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Grainyhead-like factor *Get1/Grhl3* regulates formation of the epidermal leading edge during eyelid closure

Zhengquan Yu¹, Ambica Bhandari¹, Jaana Mannik¹, Thu Pham¹, Xiaoman Xu¹, and Bogi Andersen^{1,*}

¹ Departments of Medicine and Biological Chemistry, University of California, Irvine, California 92697-4030

Abstract

Grainyhead transcription factors play an evolutionarily conserved role in regulating epidermal terminal differentiation. One such factor, the mammalian Grainyhead-like Epithelial Transactivator (*Get1/Grhl3*), is important for epidermal barrier formation. In addition to a role in barrier formation, Grainyhead genes play roles in closure of several structures such as the mouse neural tube and *Drosophila* wounds. Consistent with these observations, we found that *Get1* knockout mice have an eye-open at birth phenotype. The failure of eyelid closure appears to be due to critical functions of *Get1* in promoting F-actin polymerization, filopodia formation, and the cell shape changes that are required for migration of the keratinocytes at the leading edge during eyelid closure. The expression of TGF α , a known regulator of leading edge formation, is decreased in the eyelid tip of *Get1*^{-/-} mice. Levels of phospho-EGFR and phospho-ERK are also decreased at the leading edge tip. Furthermore, in an organ culture model, TGF α can increase levels of phospho-EGFR and promote cell shape changes as well as leading edge formation in *Get1*^{-/-} eyelids, indicating that in eyelid closure *Get1* acts upstream of TGF α in the EGFR/ERK pathway.

Introduction

The transcription factor Grainyhead was first identified in *Drosophila* where it was demonstrated to play a key role in the formation of the normal larval cuticle (Bray and Kafatos, 1991). In addition to a role in epidermal barrier formation, Grainyhead is critical for wound healing in *Drosophila* (Mace *et al.*, 2005). Studies in *C. elegans* and *Xenopus* also suggests a role for Grainyhead genes in cuticular formation and epidermal differentiation, respectively (Tao *et al.*, 2005b; Venkatesan *et al.*, 2003). In mammals, *Grainyhead-like epithelial transactivator 1* (*Get1*; also referred to as *Grainyhead-like 3*, *Grhl3*) (Kudryavtseva *et al.*, 2003; Ting *et al.*, 2003), one of the three mammalian Grainyhead genes, has been most extensively studied. This gene regulates key aspects of the terminal differentiation program and barrier formation of the mouse epidermis (Ting *et al.*, 2005; Yu *et al.*, 2006). In addition, *Get1/Grhl3* promotes epithelial closure of embryonic wounds as well as neural tube closure (Ting *et al.*, 2005; Ting *et al.*, 2003). Evolutionary sequence analysis suggests that the ancestral Grainyhead gene arose at the time of the first multicellular organisms over 500 million years ago (Venkatesan *et al.*, 2003).

*Corresponding author: Bogi Andersen, University of California, Irvine, Sprague Hall, Room 206, Irvine, CA 92697-4030, Phone: 949-824-9093, Fax: 949-824-2200, Email: bogi@uci.edu.

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A subset of mice with a mutation in *Get1* are born with eye-open phenotype (Yu *et al.*, 2006), suggesting a defect in eyelid closure during embryonic development. Eyelid closure is a normal biological event in the development of all mammals; in the mouse the processes of eyelid development and closure are completed in five steps between embryonic days (e) 11.5 and 16.5 (Findlater *et al.*, 1993; Mine *et al.*, 2005; Tao *et al.*, 2005a). First, the primitive eyelid starts forming at e11.5 with groove formation along with increased proliferation and morphological changes that generate protruding ridges of epithelium referred to as the eyelid root. This process is completed by e14.5 when in the second stage, a leading edge composed of groups of rounded cells starts extending from the tip of root epithelium. The third stage is marked by protrusion of the opposing leading edges, which extend over the cornea between e14.5 and e16. During the fourth stage, the leading edges meet together over the center of the cornea and fuse around e16. In the final stage, at e16.5, the eyelid roots appose, causing the epithelial cells of the leading edge to pile up at the center; these cells eventually slough off (Mine *et al.*, 2005).

Several interacting signal transduction pathways have been shown to control eyelid formation. FGF signaling originating from the mesenchyme is important for the formation of the primitive eyelid as well as leading edge development (Li *et al.*, 2001; Tao *et al.*, 2005a). EGF ligands acting through the EGF receptor, and activin acting through JNK and c-Jun, are critical for leading edge migration (Xia and Kao, 2004). Eyelid closure defects are also common in mice with mutations in planar cell polarity genes (Wang and Nathans, 2007).

Similar to dorsal closure in *Drosophila*, eyelid closure in mammal is thought to reproduce many features of natural wound healing. Dorsal closure, eyelid closure and wound healing are all characterized by specific morphogenetic steps that include the formation of leading edge of epithelial cells with actin stress fibers, directed cell migration and re-epithelialization (Martin and Parkhurst, 2004). The eye-open phenotype of *Get1* knockout mice is of interest because it provides an opportunity to investigate the role of Grainyhead-like genes, such as *Get1*, in closure of epithelial structures. While there is evidence for the role of Grainyhead genes in epithelial closure, both cellular and molecular mechanisms involved in *Get1*-dependent closure remain poorly defined. In this study, we have taken advantage of the eye-open phenotype to study the mechanisms of action for *Get1*-dependent epithelial closure. Our results indicate that *Get1* promotes actin polymerization, filopodia formation, keratinocyte cell shape change, and the timely formation of the leading edge during eyelid closure. Furthermore, our data suggest that *Get1* acts upstream of TGF α in the EGFR/ERK pathway to regulate migration of the keratinocyte leading edge.

Materials and Methods

Mice

The generation of *Get1*^{-/-} mice was previously described (Yu *et al.*, 2006). Genotypes of mice were determined as previously described.

RT-PCR analysis

Semi-quantitative RT-PCR was performed with cDNA generated with the High Capacity cDNA Archive Kit (Applied Biosystems), using total RNA, which was prepared from back skin of WT and *Get1*^{-/-} embryos using Trizol Reagent (Invitrogen). Reactions were sampled after 25, 28 and 30 cycles at different PCR conditions to monitor product accumulation. TGF α forward primer: CTGAAGGGAAGCTGCTTG; reverse primer: CAACCCTTTGAGGTTCGTGT.

Histology, immunofluorescence, and BrdU staining

Embryonic mouse heads were fixed in 10% formalin and paraffin-embedded. Following sectioning, 6- μ m sections were stained with hematoxylin and eosin. For immunofluorescence staining, 6- μ m-thick fresh frozen sections were air-dried and fixed in cold Acetone (-20°C) for 5 min, followed by a PBS wash. Sections were then soaked in blocking solution for 40 min, incubated with primary antibody for 2 h, washed three times with PBS, and incubated with FITC secondary IgG polyclonal antibody (Oncogene Research Products) and DAPI as a counterstain. Pregnant mice were injected with BrdU (100 μ g/g of body weight) 1 h before dissection. For BrdU detection, slides were pre-treated in 1 M HCl for 1 h at 37°C . The primary antibodies were as follows: Rabbit anti-mouse Get-1, rabbit anti-mouse TGF α (sc-9043, Santa Cruz Biotechnology), rabbit anti-mouse EGFR (sc-03, Santa Cruz Biotechnology), goat anti-mouse phospho-EGFR (sc-12351, Santa Cruz Biotechnology), mouse monoclonal antibody for phospho-ERK (9106, Cell Signaling Technology), rabbit anti-mouse phospho-JNK (9251, Cell Signaling Technology) and anti-BrdU (Roche), rabbit anti-mouse keratin 6 (PRB-169p, Covance).

In-situ hybridization

In situ hybridization studies with ^{35}S -labeled cRNA probes were performed on paraffin-embedded and frozen sections and counterstained with bisbenzamide as described previously (Andersen *et al.*, 1995; Andersen *et al.*, 1997).

Transmission electron microscopy

The eye region was dissected from e15.5 embryos and fixed by immersion in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS (pH 7.4) at room temperature followed by a PBS wash. The tissues were then postfixed with 0.2% Ruthenium tetroxide (RuO_4) and dehydrated through graded ethanol series, and embedded in agar 100 resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined on a transmission electron microscope.

Scanning electron microscopy

The eye region was dissected and fixed in 10% formalin overnight. After a PBS wash, tissues were postfixed in 1% Osmium for 1 h and dehydrated through graded ethanol series and HMDS. After gold coating, tissues were observed with scanning electron microscope.

