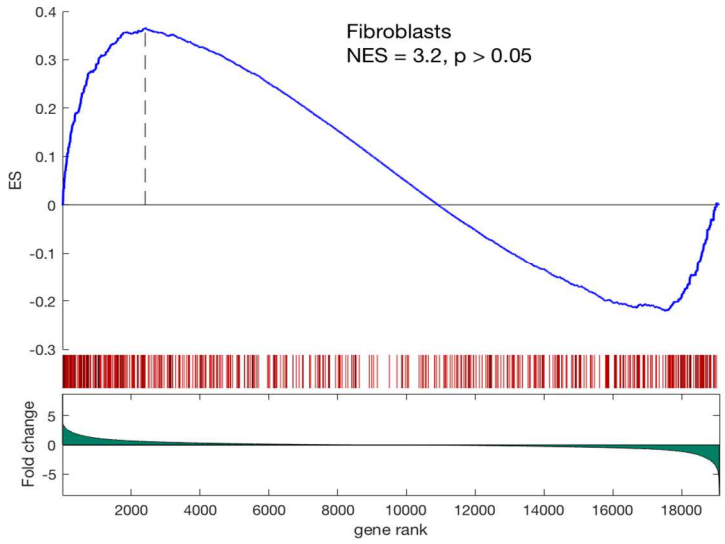
F


Dermal condensate- Schwann
NES = -0.74, p > 0.05
ES
Fold change
gene rank

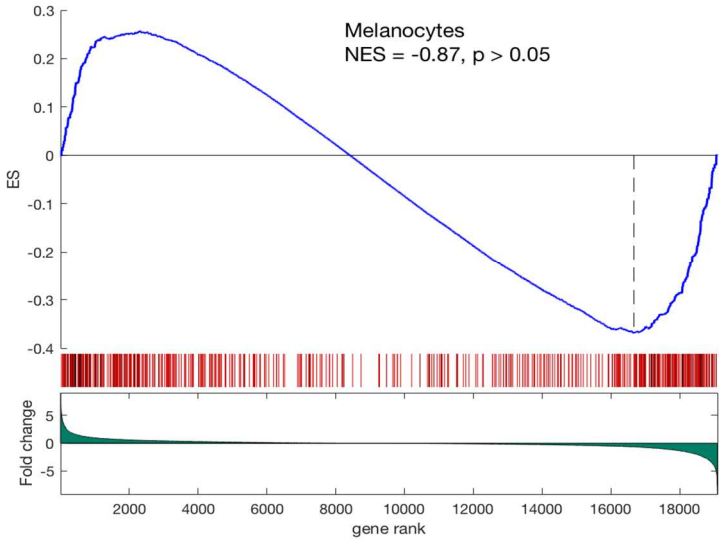
G


Dermal condensate
NES = -0.19, p > 0.05
ES
Fold change
gene rank

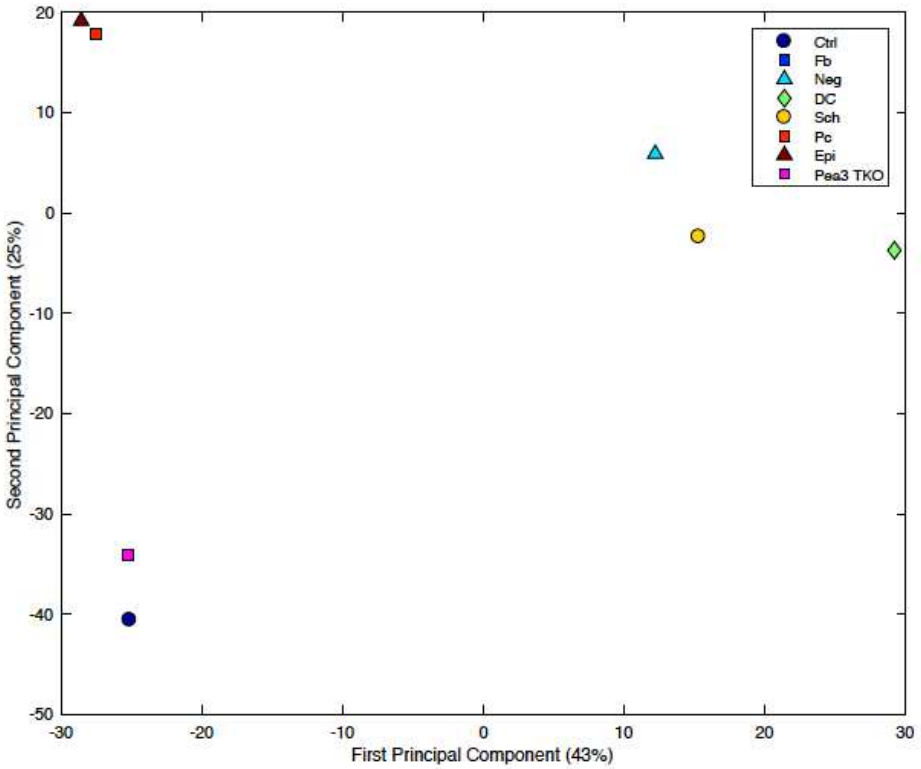
H


Fibroblasts
NES = 3.2, p > 0.05
ES
Fold change
gene rank

I


Melanocytes
NES = -0.87, p > 0.05
ES
Fold change
gene rank

J


Ctrl
Fb
Neg
DC
Sch
Pc
Epi
Pea3 TKO
Second Principal Component (25%)
First Principal Component (43%)
20
10
0
-10
-20
-30
-40
-50
-30
-20
-10
0
10
20
30

K

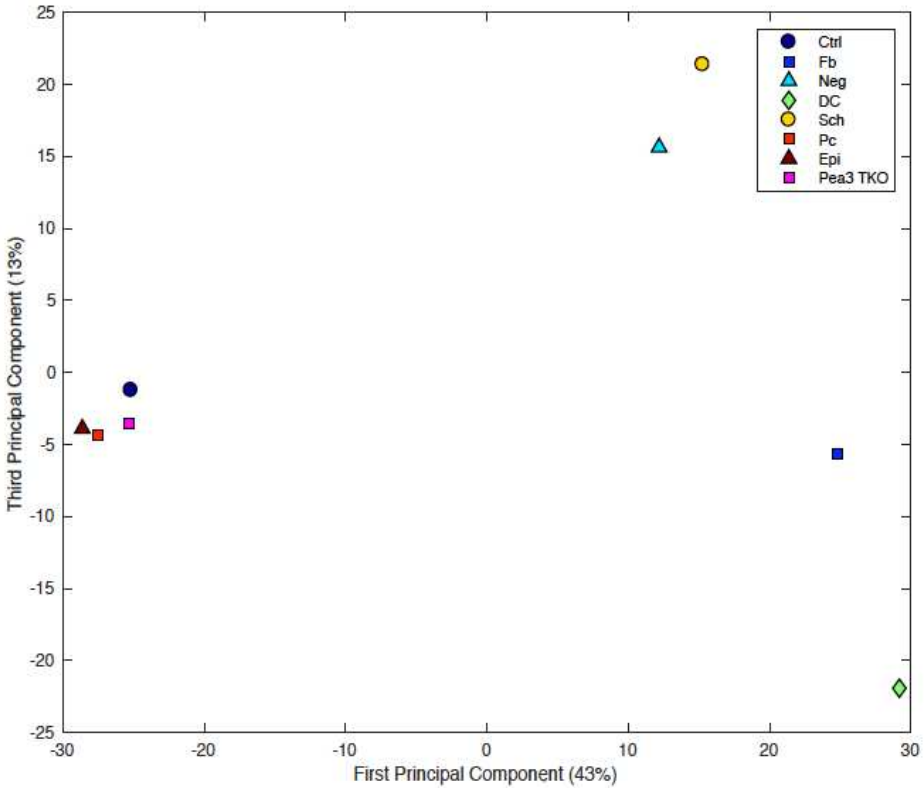
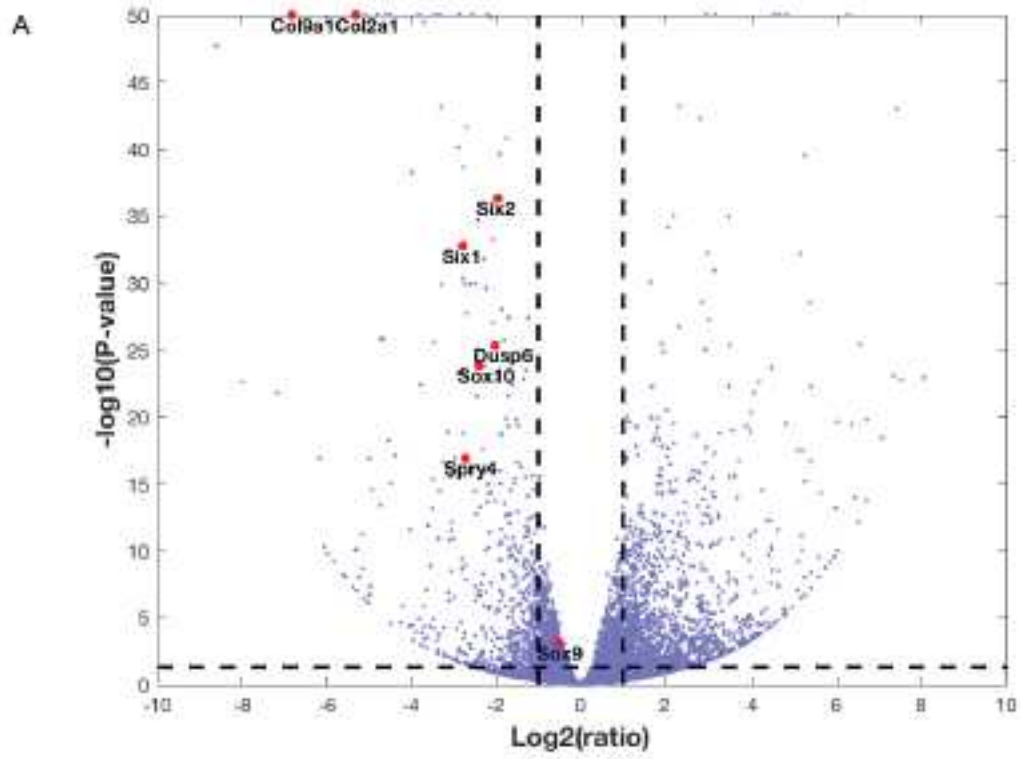

Ctrl
Fb
Neg
DC
Sch
Pc
Epi
Pea3 TKO
Third Principal Component (13%)
First Principal Component (43%)
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-20
-10
0
10
20
30

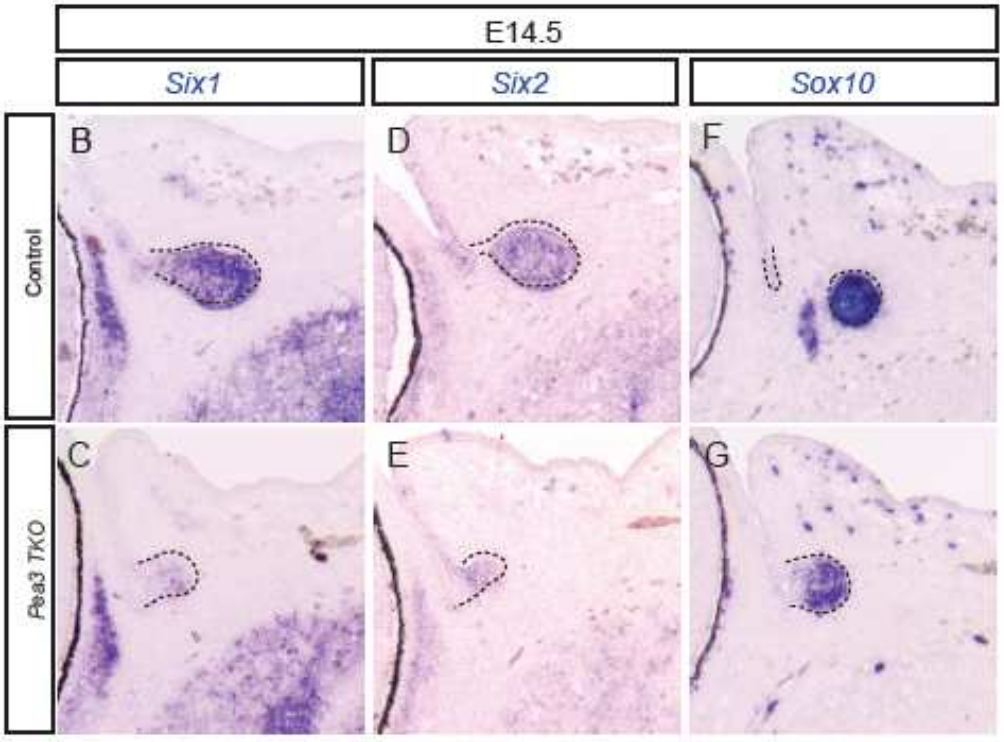
Figure 14. Pea3 genes are required for establishing the lacrimal gland cell fate. (A-B) Keratin 14, an epidermal skin marker, is upregulated in the lacrimal gland bud of *Pea3* mutants (B dotted line, arrow). (C-D) GSEA analysis of differentially upregulated genes in *Pea3* mutants shows a significant enrichment in the epidermal skin reference database. (E-I) There was no enrichment in the reference gene expression datasets from other cell types present within the skin. (J-K) Principal component analysis of RNA-seq data from different skin cell types as well as from lacrimal gland tissues from control and *Pea3* mutants. Analysis indicated that mutant lacrimal gland is more closely related to the epidermal skin compared to the control lacrimal gland along first and second as well as first and third principal axes.

ECM encoding genes- *Col2a1, Col9a1*, were down-regulated in Pea3 mutants. Interestingly, among Sox genes, which were reported to be downstream of Fgf signaling during lacrimal gland development, Sox10 was dramatically downregulated whereas Sox9 showed no significant change. This is consistent with previous finding that ablation of Sox9 downregulated phospho-Erk and Erm in the epithelium, suggesting that Sox9 is upstream of *Pea3* genes [33]. Of note, transcription factors *Six1* and *Six2* also emerged as promising candidate genes. Six1 mutant has been previously shown to have defects in duct elongation and branching during lacrimal gland development in mouse [45]. Moreover, Six2 has been shown to be downstream of Six1 in human embryonic stem cell-derived lacrimal gland cells [146].

We validated these findings by performing in situ hybridization and immunostaining for most of these genes. Indeed, in situ hybridization revealed that the expression of *Six1* and *Six2* were significantly downregulated in *Pea3* mutants (Fig. 15B-E, dotted lines). Similarly, the expression of *Sox10* and *Dusp6* were diminished in the mutants, consistent with the RNA-seq data. Immunostaining for Col2a1 revealed that its expression was downregulated in the epithelial basement membrane (Fig. 15F-K, white dotted lines, arrows). To show that the expression of Six1 and Six2 are driven by Fgf-signaling, we performed similar in situ hybridization of *Six1* and *Six2* in mice with epithelial deletion of Fgfr2. Our results demonstrated that expression of Six1/2 and Erm were completely abolished in *Le-Cre, Fgfr2*$^{fl/fl}$ embryos at the E14.5 stage (Fig. 15L-S, dotted lines, arrows) in the conjunctival tissue which still retains Pax6 expression, suggesting that Six1 and Six2 are downstream targets of Pea3-mediated Fgf signaling in the epithelium. We further explored the genetic requirement of Six genes during lacrimal gland development. Interestingly, unlike *Six1 KO* mice, *Six2 KO* mice do not have lacrimal gland defect (data not shown) suggesting that Six1 might be compensating for Six2 during lacrimal gland development.

Since Pea3 are direct responsive genes of Erk signaling, we proposed that these Fgf-responsive genes could be direct targets of Pea3 transcription factors. To analyze this, we


A
Col9a1Col2a1
Six2
Six1
Dusp6
Sox10
Spry4
Sox9
-log10(P-value)
Log2(ratio)