Wholemout Phalloidin Staining

The dissected eyes were fixed in 4% PFA, washed with PBS and incubated in Texas Red - phalloidin (Molecular Probes) overnight. Tissues were scanned with a confocal microscope.

Generation of a Get1 antibody

Rabbit anti mouse Get-1 polyclonal antibody was generated by injecting a conjugated peptide corresponding to the N-terminus of Get1 into rabbits. The peptide used for generating the antibody was CSNEDEAWKTYLENPLT where cystein was added to the N-terminus to aid in conjugation. The fifth and the terminal bleeds from the two rabbits were affinity purified using the same peptide.

Explant cultures of mouse eyelids

Eyelids were cultured according to a previously described method (Tao et al., 2006; Tao et al., 2005a). Briefly, eyelids from e14.5 mouse embryos were dissected under a sterile condition, and placed on 0.4- μ m Milli Cell-EM culture plate insert (PICM03050, Millipore) in 6-well

plates containing DK-SFM (Invitrogen) with PBS or 20 ng/mL TGF α (Sigma). The organs were incubated at 37°C with 100% humidity, 95% air and 5% carbon dioxide for 10h.

Results

Mutation of *Get1* leads to a delayed eyelid closure in mice

We previously generated a mutant allele of *Get1* by deleting exons 4 to 7, which include regions encoding the critical DNA-binding domain. At birth (e18.5), approximately 10% of *Get1*^{-/-} embryos displayed eye-open phenotype while none of wild type (WT) embryos had open eyes (Yu *et al.*, 2006). To determine whether the eye-open phenotype was due to an effect of *Get1* on eyelid closure, we investigated the eye phenotype of *Get1*^{-/-} embryos during earlier stages. As expected, we found that eyelid closure was completed in 100% of WT mouse embryos by e16. In contrast, 100% and 25% of *Get1*^{-/-} embryos had open eyes at e16 and e17, respectively (Figs. 1A–C). Thus, the eye-open phenotype of *Get1*^{-/-} mice is fully penetrant when examined during embryogenesis. These results show that a mutation of *Get1* leads to delayed eyelid closure, indicating that *Get1* functions in normal eyelid closure. Eyelid closure defects are sometimes associated with a delay in other developmental fusion processes during late embryogenesis in the mouse, including fusion of the ear pinnae to the scalp, and digit fusion (Juriloff, 1987) (Maconachie, 1979). Consistent with this notion, we found that at e15.5 the pinnae were fused to the cranium in WT embryos (Figs. 1D and F), while this fusion was incomplete in the *Get1*^{-/-} mice (Figs. 1E and G). However, we did not detect delayed digit fusion (Supplementary Fig. 1).

Get1 expression is upregulated in keratinocytes of the leading edge

To start investigating the cause of the eye-open phenotype of *Get1*^{-/-} mice, we performed immunofluorescence studies to determine the expression pattern of *Get1* during eyelid development. *Get1* transcripts are expressed throughout the developing epidermis from at least as early as e11.5 (Auden *et al.*, 2006; Kudryavtseva *et al.*, 2003), and as expected, uniform *Get1* protein expression was found in epidermal keratinocytes covering the eyelid at e13.5 (Fig. 2A). *Get1* expression becomes highly upregulated in the cluster of rounded keratinocytes that initiate the formation of the leading edge at e14.5 (Figs. 2B–C). At e15.5, when the leading edge is extending towards the center of eye, *Get1* continues to be highly expressed in keratinocytes of the outer layer of the migrating leading edge (Figs. 2E–F). At e17, when the leading edges have fused to close the eyelid, *Get1* expression is downregulated along the fusion line but persists in the suprabasal epidermal keratinocytes covering the eyelids (Fig. 2D). Interestingly, *Get1* mRNA expression is also highly upregulated in the epidermal wound front in adult mice (Supplementary Fig. 2A–B), suggesting that upregulation of *Get1* in migrating epidermal keratinocytes could be a common feature. Together, these data suggest the possibility that *Get1* may play roles in keratinocyte migration during epidermal closure.

Impaired formation of the leading edge in *Get1*^{-/-} mice

To investigate the potential role of *Get1* in eyelid closure, we compared eyelid development in *Get1*^{-/-} and WT embryos at various stages, using histological analysis. In WT embryos, the root of the primitive eyelid was fully formed at e13.5 (Figs. 3A and C), and a small leading edge protruded from eyelid root at e14.5 (Figs. 3E and G); the leading edge extended towards the center at e15.5 (Figs. 3I and K) and fused in the center of eye at e16 (Figs. 3M and O). In *Get1*^{-/-} embryos, no obvious abnormality was found at e13.5, indicating that eyelid root formation is independent of *Get1* (Figs. 3B and D). However, marked abnormalities were found in the *Get1*^{-/-} mice at e14.5 (Figs. 3F and H) and e15.5 (Figs. 3J and L); no leading edge had formed at these time points. At e16, a rudimentary leading edge was observed (Figs. 3N and P), but it did not continue to extend. These results show that both initiation and progression of the leading edge are affected by *Get1* gene deletion. At birth, eyelid closure was achieved in

90% of *Get1*^{-/-} mice, perhaps due to compensatory mechanisms or through apposition of the eyelid roots. Statistical analysis on multiple embryos at e15.5 shows that whereas the eyelid root is of normal size, the length of leading edge is strikingly shorter in *Get1*^{-/-} mice (Figs. 3Q–R). These results indicate that during eyelid closure, *Get1* functions predominantly in the formation and extension of the leading edge.

***Get1* is required for cell shape changes and filopodia formation in the leading edge**

We used scanning electron microscopy (SEM) to study the cellular appearance of the eyelid in *Get1*^{-/-} embryos. In WT mice at e15.5, we observed an accumulation of loosely associated surface keratinocytes with a rounded appearance at the eyelid margin (Figs. 4A, and C–F); the cell shape change is thought to relate to the migratory behavior of these cells. In *Get1*^{-/-} mice at e15.5, this cell shape change was much less prominent, and of the rounded cells that were present, many extended from the epidermal surface at aberrant locations away from the eyelid tip (Figs. 4B and G–J). Compared with WT eyelids, the typical clump of rounded keratinocytes at the inner and outer canthus was absent in the *Get1*^{-/-} mice (Figs. 4A, B, C, E, G, and I). Also, in the *Get1*^{-/-} eyelids, many of the rounded keratinocytes appeared to have an axis parallel to the rim of the eyelid while in WT mice, rounded keratinocytes appeared to have an axis perpendicular to the edge of the eyelid, consistent with their migration towards the center of the cornea. By e16, WT eyelids were closed with a clump of degenerating keratinocytes located at the site of closure. In the *Get1*^{-/-} mice, however, the eye remained open at e16 with disorganized and scattered rounded keratinocytes, many of them in aberrant locations (Fig. 4L). By e17, the *Get1*^{-/-} eyelids closed, apparently through apposition of the eyelid roots and only occasional scattered rounded keratinocytes were observed; a typical cell clump does not form over the fused region (Figs. 4M and N).

The rounded keratinocytes at the leading edge are known to form filopodia, which are rod-like extensions of the cell surface filled with parallel bundles of actin filament that function in cell migration and epithelial fusion (Faix and Rottner, 2006; Martin and Parkhurst, 2004). Transmission electron microscopy (TEM) revealed abundant filopodia formation in keratinocytes of the WT leading edge. In contrast, the leading edge was immature with fewer and shorter filopodia in the *Get1*^{-/-} mice (Figs. 5C–D). Consistent with the SEM findings in *Get1*^{-/-} mice, rounded keratinocytes with filopodia were present in eyelid epithelia located away from the tip of eyelid (data not shown) in *Get1* mutant mice. These data indicate that *Get1* is required for the cell shape change and filopodia formation at the leading edge during eyelid closure.

***Get1* is required for normal actin bundle formation during leading edge development**

During eyelid closure, actin filament polymerization in the leading edge generates the force required for formation of filopodia and cell migration. To test whether *Get1* regulates actin filament polymerization in the eyelid epithelium, we examined the polymerization of F-actin at various time points using wholemount and section Phalloidin staining. In WT eyelids, F-actin first becomes prominent at e14.5. Subsequently, Phalloidin staining becomes progressively stronger at e15 and e15.5, eventually decreasing at e16 as the eyelids fuse (Figs. 6A, C, E and G). By contrast, in *Get1*^{-/-} eyelids, F-actin formation is greatly decreased at all stages (Figs. 6B, D, F and H). These results are consistent with the relative absence of rounded keratinocytes, and fewer and shorter filopodia in the leading edge of *Get1*^{-/-} eyelids as observed by SEM and TEM, respectively. Similar results were obtained when eyelid tissue sections were stained with Phalloidin (Figs. 6I–N). In contrast to the striking effects on F-actin formation, *Get1* had no effect on cell proliferation (Supplementary Fig. 3A–D) or apoptosis (data not shown) in the eyelids during the same stages of eyelid development. We conclude that *Get1* regulates genes that affect actin stress fiber polymerization in the leading edge of the developing eyelid.

Get1 regulates the TGF α /EGFR/ERK signaling pathway during eyelid closure

Previous studies have shown that the EGFR/ERK signaling pathway plays a key role in eyelid closure (Xia and Kao, 2004). Mice lacking the EGFR ligand TGF α display impaired formation of the leading edge with occasionally eye-open phenotype at birth (Luetteke *et al.*, 1993). Also, similar to Get1, TGF α is highly expressed in leading edge of the eyelid (Berkowitz *et al.*, 1996) and in the suprabasal layers of the epidermis (Gibbs *et al.*, 1998). Furthermore, we noticed in a previous microarray gene expression study that TGF α was downregulated in the epidermis of *Get1* mutant mice (Yu *et al.*, 2006). The similar expression patterns and eye phenotypes of these two mouse mutants prompted us to examine the potential role of *Get1* in the TGF α pathway. TGF α transcript expression was decreased in the skin of *Get1*^{-/-} mice at both e16.5 and e18.5 (Fig. 7A). Immunofluorescence studies showed that in WT mice, TGF α was most highly expressed in the most superficial keratinocytes of the epidermis, including the tip of the eyelids at e14 and e14.5 (Figs. 7B and D), and in the leading edge of eyelids at e15 and e15.5 (Figs. 7F and H). In *Get1*^{-/-} eyelids, TGF α levels were lower and expression was especially decreased at the tip of the eyelid, which is the site where the normal leading edge forms (Figs. 7C, E, G and I).

While EGFR expression is similar in WT and *Get1*^{-/-} eyelids at e14.5 and e15.5 (Figs. 8A–D), phosphorylated EGFR fails to concentrate in the tip and the leading edge of the *Get1*^{-/-} eyelids, as is observed in the WT eyelids (Figs. 8E–H). Expression of phosphorylated EGFR in the suprabasal layers of the eyelid epidermis otherwise appears normal. Expression of phosphorylated ERK was also decreased at the tip of the developing eyelids of *Get1*^{-/-} mice (Figs. 8I–L), but appeared to be normal in the basal layer of the epidermis and in the dermis at e15.5 (Figs. 8K–L). Keratin (K) 6, which is a marker gene for epithelialization (Paladini and Coulombe, 1998) in adult skin and in embryonic eyelid closure (Mazzalupo *et al.*, 2003), plays a role in migration during wound healing (Wojcik *et al.*, 2000; Wong and Coulombe, 2003). Also, a previous study showed that TGF α induces K6 and K16 expression (Jiang *et al.*, 1993). Consistent with these studies, we found that K6 is markedly upregulated at the tip of the eyelid in WT mice (Figs. 8M,O). And consistent with the decreased TGF α /EGFR/ERK activation at the leading edge in *Get1*^{-/-} mice, K6 expression fails to focus at the tip of the leading edge (Figs. 8M,P). Increased K6 expression, however, was observed in an aberrant location away from the rim of the eyelid, consistent with the presence of rounded keratinocytes observed in this location with SEM. In contrast to the defective EGFR signaling, there was no change in the expression of phosphorylated JNK (Figs. 8Q–T), a major downstream target for TGF β /activin-MEKK1-JNK-cJun, which is also important for eyelid closure (Li *et al.*, 2003; Weston *et al.*, 2004; Xia and Kao, 2004; Zenz *et al.*, 2003; Zhang *et al.*, 2003). Taken together, these data indicate that *Get1* participates in regulation of the TGF α /EGFR/ERK signaling pathway during leading edge formation in eyelid closure.

TGF α can rescue eye-open phenotype in *Get1*^{-/-} eyes

If *Get1* acts upstream of TGF α during eyelid closure, then TGF α should be able to promote eyelid closure in *Get1*^{-/-} eyes. To test this prediction, we performed TGF α rescue experiments in *Get1*^{-/-} mice, using eyelid explant culture methods previously developed by others (Tao *et al.*, 2006; Tao *et al.*, 2005a). TGF α added to the cultures significantly promotes eyelid closure in *Get1*^{-/-} explant cultures after 10 hours (Figs. 9A–D). In response to TGF α treatment, we observed accelerated closure along both the short and long axis of the *Get1*^{-/-} eyelids (Figs. 9E–F). TGF α does not affect WT eyelid closure significantly (Supplementary Fig. 4), indicating specificity of the TGF α effect. Histological analysis showed that while with PBS treatment, no leading edge formed over 10 hours (Fig. 9G), TGF α induced the formation of a leading edge in *Get1*^{-/-} eyelids (Fig. 9H). Consistent with these findings, SEM showed limited accumulation of rounded cells in the rim of *Get1*^{-/-} eyelids treated with PBS (Fig. 9I). In contrast, TGF α treatment induces accumulation of rounded cells at the eyelid rim (Fig. 9J),

although the number of rounded cells is higher in WT eyelids (data not shown), suggesting incomplete rescue. These results indicated that TGF α can induce cell shape changes and the formation of the leading edge on a *Get1*^{-/-} background. Furthermore, the downstream mediators of TGF α signaling, phospho-EGFR and phospho-ERK were upregulated in *Get1*^{-/-} eyelids after addition of TGF α (Figs. 9K–N). Taken together, these results suggest that during leading edge formation in the eyelid, *Get1* functions upstream of TGF α in the TGF α /EGFR/ERK signaling pathway.

Discussion

In this manuscript we report a novel role for *Get1* in eyelid development; deletion of the *Get1* gene leads to delayed eyelid closure. *Get1* is strongly expressed in the leading edge of the developing eyelid and in the leading edge of wounds. Consistent with the expression pattern, the formation of the eyelid leading edge was impaired in *Get1* mutant mice. The number of migrating round-shaped keratinocytes was greatly decreased and many of the rounded cells that were present were aberrantly localized to a region away from the eyelid tip in *Get1* mutant mice. In addition, filopodia formation was impaired and actin bundles, which create the force for cell migration and formation of filopodia, are strikingly decreased in the eyelid tip of *Get1* mutant mice. Actin fiber polymerization is known to be regulated by the TGF α /EGFR/ERK signaling pathway and TGF α and levels of its downstream targets, phosphorylated EGFR and phosphorylated ERK, were decreased in the tip of the eyelid in *Get1* mutant mice. In explant cultures, the addition of TGF α to *Get1*^{-/-} eyelids promoted cell shape changes and induced formation of a leading edge, leading to accelerated eyelid closure. These results indicate that *Get1* acts upstream of TGF α in the EGFR/ERK signaling pathway to regulate eyelid closure.

Get1 regulates leading edge formation and keratinocyte sheet migration

Mammalian eyelid development and fusion follows a stereotypical stepwise pathway in which the primitive eyelid root forms first (Findlater *et al.*, 1993; Mine *et al.*, 2005; Tao *et al.*, 2005a). Then, a leading edge of keratinocytes extends centripetally from the rim of the eyelid, ultimately covering the eye through keratinocyte sheet migration. After fusion of the eyelid, the eyelid roots appose and most of the leading edge epithelial sheet is shed. In addition to migration, keratinocyte proliferation in the basal layer of the eyelid root is required for normal eyelid closure. Our data shows that the eye-open phenotype of *Get1*^{-/-} mice is due to a defect in the initiation of the leading edge and migration of the epithelial sheet (Figs. 3A–P). *Get1* does not appear to affect eyelid root formation or keratinocyte proliferation (Supplementary Fig. 3). Therefore, we conclude that the likely role of *Get1* in eyelid closure involves keratinocyte migration. This notion is also consistent with the upregulation of *Get1* in wound front keratinocytes in adult skin (Supplementary Fig. 2). Interestingly, in *Get1*^{-/-} mice, we observed the formation of rounded keratinocytes at the superficial surface of the eyelid, in an aberrant location away from the eyelid rim. These cells were observed with SEM (Fig. 4J) and with K6 upregulation (Figs. 8S and U). Therefore, *Get1* may play a role in focusing the site of the initial leading edge.