E14.5
Six1
Six2
Sox10
Control
Pea3 TKO
B
C
D
E
F
G

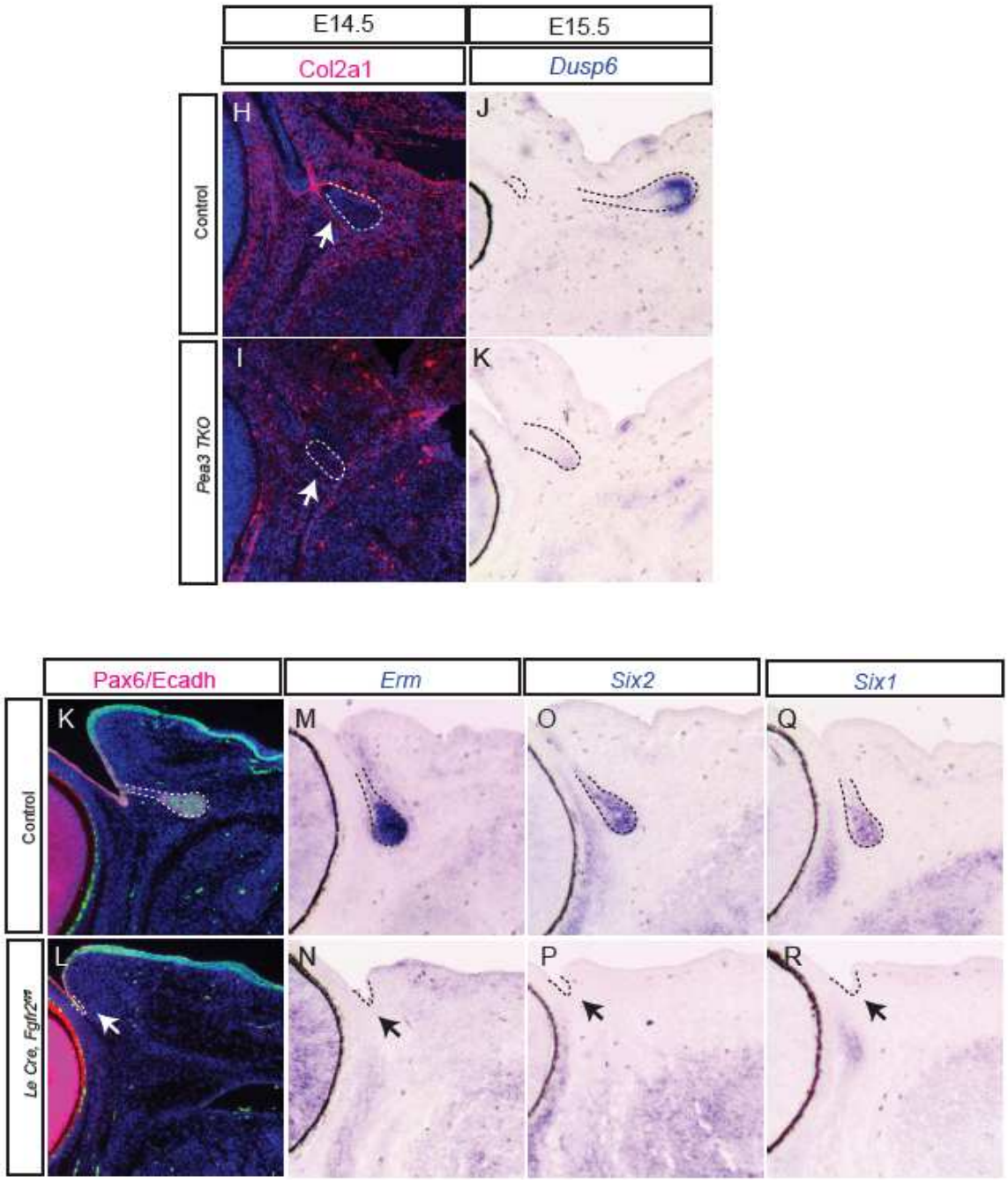


Figure 15. Six1 and Six2 are downstream targets of Fgf signaling mediated by Pea3 transcription factors. Volcano plot demonstrating the relative distribution of differentially expressed genes, with respect to fold change and statistical significance, red dots highlighting Six family genes, Six1, Six2, and previously published downstream targets of Fgf signaling. (A-J) In situ hybridization against Six1 and Six2 show that

expression of these *Six* genes are downregulated in *Pea3 TKO* (A-D, dotted lines). (E-J) Expression of *Sox10*, Col2a1 and *Dusp6* reported to be downstream targets of Fgf signaling were downregulated in *Pea3 TKO* mutants (dotted lines, arrows). (K-R) Expression of *Six1* and *Six2* were also downregulated in the *Le-Cre, Fgfr2*$^{fl/fl}$ mutant lacrimal gland epithelium (O-R, dotted lines) which retained progenitor differentiation marker Pax6 and epithelial marker E-cadherin in mutants (K, L, dotted lines). As expected, expression of *Erm* was also abolished in Fgfr2 conditional knockout epithelium (M-N, dotted lines).

used published ChIP–seq data for targets of Pea3 and Er81 in cancer cell lines and used intersection-of-list approach to find the common targets among significantly downregulated genes in *Pea3 TKO* mutants [147, 148]. We found that, among direct targets of Pea3, 61 genes are upregulated in *Pea3 TKO,* whereas 57 genes are downregulated, including Six1, Col2a1, Dusp6 and Etv4 itself. Similar analysis with direct targets of Er81 transcription factor revealed that 31 gene targets are upregulated in *Pea3 TKO* and 39 gene targets are downregulated, including Six2 and Col8a2 (Fig. 16A, B). Since this information is based on the human cell line data, we further performed Transfac analysis under stringent criteria and identified the binding sites of Pea3, Erm and Er81 in the promoter regions (5kb upstream) of these genes (Fig. 16C) in mouse genome. Taken together, these data demonstrate for the first time that Six1 and Six2 are downstream of Pea3 signaling important for lacrimal gland development and potentially regulate the lacrimal gland cell fate.

3.2.5 Pea3 transcription factors suppress Notch signaling during lacrimal gland development

With the observed upregulation of epidermal markers and downregulation of *Six1* and *Six2*, we hypothesized that *Six* genes are important for regulating the lacrimal gland cell identity. To test this hypothesis, we analyzed the genes and signaling pathways which were upregulated in *Pea3* mutants. DAVID-KEGG pathway analysis of the RNA-seq data revealed that Hippo, Wnt and Notch signaling pathways were upregulated in *Pea3 TKO* mutant glands (Fig. 17A). To validate these findings, we performed GSEA to find enrichment of genesets from these upregulated signaling pathways in our dataset. Our analysis indicated that Notch target genes were significantly enriched in our dataset but not those from the Hippo or Wnt signaling pathways (NES = 2.56, $p<0.01$, Fig. 17B-D). Plotting differential gene expression values, Log_2 (fold change) vs corresponding Log_2(p-value) on y-axis revealed that several genes associated with Notch pathway were upregulated in *Pea3 TKO* mutants as highlighted in the volcano plot (Fig. 17E). We confirmed these results by immunostaining and observed an increased expression of Jag1 and Notch1-ICD in *Pea3 TKO* (Fig. 17F-I, dotted lines, arrows).

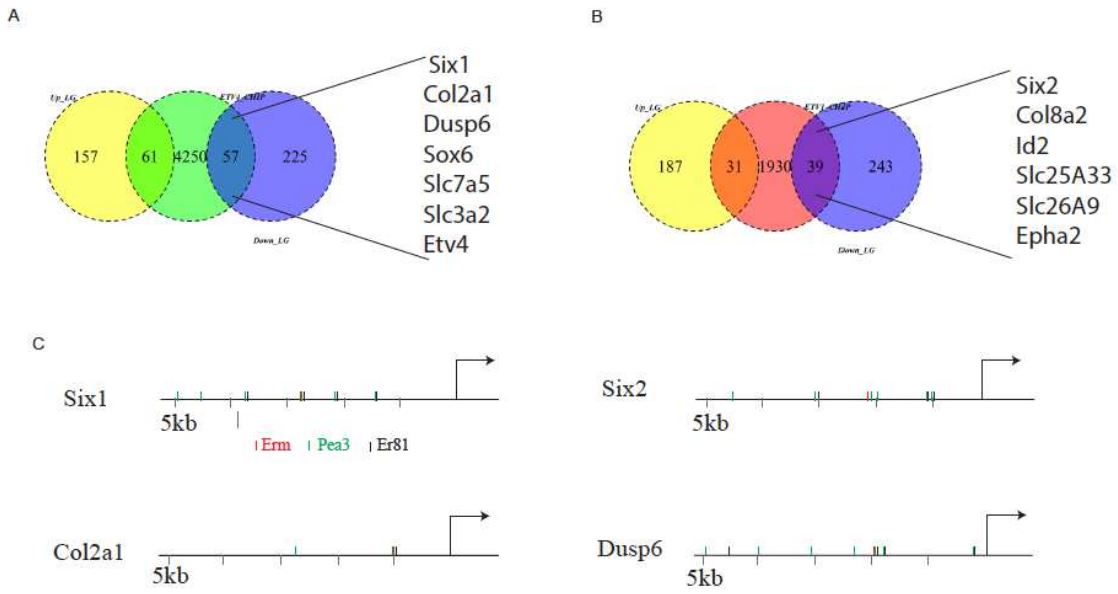


Figure 16. Six1 and Six2 can potentially be direct targets of Pea3 transcription factors. (A-B) Intersection-of-list analysis was performed with published Pea3 and Er81 ChIP on tumor cell lines and differentially regulated genesets in *Pea3* mutants, indicating that Six1 and Six2 can be direct targets of Pea3 genes during lacrimal gland development. (C) Transfac analysis of genomic region 5 kb upstream of murine *Six1*, *Six2*, *Col2a1* and *Dusp6* genes showed putative binding sites of Pea3 transcription factors.

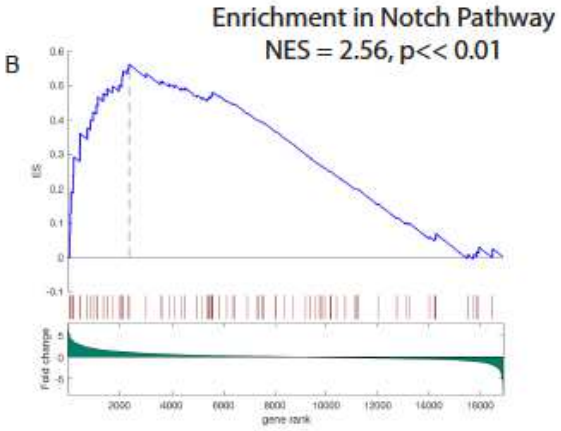
Based on these results, we concluded that for acquiring the lacrimal gland cell fate, Notch signaling must be downregulated in the lacrimal gland epithelium, suggesting that it was under negative regulation by Pea3-mediated Fgf signaling. To confirm these findings in vivo, we overexpressed Notch1-ICD (NICD) allele in the lacrimal gland using *Le-Cre* and found that lacrimal gland formation was completely disrupted (n=10, Fig. 18A-C). To further investigate whether Six1 and Six2 gene expression also depend on suppression of Notch signaling, we performed in situ hybridization for *Six1* and *Six2* to find that the expression of these genes were indeed ablated in *Le Cre, Rosa-NICD* embryos, while the expression of lacrimal gland progenitor cell marker Pax6 was retained (Fig. 18D-G, P-Q, n=4). Furthermore, expression of target genes downstream of Fgf signaling such as *Sox10*, *Erm*, *Pea3* and *Dusp6* were also downregulated in these mutants (Fig. 18H-O, n=4), consistent with the loss of lacrimal gland cell fate. Interestingly, we found that Jag1 was upregulated in these mutants, suggestive of the lateral activation of Notch signaling acting as a positive feedback loop to increase Jag1 expression (Fig. 18R-S, dotted lines). Taken together, these results show that Pea3 is responsible for suppression of Notch signaling in maintaining the lacrimal gland cell fate (Fig. 18T).

A

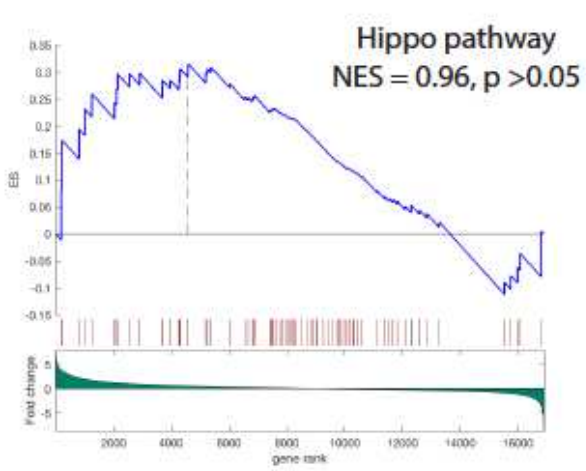
B

DAVID- KEGG Pathway analysis|Upregulated genes	p-value
Signaling pathways regulating pluripotency of stem cells	3.60E-02
Wnt signaling pathway	4.00E-02
Calcium signaling pathway	4.60E-02
p53 signaling pathway	4.80E-02
Hippo signaling pathway	5.40E-02
MAPK signaling pathway	1.90E-01
Notch signaling pathway	2.40E-01
Fatty acid degradation	2.40E-01
HIF-1 signaling pathway	2.40E-01
FoxO signaling pathway	3.40E-01
cAMP signaling pathway	3.30E-01
Tight junction	3.70E-01
PI3K-Akt signaling pathway	4.00E-01

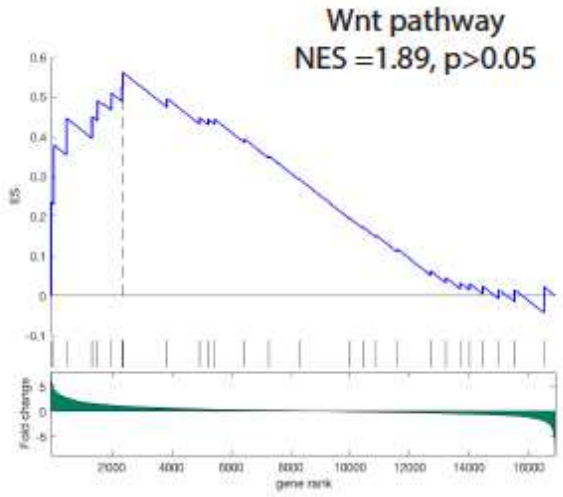
B



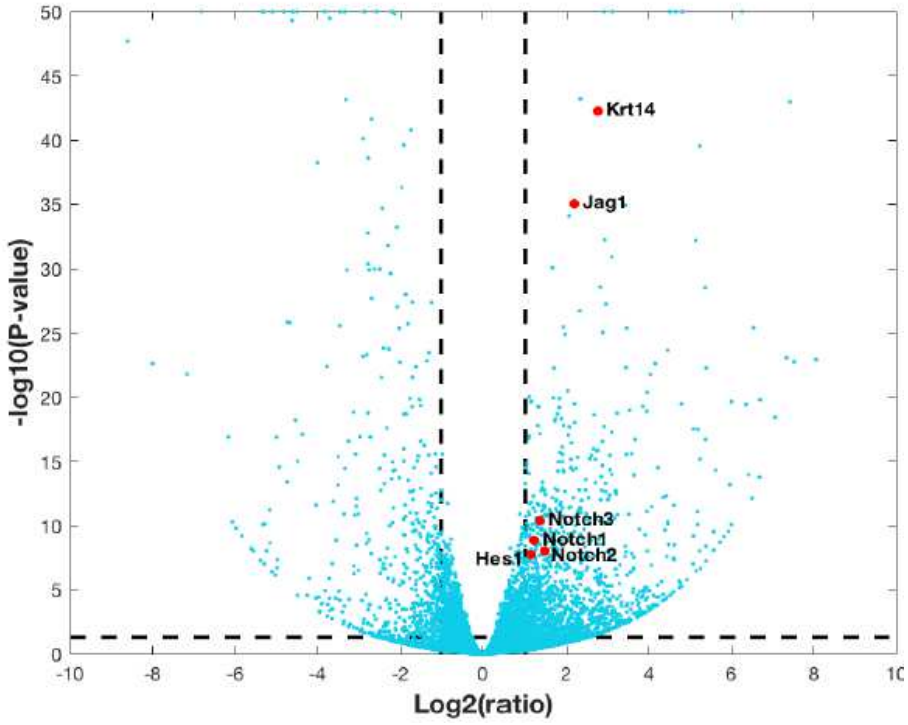
C



D


Wnt pathway
NES =1.89, p>0.05
ES
Fold change
gene rank

E


Krt14
Jag1
Notch3
Notch1
Notch2
Hes1
-log10(P-value)
Log2(ratio)

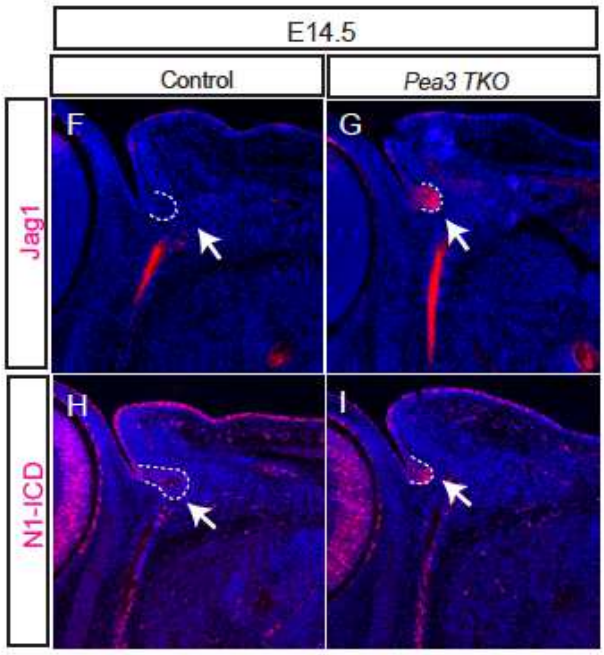
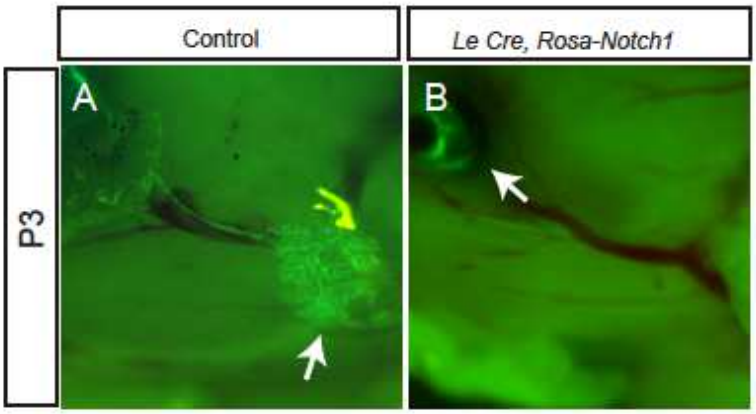
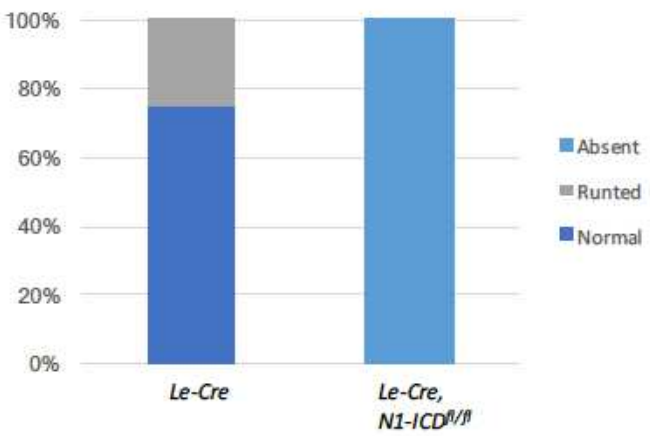
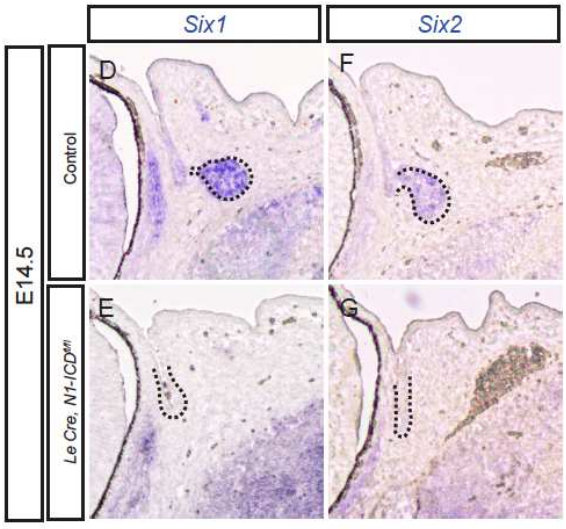