Get1 deletion affects F-actin polymerization, keratinocyte shape change, and filopodia formation during eyelid closure

During eyelid closure, F-actin polymerizes to form a contractile cable at the leading edge (Zhang *et al.*, 2003), which has been proposed to drive epithelial sheet movement in a purse string manner (Martin and Parkhurst, 2004; Shimizu *et al.*, 2005). Similar actin cables are thought to generate the contractile force required for epithelial fusion during dorsal closure in *Drosophila* (Kiehart *et al.*, 2000). In addition to actin cable formation, radial F-actin fibers form, which extend with an axis in direction of the leading edge (Zhang *et al.*, 2003). Wholemount Phalloidin staining showed that both types of F-actin reorganization are greatly

decreased in the developing eyelid of *Get1*^{-/-} mice (Figs. 6A–H), indicating that *Get1* regulates F-actin fiber formation. Actin reorganization is also thought to be required for the striking changes in keratinocyte cell-shape in the migrating leading edge (Kodama *et al.*, 2004). During normal eyelid closure, keratinocytes take on a rounded appearance and in the *Get1*^{-/-} mice this phenomenon is markedly decreased (Fig. 4). Most likely this defect relates to the lack of actin polymerization.

Filopodia are rod-like extensions of cell surface filled with parallel bundles of actin filaments (Faix and Rottner, 2006). Filopodia-mediated epithelial migration and fusion are common phenomena in vertebrate development fusion events (Martin and Parkhurst, 2004). Filopodia can be observed in the opposing eyelid epithelial cells, as the eyelids transiently fuse in late mammalian gestation (Zenz *et al.*, 2003). Compared with WT mice, filopodia appear much fewer and shorter in the leading edge of *Get1*^{-/-} mice (Figs. 5C–D). It is likely that this defect also relates to lack of F-actin polymerization, and contributes to decreased keratinocyte migration and delayed eyelid closure.

Get1 functions upstream of the TGF α /EGFR signaling pathway

While we have gained insights into the molecular mechanisms whereby *Get1* regulates barrier formation, the molecular mechanisms underlying the role of *Get1* in re-epithelialization remain unclear. An important regulator of eyelid closure is the EGFR signaling pathway. Targeted disruption of the EGFR gene leads to an eye-open phenotype at birth with 100% penetrance (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). A critical event in EGFR activation during eyelid closure appears to be binding by its ligands TGF α and HB-EGF. Thus, both TGF α and HB-EGF are highly expressed in the leading edge of the mouse eyelid, and mice deleted for either TGF α or HB-EGF exhibit eye-open phenotype at birth, although with incomplete penetrance (Berkowitz *et al.*, 1996; Luetke *et al.*, 1993; Mann *et al.*, 1993; Mine *et al.*, 2005). EGFR signaling activates ERKs, leading to the induction of actin filament polymerization (Xia and Kao, 2004; Zhang *et al.*, 2003). The actin stress fibers form actin purse-string at the leading edge of the migrating epithelial sheet of eyelid, which drives epithelial cell closure (Martin and Parkhurst, 2004). Furthermore, TGF α /EGFR induces expression of K6 and K16 (Jiang *et al.*, 1993), keratins which are markers of activated keratinocytes and facilitate their migration in the developing eyelid and wound healing (Paladini and Coulombe, 1998) (Mazzalupo *et al.*, 2003) (Wong and Coulombe, 2003).

The decreased TGF α expression (Figs. 7B–I), as well as decreased EGFR and ERK activation (Figs. 8E–L) in the tip of *Get1*^{-/-} eyelids suggests that *Get1* may act upstream of TGF α in this pathway. Consistent with this model, the severity of the eye-open phenotype of *Get1* and TGF α mutated mice is similar, and TGF α was able to rescue the phenotype in *Get1*^{-/-} eyelids (Figs. 9A–F). Under these conditions, TGF α was able to increase EGFR (Figs. 9I–J) and ERK (Figs. 9K–L) activation, and induce cell shape changes (Figs. 9M–N) and leading edge formation (Figs. 9G–H). These data lend further support for our model that *Get1* acts upstream of TGF α . Since decreased TGF α expression in *Get1*^{-/-} mice is observed prior to formation of the leading edge in WT mice, it is unlikely that this decrease is due to the absence of TGF α expressing cells (Figs. 7B–E).

We also noticed that the rescue of TGF α in *Get1*^{-/-} eyelids was incomplete. While this may be due to limitations of the organ culture model, we cannot rule out the possibility that *Get1* also acts downstream in the EGFR pathway. In this respect, it is important to note that in *Drosophila* wound healing, Grainyhead acts downstream of ERK signaling. Other pathways important for eyelid closure include the TGF β /activin-MEKK1-JNK-cJun signaling pathway (Weston *et al.*, 2004; Xia and Kao, 2004; Zhang *et al.*, 2003), which crosstalk with the TGF α /EGFR/ERK pathway because c-Jun can induce EGFR and HB-EGF expression (Li *et al.*, 2003; Zenz *et al.*, 2003). Our data showing normal JNK activation in the *Get1*^{-/-} eyelids argues

against a major role for *Get1* in the activation of the TGF β /activin-MEKK1-JNK-cJun signaling pathway.

The eye open at birth phenotype is also common in mice with mutations in core planar cell polarity genes and it has been argued that eyelid closure represents a planar cell polarity process (Wang and Nathans, 2007). Interestingly, inhibition of WNT signaling in the developing epidermis leads to an eye open phenotype (Andl *et al.*, 2002). However, it is unclear whether this relates to activation of the canonical WNT pathway or the planar cell polarity pathway. In *Drosophila*, Grainyhead affects planar polarity in the wing (Lee and Adler, 2004), and the *Get1*^{-/-} mice (Ting *et al.*, 2005; Yu *et al.*, 2006) have many features in common with mice mutated for core planar cell polarity genes (Jones and Chen, 2007; Seifert and Mlodzik, 2007; Wang and Nathans, 2007). Therefore, if the planar cell polarity pathway is important in eyelid closure, *Get1* might modulate this pathway. However, the link between planar cell polarity genes and eyelid closure remains to be defined.

Get1 has sequential distinct roles in epithelial closure

Previous studies have demonstrated that Grainyhead genes play important roles in epithelial barrier formation and that this function is conserved in evolution across multiple species. In *Drosophila*, Grainyhead, activates the Dopa Decarboxylase (Ddc) gene, which is required for hardening of the larval cuticle (Bray and Kafatos, 1991). In the mouse, the Grainyhead homologue *Get1* activates a broad set of terminal differentiation genes that are required for epidermal barrier formation. The genetic program activated by *Get1* during terminal differentiation of the epidermis includes lipid-producing enzymes, adhesion molecules, structural genes and protein crosslinking enzymes (Fig. 10) (Ting *et al.*, 2005; Yu *et al.*, 2006).

In addition to a role in epidermal barrier formation, there is previous evidence indicating a role for *Get1* in re-epithelialization. In *Drosophila*, Grainyhead is required for wound healing (Mace *et al.*, 2005), and in mice, *Get1* mutation impairs embryonic wound healing (Ting *et al.*, 2005). Consequently, similar to the barrier function, the wound-healing role of Grainyhead also appears to be evolutionarily conserved. Our experiments strongly indicate that *Get1* regulates eyelid closure by affecting F-actin polymerization through the TGF α /EGFR/ERK signaling pathway (Fig. 10). These observations suggest that in mice *Get1* employs distinct mechanisms to execute the two functions in re-epithelialization and barrier formation. Therefore, during the epithelial closure process in mice, *Get1* is likely to act at two distinct steps: early in epithelial migration to close the epithelial defect, and later after completion of re-epithelialization, in barrier formation of the epithelial sheet (Fig. 10). It has been proposed that during evolution Grainyhead genes arose in the first epithelia (Venkatesan *et al.*, 2003). Since the early epithelia may not have evolved complex barrier mechanisms, it is tempting to speculate that the re-epithelialization function of Grainyhead may be more ancient than its barrier role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

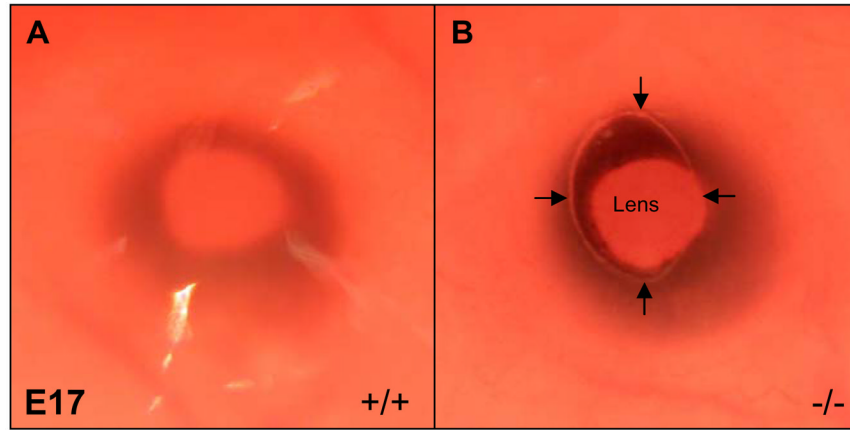
Acknowledgements

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C.