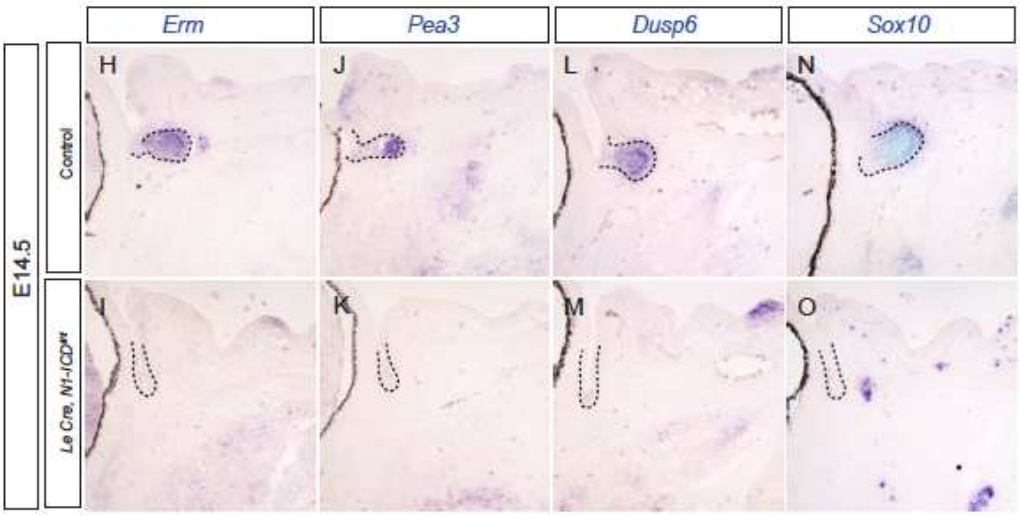
Figure 17. Fgf signaling mediated by Pea3 genes suppresses notch signaling during lacrimal gland development. A. KEGG pathway analysis of upregulated genes indicated that several signaling pathways such as Notch, Hippo and Wnt were upregulated. (B) Gene set enrichment analysis of published Notch signaling geneset with reference to Pea3 differential gene expression dataset indicated an enrichment of Notch signaling genes but not Hippo and Wnt signaling genes (C-D). (E) Log2(Fold change) vs Log2(p-value) indicated that several Notch pathway genes were upregulated, highlighted by red dots. (F-I) Antibody staining against Jag1 and N1-ICD showed an ectopic increase in the expression of these Notch signaling activators in *Pea3* mutant lacrimal glands (G, I, dotted lines, arrows).

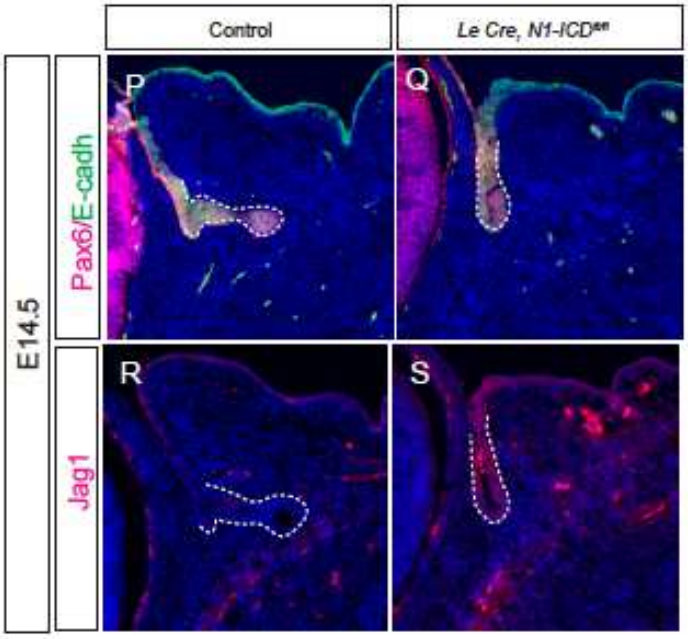

Control
Le Cre, Rosa-Notch1
P3
A
B

C


100%
80%
60%
40%
20%
0%
Le-Cre
Le-Cre, N1-ICDfl/fl
Absent
Runted
Normal


Six1
Six2
E14.5
Control
Le Cre, N1-ICDfl/fl
D
F
E
G


Erm
Pea3
Dusp6
Sox10
E14.5
Control
Le Cre, N1-ICD
H
J
L
N
I
K
M
O


Control
Le Cre, N1-ICD
E14.5
Pax6/E-cadh
Jag1
P
Q
R
S

T

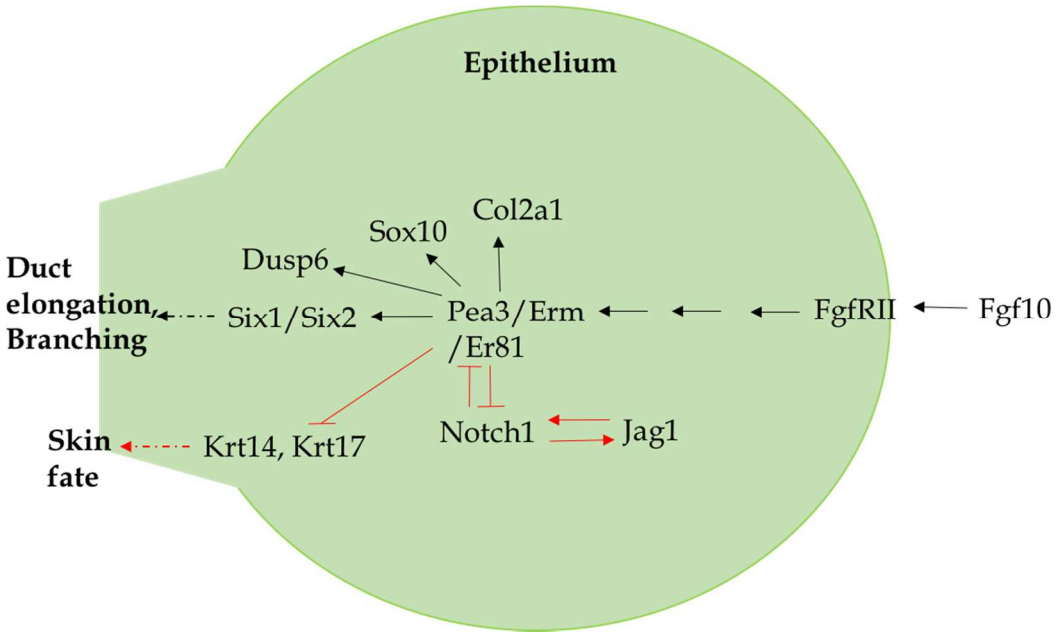


Figure 18. Ectopic activation of Notch signaling in the lacrimal gland epithelium aborts gland development. (A-C) Analysis at pups at P1 stage showed that mice with overexpression of N1-ICD in the lacrimal gland epithelium failed to form lacrimal gland (B, arrow). (D-O) Analysis of embryos at the E14.5 stage using in situ hybridization showed that the expression of Six genes was abolished (D-G, dotted lines), concomitant with the loss of expression of other Fgf signaling target genes- *Erm*, *Pea3*, *Dusp6* and *Sox10* (H-O, dotted lines). (P-S) Expression of N1-ICD maintained lacrimal gland progenitor cell population as shown by Pax6 and E-cadherin expression (P, Q, dotted) and it also resulted in activation of auto-stimulatory loop via upregulation of Jag1 expression (R, S, dotted lines). (T) Graphical summary of the gene regulatory network summarizing the signaling mechanisms associated with Pea3 transcriptional network during lacrimal gland epithelial development.