Analysis of open eye phenotype in *Get-1* KO mice

	E16	E17	E18.5
WT	0% (0/32)	0% (0/36)	0% (0/70)
KO	100% (24/24)	25% (6/24)	10% (5/48)
	$P=2.30E-16$	$P=0.0027$	$P=0.0098$

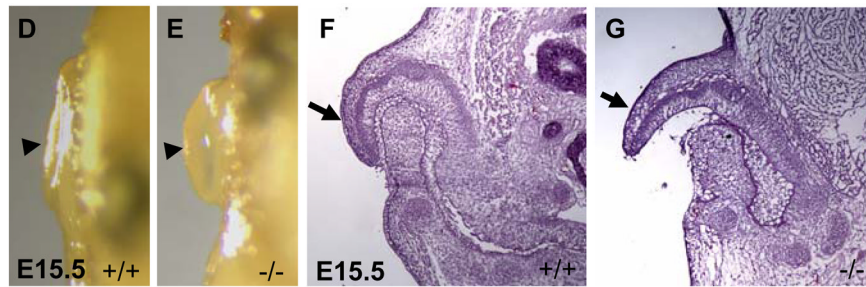


Fig. 1. Delayed eyelid closure and delayed pinna fusion in *Get1* mutant mice

(A) Eyelid is closed in WT embryos at e17. (B) Open eye in *Get1* mutant mice at e17. (C) Statistical analysis of the eye-open phenotype in WT and *Get1* mutant mice. *P*-values were determined using fisher exact probability test (one tailed). (D) Ear pinna is fused to the scalp in WT embryos at e15.5. (E) Unfused ear pinna in *Get1* mutant embryos at e15.5. Arrowheads point to the ear pinnae in panels D and E. (F and G) H&E staining of the ears from WT and *Get1* mutant mice at e15.5. Arrows point to the tip of the pinnae in panels F and G.

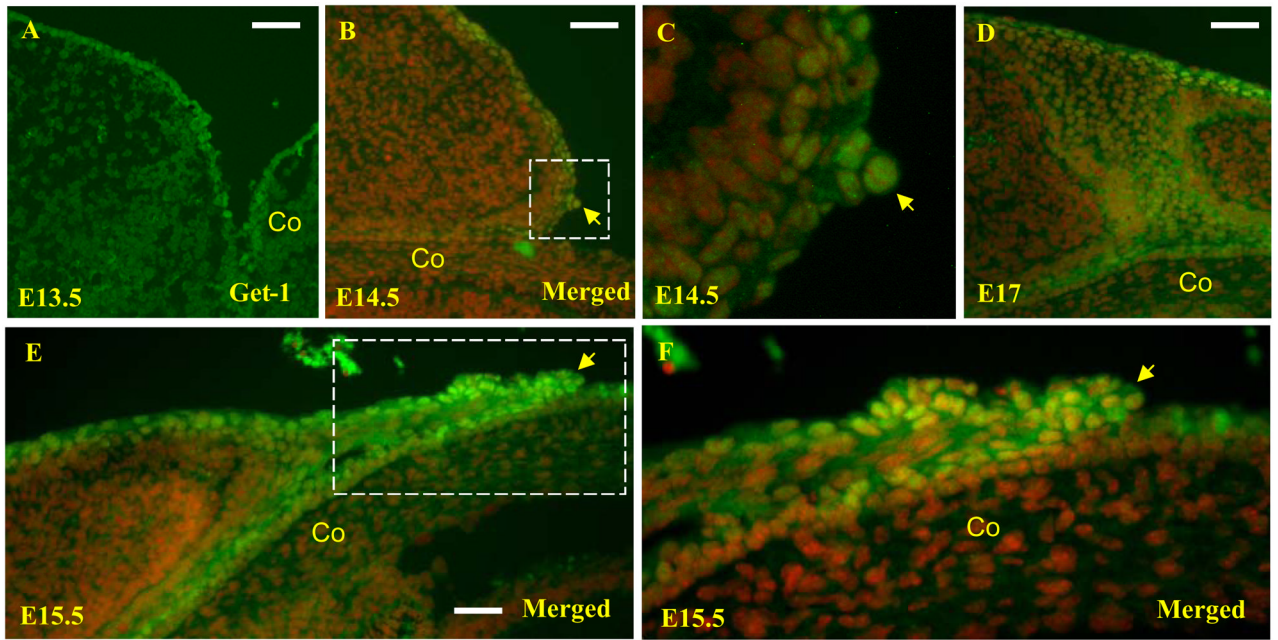


Fig. 2. Get1 expression during eyelid development

(A to F) Immunofluorescence analysis of Get1 expression in eyelids at e13.5 (A), e14.5 (B and C), e15.5 (E and F) and e17 (D). (B and C) The arrow points to the beginning of the leading edge at e14.5, where Get1 is highly expressed. (C) High magnification of the boxed region in panel B. (E and F) The arrow indicates the leading edge of the eyelid which expresses Get1 at high levels. (F) High magnification of the boxed region in panel E. Red represents DAPI staining. Co, cornea. Scale bar: 50 μ m.

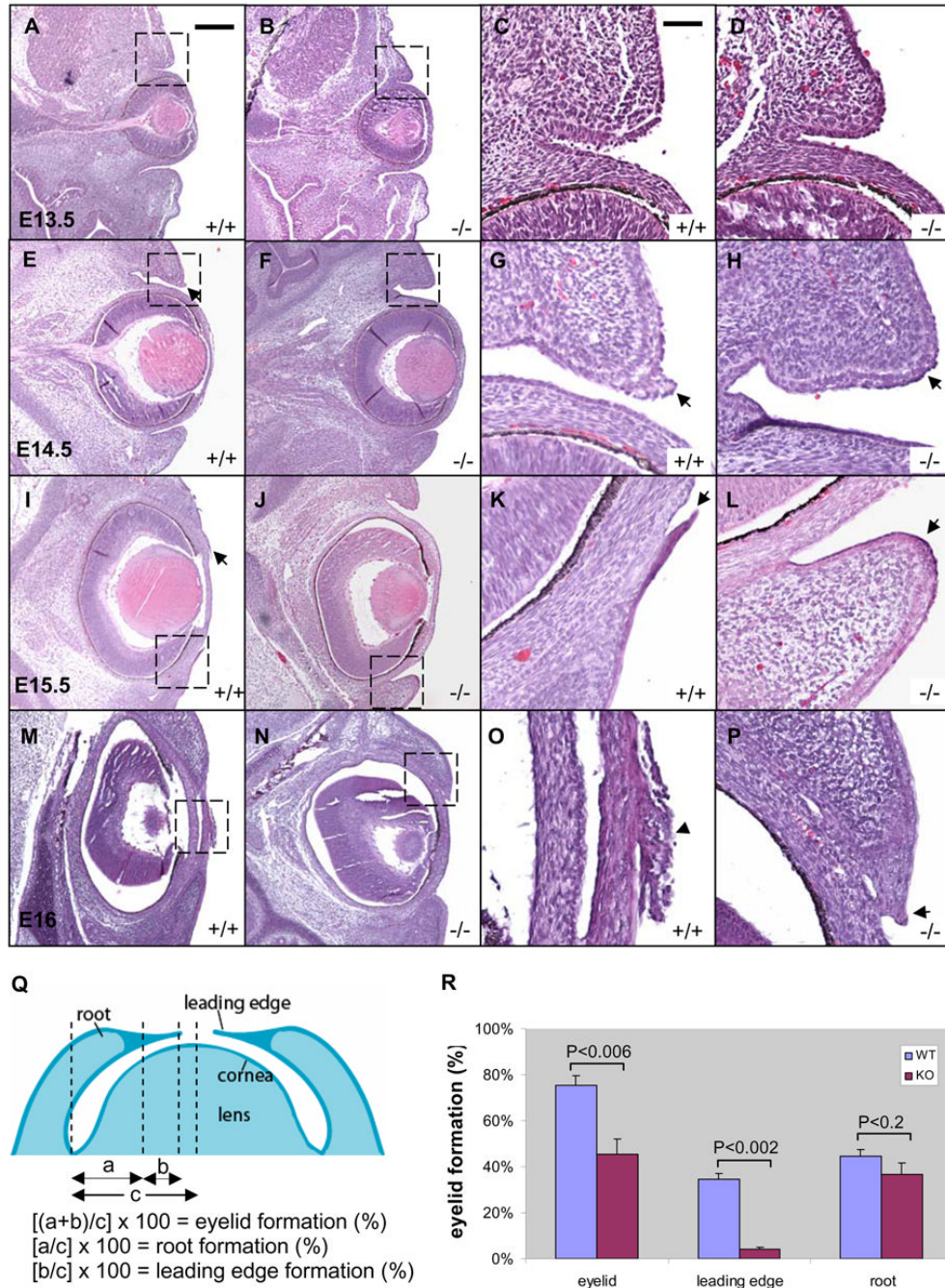


Fig. 3. Defective formation of the eyelid leading edge in *Get1*^{-/-} mice

(A to P) H&E staining of the eyes from WT and *Get1* mutant mice during eyelid closure at e13.5 (A to D), e14.5 (E to H), e15.5 (I to L) and e16 (M to P). The panels in the last two columns are high magnification pictures of the corresponding boxed regions in the panels in the two columns to the left. Arrows point to the tip of the eyelid. The arrowhead in panel O points round keratinocytes after eyelid fusion. (Q) Schematic diagram showing how eyelid root, leading edge and total eyelid were measured during eyelid closure at E15.5. Adapted from Mine et al. (Mine *et al.*, 2005). (R) Quantification and statistics of eyelid closure in WT and *Get1* mutant mice at e15.5. Results represent the mean and SEM for 5 embryos. *P*-values were

determined using T-test (one tailed). Scale bars 200 μm for A, B, E, F, I, J, M, N; 50 μm for C, D, G, H, K, L, O, P.

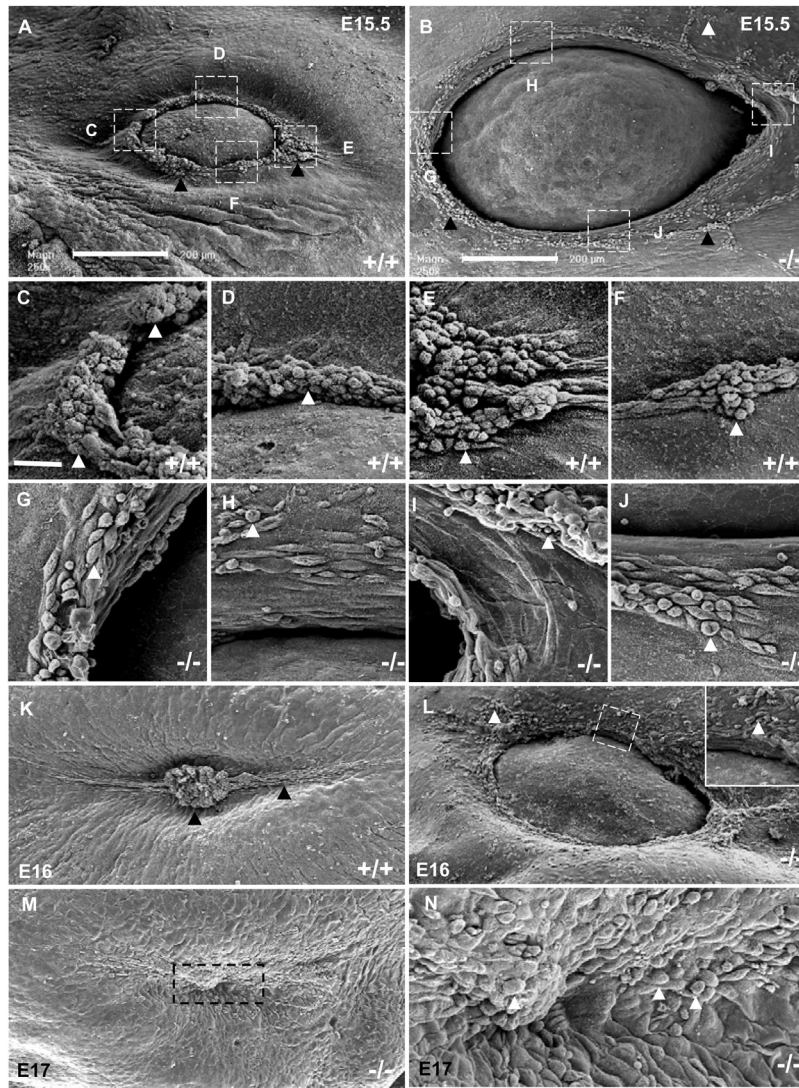


Fig. 4. Defective cell shape changes during leading edge formation in *Get1* mutant mice
 Scanning electron microscopy of the eyelid region of WT (A, C to F and K) and *Get1*^{-/-} (B, G to J and L to N) mice during the indicated time points. (C to J) Higher magnification pictures of the corresponding boxed regions in panels A and B. The arrowheads point to rounded cells. The boxed region in panel L is shown in higher magnification in the insert. High magnification picture of the fusion region contained in the boxed region in panel M is shown in panel N. Scale bar is 200 μm for A, B, K, L, M; 25 μm for C-J.

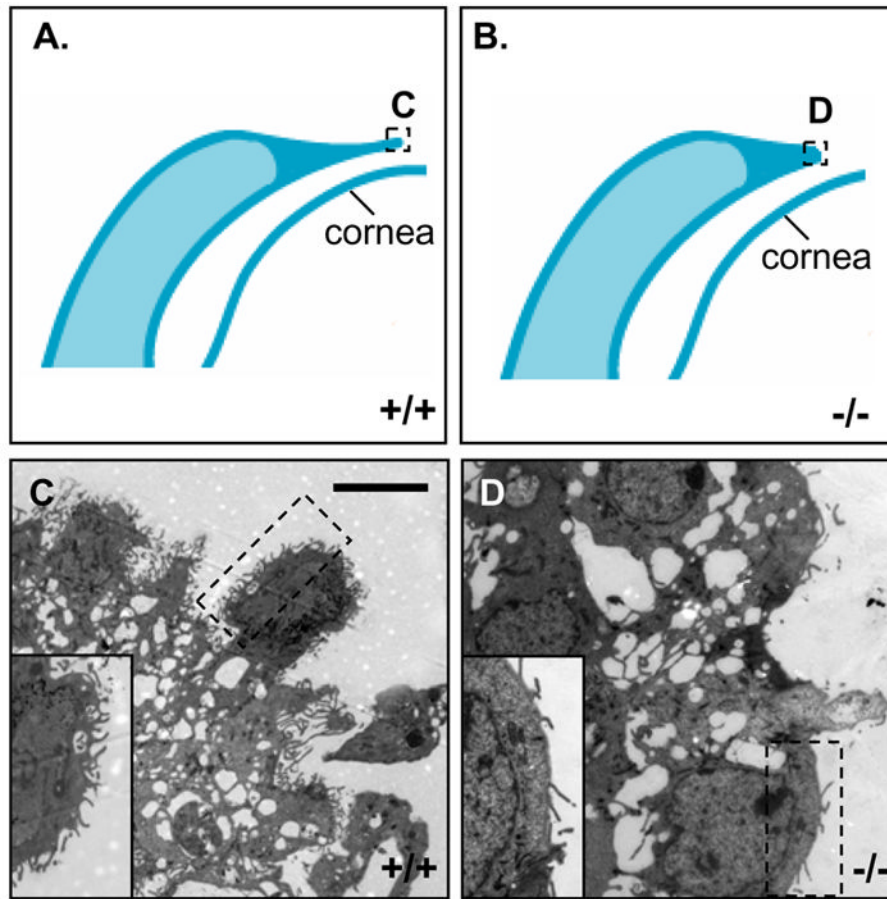


Fig. 5. Defective filopodia formation in *Get1* mutant mice

(A and B) Schematic diagram showing the tip region of WT and *Get1* mutant eyelids at e15.5. The boxes C–F indicate the regions imaged by transmission electron microscopy and shown in the corresponding panels. (C) The tip of the WT leading edge. The high magnification insert of the boxed area shows numerous well developed filopodia. (D) The tip of the *Get1* mutant eyelid. The insert shows high magnification picture of the boxed region, highlighting paucity of filapodia.