4 Discussion

Lacrimal gland development is a highly-regulated process driven by extrinsic and intrinsic signaling interactions, often involving multiple tissue types which orchestrate to form the functional structure. Lacrimal gland formation is based on the cross-talk between the surrounding neural crest-derived mesenchyme and the conjunctival epithelium tissue. During organogenesis of the lacrimal gland, multiple signaling pathways, most notably, Fgf, BMP, Notch and Wnt are involved in establishing different cell fates. These signaling interactions often strive for maintaining just the right level of signaling activity of the respective pathways by either supporting or antagonizing each other in tissue-specific manners.

Our current knowledge of the lacrimal gland is largely restricted to epithelium rather than in the mesenchyme, in part due to the fact that over 90% of the mature lacrimal gland is of the epithelial origin. Mesenchymal condensation is one of the earliest events in lacrimal gland development, but its mechanism and functional significance remain poorly understood. The focus of this thesis study is two folds- one is to understand the development of the lacrimal gland mesenchyme, more specifically, the signaling mechanisms which are important for regulating Fgf10 production. The other is to understand the role of direct downstream targets of sequential Fgf signaling pathway triggered within the epithelium during the development of lacrimal gland.

4.1 Understanding the development of lacrimal gland mesenchyme

In this study, we show that FGF signaling in the neural crest is required for *Fgf10* production within the periocular mesenchyme, which triggers a second round of FGF signaling in the conjunctival epithelium to form the lacrimal gland (Fig. 7D). This is mediated by Frs2α and Shp2, which activates Ras-MAPK pathway to control the survival, migration and differentiation of the cranial neural crest. The downstream effector of Shp2 signaling in the periocular mesenchyme is homeodomain transcription factor Alx4, which acts as a relay to transmit earlier FGF signaling in the neural crest to induce *Fgf10* signaling in the lacrimal gland. Our results highlight the role of Alx4 in

inducing reiterative FGF signaling in neural crest development and reveal the etiology of lacrimal insufficiency in *ALX4*$^{-/-}$ patients.

The source of the FGF signaling in the neural crest for lacrimal gland development is an intriguing question. It is unlikely autocrine signaling of Fgf10, because deletion of Fgfr2, the cognate receptor for Fgf10, in the neural crest only produced minor defects in lacrimal gland development (Fig. 2). We have tested *Fgf9*, which is expressed in the periocular mesenchyme, but lacrimal gland development was unaffected in *Fgf9* knockout animals (data not shown). *Fgf8* is known to play important roles in neural crest development [126, 127, 136, 137, 139], but deletion of *Fgf8* in the midbrain-hindbrain junction, branchial arches or the forebrain failed to disrupt lacrimal gland development (data not shown). Considering the complexity of FGF family, additional work is needed to identify the relevant FGF ligands in the neural crest.

RASopathies represent a spectrum of congenital abnormalities caused by aberrant Ras-MAPK signaling, but the relevant RTK signaling pathway mediated by Ras in normal development is not always clear [149, 150]. Using mouse genetics, we showed that defective FGF signaling, but not PDGF signaling, in the neural crest reproduced the Shp2 deletion phenotype in the lacrimal gland, thereby positioning FGF receptors as the primary regulators of Shp2 in neural crest and lacrimal gland development. Contrary to a previous study in zebrafish, we did not find Shp2 acts upstream to p53 to suppress neural crest cell apoptosis [134]. This discrepancy could be due to difference in species or the experimental approaches as we used genetic knockout whereas the zebrafish study used morpholinos knockdown. Instead, our genetic evidence demonstrates a fundamental role for the Shp2-Ras-Mek-Erk signaling cascade in neural crest survival and development. MAPK is known to phosphorylate and induce the ETS domain transcription factors, which act as downstream effectors in gene regulation. In particular, the expressions of *Pea3* family genes *Etv1/4/5* correlate closely with FGF signaling activities during embryonic development [25]. However, deletion of all three *Pea3* family genes in the neural crest failed to produce any craniofacial or lacrimal gland defects, but overexpression of a dominant-negative *Etv4* indeed stunted lacrimal gland growth. This

suggests that other members of the ETS domain transcription factors, which recognize similar binding sites as Etv1/4/5, can play redundant roles in transmitting FGF-MAPK signaling in the neural crest development.

With the prior understanding of the role of Fgf signaling in proliferation and differentiation of gland epithelium, our study demonstrated that Fgf signaling also plays a role in differentiation of neural crest-derived mesenchyme to produce Fgf10. However, our current study does not demonstrate the spatiotemporal requirement of Fgf signaling. Based on the expression of Pea3, Erm, Er81 and previously reported phospho-ERK staining data [25], it appears that neural crest cells may not experience active Erk signaling at that time of expressing Fgf10. Activation of Erk signaling is very dynamic during the embryonic development. Further development of mouse models will be necessary to precisely determine the timing and site of Fgf signaling resulting in differentiation of neural crest cells to produce Fgf10 during lacrimal gland development. With the *Wnt-1 Cre* specific deletion, one could argue that Fgf signaling might be required in the pre-migratory phase as the expression of *Wnt-1 Cre* discontinues at the onset of migration. However, Sox10-Cre driven deletion of Shp2 in migrating neural crest cells suggests that the window for Fgf signaling requirement must be between the migratory and differentiation phases (E10.5- E12.5). Ongoing studies is taking advantage of Pdgfrα–CreEr mouse line, as Pdgfrα continues to be expressed by neural crest cells even after reaching their destination to produce Fgf10. Hence, deletion of Fgfr1/2 at different time-points by activating Pdgfrα-CreEr with tamoxifen injections to pregnant females, followed by determining the lacrimal gland phenotype in embryos, can help identify the specific time-point when Fgf signaling is required.

Our study demonstrates that *Alx* genes are the ultimate downstream effectors of Shp2 signaling in the periocular mesenchyme. Alx4 shares sequence and structural homologies of paired-type homeodomain and C-terminal aristaless domain with two other transcription factors, Alx1 and Alx3. These proteins are present within the craniofacial mesenchyme and limb bud, showing overlapping expression patterns [112]. Members of this family of transcription factors also display functional redundancies as shown by

genetic interactions in specific tissues. *Alx3* knockout mice were morphologically normal, but *Alx3/4* double mutants displayed more severe defects in the neural crest-derived craniofacial structures than *Alx4* knockout alone [112]. *Alx1* null mice showed craniofacial defects distinct from *Alx4* mutants and combined deletion of both genes led to developmental abnormalities not found in either of the single mutants, indicating that *Alx1* and *Alx4* have both unique and redundant roles [143]. The lacrimal gland mesenchyme expresses *Alx1* and *Alx4*, but not *Alx3*. Although we did not observe a synergistic effect of *Alx1* and *Alx4* in our in vitro experiment, it remains possible that *Alx4*/*Alx1* double knockout mice will present more severe lacrimal gland defects as the neural crest *Shp2* mutant.

The main and accessory lacrimal glands secrete the aqueous component of the tear film, and play an important role in maintaining the health and transparency of the ocular surface. Because the tear is only necessary for land animals whose eyes are constantly exposed to the air, but not for animals living in an aquatic environment, the lacrimal gland emerges relatively late in tetrapods during vertebrate evolution. In this study, we show that the *Alx4* binding site in the *Fgf10* locus lies within a region conserved from human to alligator, but not to frog and fish. This suggests that, although both *Alx4* and *Fgf10* arise in more primitive organisms, these two genes are probably not functionally linked until the emergence of the lacrimal gland in reptiles. Considering that *Fgf10* lies at the top of the genetic cascade for inducing branching morphogenesis in many glandular organs, this represents an example of evolution that coopts an existing genetic circuitry to develop new organs to adapt to new environment. By showing that the Alx4-Fgf10 axis is conserved from mouse to human, our study contributes to the understanding of the role of Alx4 in human neural crest and lacrimal development and points the direction to generate the lacrimal gland from pluripotent stem cells.

Our data suggests that Alx4 is downstream of Shp2-mediated Fgf signaling as a potentiating factor for the production of Fgf10. Again, considering Erm/Pea3/Er81 as Fgf signaling readout, it is still unclear at what stage during development Alx4 is regulated by Fgf signaling. Previous studies have shown that Alx4 is genetically linked to activators of

BMP pathway during limb development [151] and Foxc1 mediates BMP activation of Alx4 during calvarial bone development [152]. Another study showed that during pubescent mammary gland development, Alx4 regulates the expression of MMP2 and MMP9 in the stroma [153]. During skull vault differentiation, Alx4 and Msx2 genetically interact to regulate the expression of Fgfr1/2 and Spp1, an ossification marker [154]. Alx4 and Lef1 showed physical interactions in vitro and showed genetic interaction during vasculogenesis [155, 156]. However, we did not detect expression of Lef-1 in the lacrimal gland mesenchyme. In our study, the rescue of Alx4 expression by expressing constitutively active Kras in Shp2-deleted mice, suggests that Alx4 is regulated by Shp2-Ras in the neural crest-derived lacrimal gland mesenchyme, which in turn regulates Fgf10 transcription. Alx4 is relatively understudied transcription factor in terms of its direct binding sites and regulation of the target genes. Through bioinformatics and ChIP analyses, we confirmed that a site within the intron 1 of Fgf10 is regulated by Alx4. Functional assays either by cloning that site upstream of a luciferase reporter, followed by transfection in the mesenchyme cells or by generating a mouse mutant through CRISPR-Cas9 to delete the reported site, will further confirm Ax4-Fgf10 interaction in vivo.

Alx4, a homeodomain transcription factor, has been found to be expressed in mesenchymal regions in a number of developing tissues in wide range of species. Interestingly, Alx4 has also been reported to be acting as either tumor promoters or suppressors [157-159]. The epigenetic methylation pattern in Alx4 has been suggested as a potential blood-based biomarker for colon and GI cancers [160, 161]. The revelation that a human patient harboring an autosomal recessive mutation in *ALX4* has lacrimal gland defects informs us the cause of the dry eye symptoms [144]. This would help clinicians to better manage the symptoms of such patients and adopt appropriate treatment strategies. Our findings not only contribute towards understanding the Alx4 associated signaling pathways in normal development but may also shed a light on tumorigenesis pathways.

4.2 Understanding the development of lacrimal gland epithelium

In this study, we showed that mediated by Pea3 family of transcription factors are not only critical for lacrimal gland duct elongation and branching but also for establishing the identity of the lacrimal gland epithelium derived from the progenitor conjunctival epithelium. High-throughput gene expression analysis revealed that, apart from regulating previously reported Fgf-responsive genes, Pea3 genes also regulate the expression of *Six1* and *Six2* during lacrimal gland development in mice. Our data also indicated that Pea3 transcription factors are important for establishing the tissue identity of the lacrimal gland cell by preventing the cell fate switch to the epidermal skin-like cells. Further analysis revealed that lack of *Pea3* genes resulted in activation of Notch signaling. Constitutive activation of Notch signaling in the conjunctiva abrogated lacrimal gland development concomitant with the loss of *Six1*/*Six2* expression. These results indicated that *Pea3* genes suppresses Notch signaling to promote the expression of *Six1*/*Six2* in order to determine the lacrimal gland cell fate and subsequent gland development.

Transcription factor Six1 has been previously shown to regulate lacrimal gland development in humans as well as in mice, however, genetic regulation of this transcription factor was not understood. In humans, heterozygous missense mutation in *SIX1* gene is autosomal dominant and causes lacrimal gland stenosis whereas *Six1* knockout mouse embryos have small lacrimal glands exhibiting duct elongation and branching defects [45]. *Six1* is widely expressed in the head mesenchyme in the early stages of development, in addition to its expression in the ducts and acini of the lacrimal gland. Hence, Six1 transcription factor could be important for regulating the lacrimal gland mesenchyme as well as the epithelia. It is interesting that lacrimal gland formation is not disrupted completely in these *Six1 KO* mice which could be attributed to the presence of *Six2* compensating for the loss of *Six1*. Six2 has been shown to be downstream of Six1, which contraindicates the compensation mechanism [146, 162]. However, it is also possible that Six2 is regulated via a separate mechanism independent of Six1. In addition, we found that *Six2 KO* does not have lacrimal gland phenotype, indicating that perhaps Six1 is compensating for Six2. Piecing these information together,

it is likely that Six1 and Six2 interact genetically and Six1/Six2 double knockout mice would exhibit more severe lacrimal gland phenotype. To address this question, ongoing studies involve carrying out lacrimal gland organ culture in the presence of *Six1* siRNA alone, *Six2* siRNA alone and *Six2* siRNA in the presence of hypomorphic *Six1* siRNA conditions to understand the genetic interactions between *Six1* and *Six2*. Preliminary results suggest that *Six1* is more critical gene with *Six2* genetically redundant and these genes interact genetically in ex-vivo organ cultures but these results need further confirmation.

There has been very limited understanding in terms of signaling mechanisms regulating Six1/Six2 expression. Apart from lacrimal gland defects, *Six1* deficiency causes defects in other organs. Development of some of these organs such as the inner ear and kidney are based on the epithelial-mesenchymal interaction. *Six1* has been further shown to have synergistic genetic interactions with *Eya1* in mice, consistent with both genes as causes of human BOR/BO syndromes [162, 163, OMIM 608389, OMIM, 113650, 164]. Similar genetic interaction experiments in mice showed that *Six1* acts upstream of *Jag1* in Notch signaling pathway during inner ear development [165]. Systems-level analysis of inner ear development showed that Pea3 negatively regulates pre-placodal genes *Six1* and *Eya2* [166]. The role of Six2 has also been implicated in kidney development in zebrafish, mice and humans [167-169]. During kidney development, both Six1and Six2 are expressed in cap mesenchymal tissue and are required for the proper ureteric budding and branching process [162, 170, 171], whereas Pea and Erm transcription factors are expressed in both the ureteric bud as well as the metanephric mesenchyme, however, only the epithelial requirement of *Pea3* genes was demonstrated during embryonic kidney development [76]. Given the understanding of the specific roles of *Six1*/*Six2* genes during development of different organs, the signaling mechanisms and transcription factors regulating *Six* gene expressions are not clear. Our data revealed that *Six1/2* are not expressed in the lacrimal gland mesenchyme but are highly expressed in lacrimal gland epithelium. The expression of *Six1/Six2* is potentially regulated directly by Pea3 transcription factors downstream of Fgf signaling pathway in the lacrimal gland epithelium. In contrast to the findings within the inner ear tissue, *Pea3* genes negatively

regulate Jag1 and prevent the Notch activity in the growing lacrimal gland epithelial bud. As a proof of principal, we showed that ectopic activation of Notch signaling resulted in upregulation of Jag1 with concomitant downregulation of *Six* genes. These findings highlight the context-dependent prevalence of gene regulatory networks.

We have shown that Pea3 transcription factors downregulate Notch signaling in the lacrimal gland epithelium, however, the mechanism of this process remains to be understood. Analysis of our RNA-seq data for the modulators of Notch signaling showed that Lfng was the only Fringe family gene expressed in the control lacrimal glands, and it is significantly downregulated in *Pea3* mutants. Lfng is the glycosyltransferase which can glycosylate O-linked fucose residues on the extracellular domain of Notch receptor and hence, modulate the ligand binding [172]. Lfng has been shown to modulate Notch 1 signaling by potentiating the Dll-mediated and inhibiting Jag1-mediated signaling [173-175]. During sensory hair cell development in the inner ear, Lfng co-expresses with Jag1 and partially rescues the phenotype when mutated in *Jag2* knockout mice [176, 177]. During lacrimal gland development, one possibility is that activation of Pea3 transcription factors turns on the expression of Lfng, which prevents Jag1-mediated Notch signaling. In the absence of Pea3 factors, down-regulation of Lfng levels results in Notch activation which is further potentiated by increasing Jag1 expression. Further investigation is required to establish the molecular link between the *Pea3* genes and Notch signaling i.e. how is Notch signaling suppressed upon activation of *Pea3* genes in the lacrimal gland bud.

Our data has shown that the lacrimal gland cell fate is established by Pea3 transcription factors, absence of which result in the cell fate switch in the epithelium marked by the ectopic expression of epidermal skin markers. We have validated this cell fate conversion by bioinformatics approaches as well as immunostaining of Keratin 14. Interestingly, Keratin 14 is highly expressed in skin but only a few cells in the conjunctiva express Keratin 14. Specific upregulation of this keratin in the lacrimal gland bud of the mutants suggests that the lacrimal gland acquires the skin-cell fate as opposed to the conjunctival cell fate. This model was consistent with preliminary finding with respect to the

expression of Klf5 and Keratin 15, which are expressed by both the conjunctiva and skin cells and do not show any differential expression in the lacrimal gland bud of the mutants. Ongoing research include further validation of our findings with immunostaining of other epidermal skin biomarkers such as plakin family of genes- Envoplakin and Periplakin which form the component of desmosomes and the epidermal cornified epithelium, and Keratins 17-19. This novel phenomenon of cell-fate switch in the lacrimal gland demonstrates the role of *Pea3* genes in establishing the identity of lacrimal gland cells by not only regulating the expression of lacrimal gland specific genes and but also suppressing Notch signaling as well as preventing the expression of genes driving the skin cell fate.