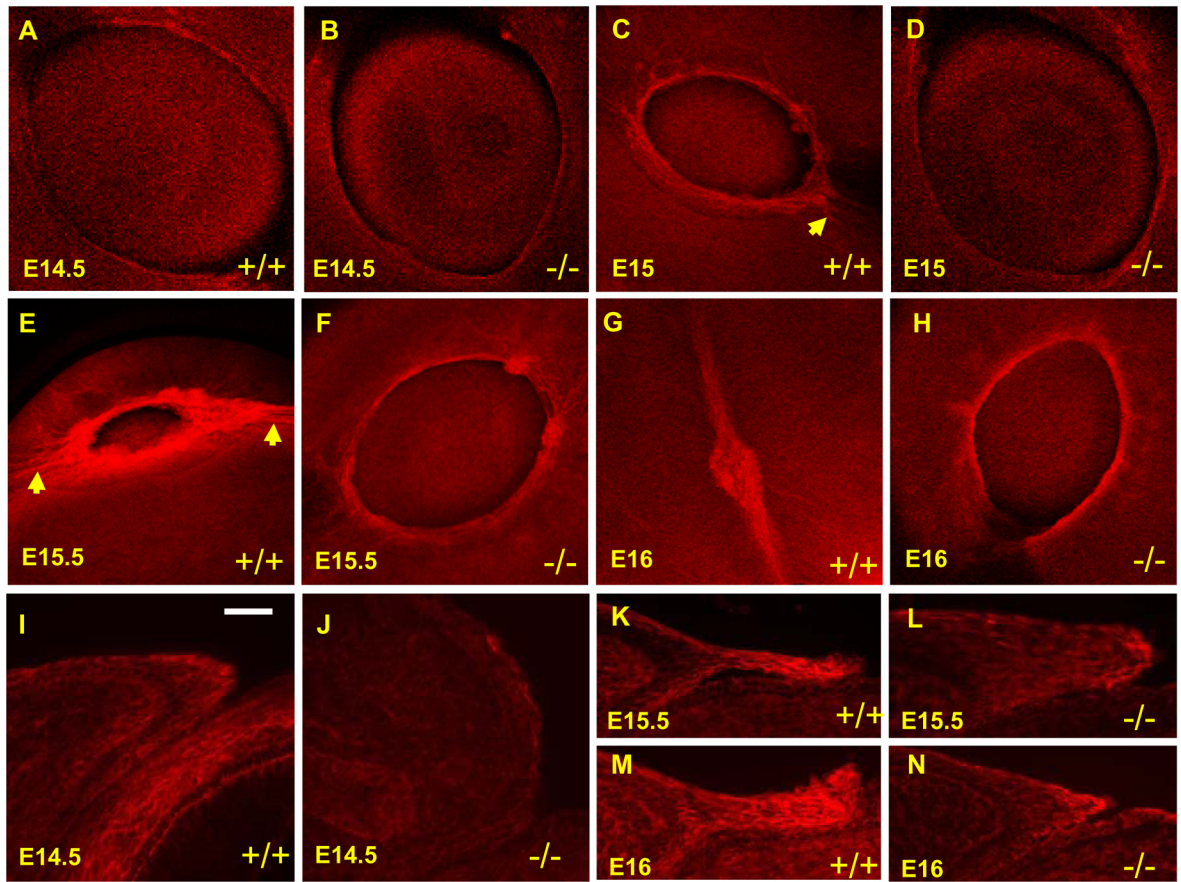


Fig. 6. *Get1* promotes actin filament polymerization in the developing eyelid epithelium
 (A to H) Wholemount phalloidin staining of WT and *Get1* mutant eyes at e14.5 (A and B), e15 (C and D), e15.5 (E and F) and e16 (G and H). (I to N) Phalloidin staining of eyelid sections from WT and *Get1* mutant mice at e14.5 (I and J), e15.5 (K and L) and e16 (M and N). Arrows in panel C and E indicate radial actin fibers. Scale bar: I–N, 50 μ m.

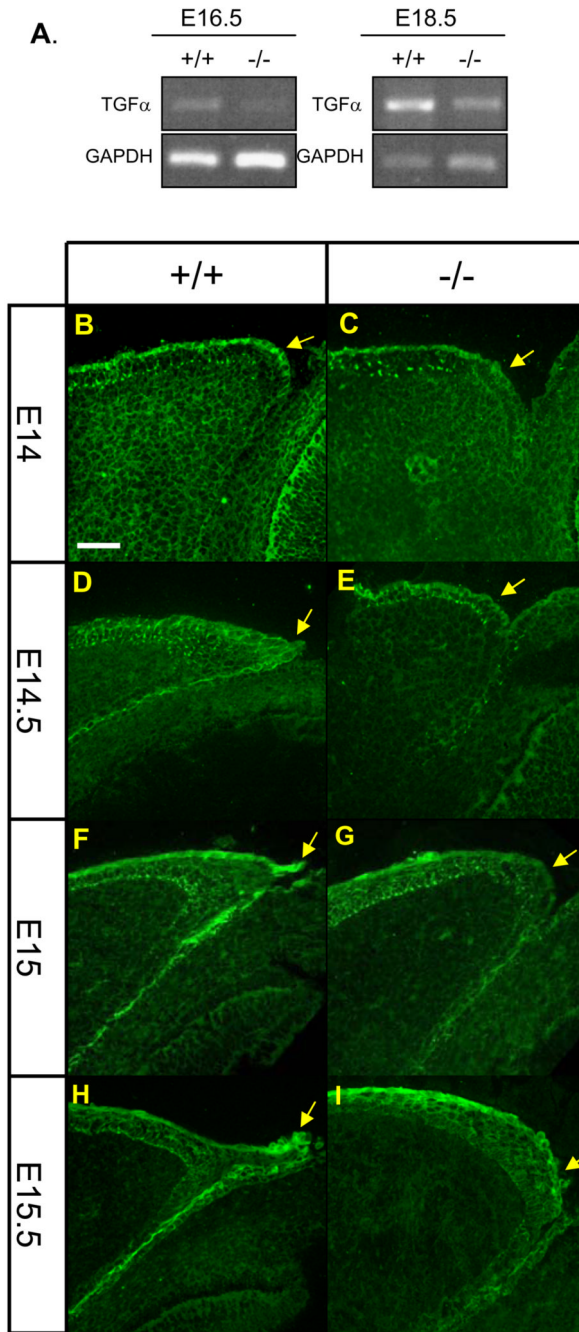


Fig. 7. Impaired TGF α expression in *Get1* mutant eyelids

(A) RT-PCR analysis of TGF α expression in skin from WT and *Get1*^{-/-} mice. (B to I) TGF α expression pattern in eyelid epithelium of WT and *Get1* mutant mice at e14 (B and C), e14.5 (D and E), e15 (F and G) and e15.5 (H and I). Arrows point to the tip of the leading edge. Scale bar: 50 μ m.