Since lacrimal gland induction was not aborted in *Pea3* mutants similar to *Fgfr2* knockout phenotype, one can argue that there are other responsive genes apart from *Pea3* downstream of Fgf signaling. Pea3 transcription factors belong to the large family of Ets transcription factors. It is a possibility that other Ets genes are also relaying the Fgf10 signal. To address this, we expressed dominant negative-Etv4 in lacrimal gland epithelium, but did not find any lacrimal gland defect (data not shown). This suggests that even the reduced amounts of Ets transcriptional activity is enough for relaying the Fgf10 signaling activity. Sox9 is another transcription factor downstream of Fgf signaling pathway which remains unchanged in *Pea3* mutants. Sox9 was reported to influence the availability and receptor binding of Fgf10, by regulating heparan sulphate synthesis in a positive feedback loop. Genetic deletion of Sox9 in the lacrimal gland epithelium resulted in a complete abortion of lacrimal gland development and down regulation of Erm and phospho-Erk in the epithelium, which means that Sox9 is activated in a parallel pathway mechanism upstream to *Pea3* genes. Unlike Sox9, other transcription factors such as Sox10, Six1, Six2 are all downregulated in *Pea3* mutants. Our data is consistent with the previously published results of *Sox* gene regulation and uncovers the previously uncharacterized gene regulatory network involving *Six* genes and Notch signaling pathway during the development of the lacrimal gland (Fig. 14T). This knowledge will help better understanding and advancement of stem cell therapeutic approaches for the treatment of lacrimal gland-associated dry eye diseases.

4.3 Current status of therapeutic advances in regenerative medicine and future research

Aqueous-deficient dry eye disease is a major health challenge that lacks effective treatment. Although lacrimal gland transplantation is potentially feasible, it is plagued by immunological complications and donor shortage. To overcome donor shortage, xenogeneic porcine organ transplantation has been proposed, however, it has not been tested functionally and immune rejection issues will still prevail. Given the lack of curative treatments for dry eye disease, regenerative medicine has emerged as a promising approach to provide more permanent and sustainable therapy. To this end, our understanding of developmental biology, stem cell biology, and the regenerative capacity of the lacrimal gland is important for advancing this field of medicine. Currently, two main strategies are being developed for lacrimal gland repair and regeneration: i) capitalizing on the intrinsic regenerative capacity of the lacrimal gland, ii) developing bioengineered lacrimal gland for tissue replacement.

Development of fully functional bioengineered gland using in vitro cell manipulation technique using collagen as scaffold, can serve as a viable alternative to repair damaged lacrimal glands [178], however, embryonic lacrimal gland cells will be difficult to procure for clinical use. With the wealth of knowledge about advantages and limitations of using rodent and porcine lacrimal gland cells, their ability to grow on various biological or non-biological scaffolds in 2D or 3D formats, porcine decellularized matrix seeded with human lacrimal gland cells seems to be a clinically feasible option for developing bioengineered implants at this point. These matrices would provide the support structure needed for vascular, nerve and ductal supply which makes it ideal substitute for tissue replacement. These lacrimal gland cells need to be derived from human IPS cells or ex-vivo expanded autologous lacrimal gland stem cells if possible. In this setting, proliferation of lacrimal gland progenitor cells and differentiation to various cell types will be an important phenomenon aiding the development of these tissue-derived bioengineered gland. Although these findings are highly promising, there are still concerns regarding the functional sustenance, cannulation, innervation and

vascularization of bioengineered organs, which need to be resolved and validated before this therapeutic approach can be applicable for clinical use [179].

On the other hand, the lacrimal gland has also been shown to exhibit robust regeneration program as a part of wound healing process, raising the possibility that it may be feasible to design therapeutic strategies that take advantage of this feature. Important issues to consider in this context include the molecular cues that trigger regeneration, the involved signaling pathways, the nature and origin of the stem or progenitor cells, and whether these cells can be isolated and expanded in culture. Stimulation of the intrinsic regenerative potential of lacrimal gland via drug or direct stem cell transplantation would be far less invasive and technically less challenging [180]. The emerging consensus is that adult lacrimal gland indeed harbors endogenous stem or progenitor cells, but their identity and location remains controversial. Because the existing studies are limited to in vitro cultures and putative stem cell markers, they may not accurately characterize lacrimal gland stem cells in vivo. We suggest that studies of lacrimal gland regeneration would benefit from the genetic approaches that have propelled the studies of lacrimal gland development. We have previously used the *Le-Cre* driver to trace the lineage of the Pax6-expressing cells during lacrimal gland development, showing that they strictly reside in the lacrimal gland epithelium, at least in new born mice [29]. With the increasing repertoire of inducible Cre lines, similar lineage tracing techniques should be readily applicable in resolving the location and nature of the lacrimal gland stem cells.

With both approaches being in nascent stages, deeper understanding of lacrimal gland development and its regenerative potential will be required. From the development perspective, we still have several unanswered questions: 1) how does the common epithelial progenitor cell gives rise to the acinar, ductal and myoepithelial cells, 2) whether neural crest cell lineage is still maintained or other cell population potentially epithelium stem cells contribute to mesenchyme at later stages 3) whether mesenchyme plays any role during adult lacrimal gland maintenance, 4) what factors are required for adult lacrimal gland homeostasis? Future studies are needed to reveal the signaling cascades that underlie lacrimal gland morphogenesis and the transcription network that

determine the tissue identity and cell lineage for repair and regeneration. These research will pave the ground for achieving the full potential of regenerative medicine for the dry eye disease.

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Curriculum Vitae

Ankur Garg

EDUCATION

Ph.D., Biochemistry and Molecular Biology 2011- 17
Indiana University, Indianapolis, Indiana

Visiting research student, Ophthalmology dept. 2013-17
Columbia University, New York

Masters of Technology, Biochemical Engineering and Biotechnology 2011
Indian Institute of Technology Delhi, India

Bachelor of Technology, Biochemical Engineering and Biotechnology 2010
Indian Institute of Technology Delhi, India

RESEARCH EXPERIENCE

Intern, Molecular Biology Department, Genentech, California June-Aug 2016

- Development of ELISA assay for FGF21 protein, a drug candidate and biomarker for metabolic diseases
- Determine the effects of various growth factors on browning of white fat in vitro

Neuro STARTUP Challenge, organized by Centre for Advancing Innovation Inc., NIH, U.S. Jan-April 2015

- Led a team of 5 people to participate in a global Business plan competition
- Developed a commercialization strategy for anti-angiogenic drug targeting brain tumor
- Spearheaded the project, analyzed market size, competitive landscape, product development and finances

Researcher, Institute Curie, CNRS, Paris May-July 2008
Optimized the protocol for the purification of protein from rat brains, a protein essential for endocytosis

Intern, Analytical R&D, Biocon Ltd., India May-July 2009
Analysis of Biomolecules using Weak Cation Exchange and Reverse Phase Chromatography

- Analyzed the composition of a major ingredient of cancer drug formulations using 3 different techniques
- Assessed the limitation of operational unit on analyses of drug samples

Research Assistant, Indian Institute of Technology Delhi, India Aug-Dec 2010

- Designed a bioprocess plant for the production of cellulase with yield of 450 tonnes/year
- Performed cost evaluation to assess the financial feasibility of the plant
- Assessed various parameters to design the equipment for production plant

HONORS AND AWARDS

- Starr foundation scholarship, Columbia University for Ph.D. research (2013- 17)
- Travel Award from Society of Development Biology (2017)
- Paul D. Henion Graduate Student Travel Award, Society of Developmental Biology (2017)
- Indiana University School of Medicine Travel Grant Award (2017)
- Ministry of Human Resource Development scholarship, Govt. of India (2010-11)
- Scholarship by EGIDE, French Government to pursue research at Institute Curie, Paris (2008)
- Semester merit award for being amongst top 7%, Indian Institute of Technology Delhi (2008)

PUBLICATIONS

- **Garg A**, et al. Pea3 transcription factors establish cell fate and regulate branching morphogenesis during lacrimal gland development (manuscript in preparation)

- **Garg A**, et al. Pea3 transcription factors are important for proper lens development (manuscript in preparation)

- **Garg A**, et al. Shp2 mediated activation of Alx4 in the neural crest is critical for lacrimal gland morphogenesis in human and mouse. *PLoS Genet*. 2017 Oct 13;13(10)

- **Garg A,** Zhang X. Lacrimal gland development: from signaling interactions to regenerative medicine (Review). *Dev Dyn*. 2017 Dec;246(12):970-980

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- Gupta S, Larsen E, **Garg A**, John F, Bansal M, Meckman P, Sembrano R. Functional Effectiveness of Inpatient Rehabilitation after Heart Transplantation. *PM&R* 2016 Sep; 8(9): 855-9

EDUCATIONAL OUTREACH

- NYAS Afterschool STEM Mentoring Fellowship, 12-week program: Educated middle-aged school children 2014
- Mentored high-school student for eye research and molecular biology experimental skills 2014
- Science Saturday Starters: Mentored in a science outreach initiative by Columbia University 2013
- Intel international Science and Engineering Fair: Judged science posters presented by high school students 2015
- Late Night Science Club: Communicated my research findings to lay audience to spread awareness 2017