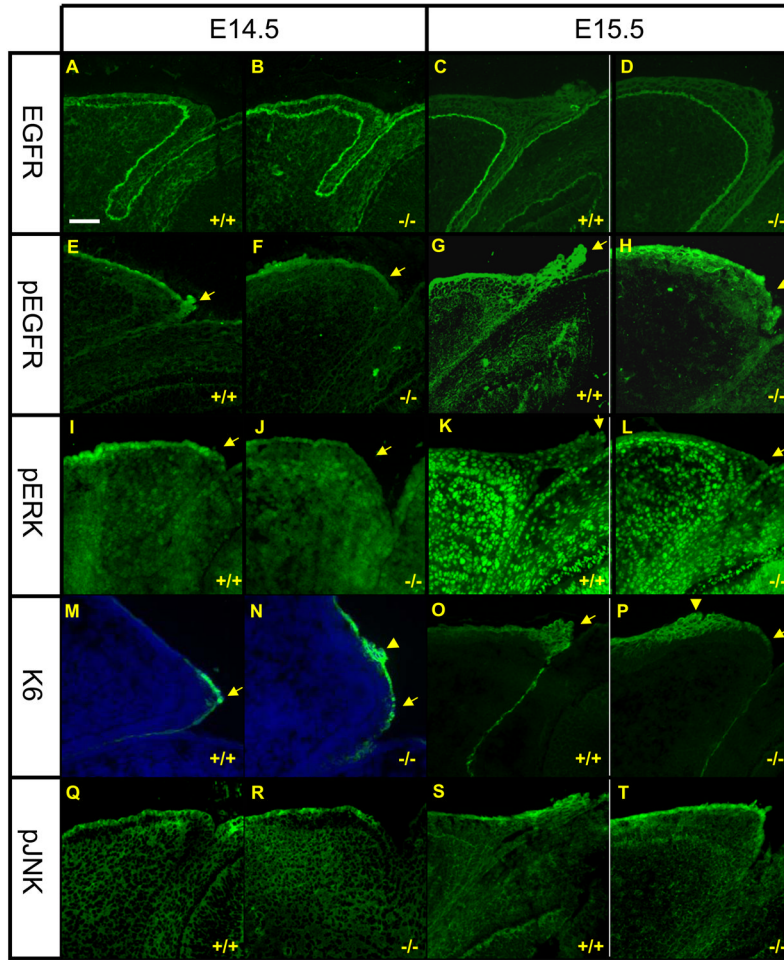


Fig. 8. Impaired EGFR/ERK signaling in *Get1* mutant eyelids

Expression of EGFR (A to D), pEGFR (E to H), pERK (I to L), K6 (M to P), and pJNK (Q to T) in WT and *Get1*^{-/-} eyelids at e14.5 (n=3) and e15.5 (n=3). Arrows point to the tip of the leading edge. The arrow heads in panels N and P point to K6 upregulation in an aberrant location of the eyelid in *Get1*^{-/-} mice. Scale bar: 50µm.

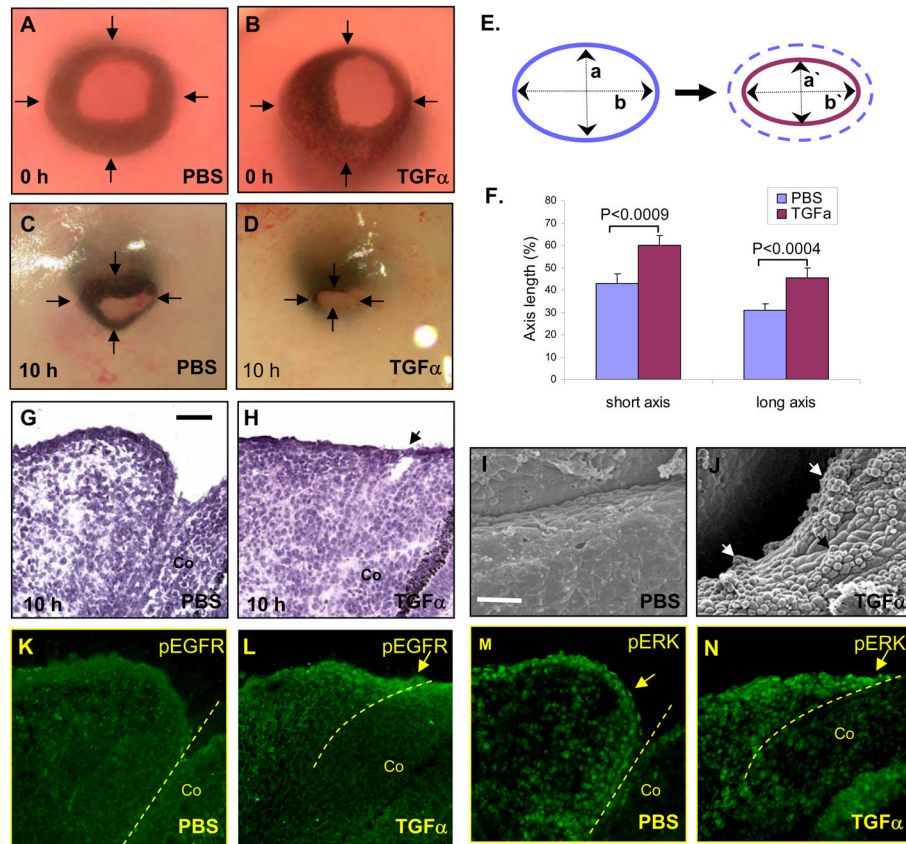


Fig. 9. TGF α can rescue eyelid closure defect in *Get1* mutant mice

(A to D) Gross pictures of *Get1*^{-/-} eyes before (A and B) and after (C and D) 10 h of PBS or TGF α treatment. Arrows point to the eyelid borders. (E) A schematic showing measurements of long and short axes, which was used to assess eyelid closure as previously described (Tao *et al.*, 2005a), using the formula: % eyelid short axis closure = $(1 - a'/a) \times 100$, and % eyelid long axis closure = $(1 - b'/b) \times 100$ where “a” and “b” represent short axis and long axis of open eye, respectively, at time 0 h, and “a'” and “b'” represent short axis and long axis of open eye, respectively, at time 10 h. (F) Analysis of the effect of TGF α treatment on eyelid closure in *Get1*^{-/-} eyes. The bars represent means and SEM, based on 15 experiments. (G and H) H&E staining of *Get1*^{-/-} eyelids treated with PBS (G) and TGF α (H) for 10 h (n=4). The arrow points to the leading edge forming in response to TGF α treatment. (I and J) SEM analysis of *Get1*^{-/-} eyelids after treatment with PBS (I) and TGF α (J) for 10 h (n=4). Arrows in panel J point to rounded cells forming in response to TGF α . (K and L) pEGFR staining in *Get1*^{-/-} eyelids after treatment with PBS (K) and TGF α (L) for 10 h (n=4). The arrow points to increased pEGFR staining in the leading edge in response to TGF α treatment. The broken line indicates the eyelid-cornea junction. (M and N) pERK staining in *Get1*^{-/-} eyelids after treatment with PBS (M) and TGF α (N) for 10 h (n=4). The arrow points to increased pERK staining in the leading edge in response to TGF α treatment. The broken line indicates the eyelid-cornea junction. Co, cornea. Scale bars: G-L, 50 μ m; M-N, 25 μ m.

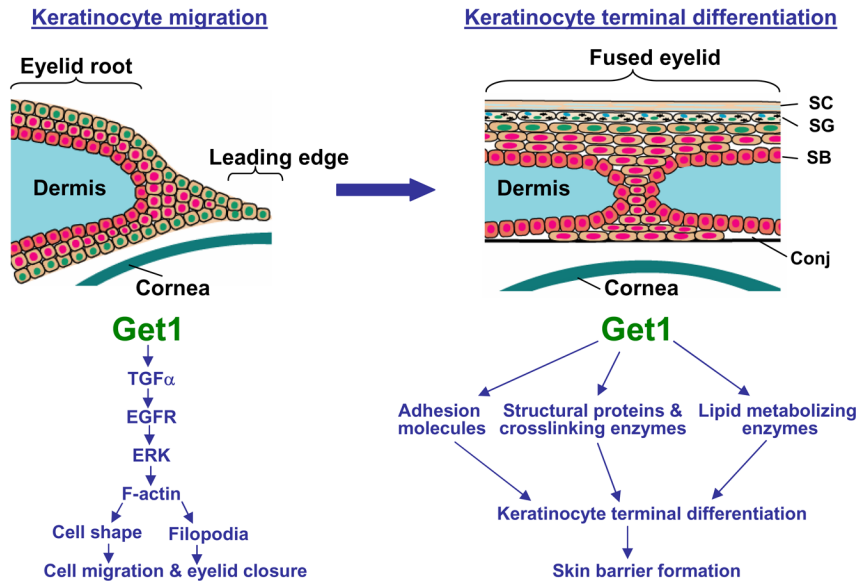


Fig. 10. The sequential distinct roles of *Get1* in eyelid development

A schematic representation of the distinct roles of *Get1* in eyelid development. *Get1* plays an early role in formation and migration of the leading edge keratinocyte sheet (left panel), and a later role in the formation of the epidermal barrier after eyelid closure (right panel). During leading edge formation, *Get1* activates F-actin polymerization through the TGF α /EGFR/ERK signaling pathway. F-actin fibers further regulate cell shape change and filopodia formation to promote keratinocyte migration and eyelid closure. *Get1* promotes skin barrier formation by directly or indirectly regulating genes encoding structural proteins and crosslinking enzymes, adhesion molecules, and lipid metabolizing enzymes (Ting *et al.*, 2005; Yu *et al.*, 2006). SC, cornified layer; SG, granular layer; SB, basal layer; Conj, Conjunctiva.