ABSTRACT

Interferon regulatory factor 6 (Irf6) regulates keratinocyte proliferation and differentiation. In this study, we tested the hypothesis that Irf6 regulates cellular migration and adhesion. Irf6-deficient embryos at 10.5 days post-conception failed to close their wound compared with wild-type embryos. In vitro, Irf6-deficient murine embryonic keratinocytes were delayed in closing a scratch wound. Live imaging of the scratch showed deficient directional migration and reduced speed in cells lacking Irf6. To understand the underlying molecular mechanisms, cell–cell and cell–matrix adhesions were investigated. We show that wild-type and Irf6-deficient keratinocytes adhere similarly to all matrices after 60 min. However, Irf6-deficient keratinocytes were consistently larger and more spread, a phenotype that persisted during the scratch-healing process. Interestingly, Irf6-deficient keratinocytes exhibited an increased network of stress fibers and active RhoA compared with that observed in wild-type keratinocytes. Blocking ROCK, a downstream effector of RhoA, rescued the delay in closing scratch wounds. The expression of Arhgap29, a Rho GTPase-activating protein, was reduced in Irf6-deficient keratinocytes. Taken together, these data suggest that Irf6 functions through the RhoA pathway to regulate cellular migration.

KEY WORDS: Interferon regulatory factor 6, Migration, Keratinocytes

INTRODUCTION

Cutaneous wound healing requires the coordination of inflammation, epithelialization, angiogenesis and dermal repair (Baum and Arpey, 2005). Epithelialization is ultimately dependent on the migration, proliferation and differentiation of keratinocytes (Coulombre, 2003). The growth and differentiation of keratinocytes is tightly regulated by transcription factors, with the transcription factor interferon regulatory factor 6 (Irf6) playing a crucial role (Biggs et al., 2012; Botti et al., 2011; Ingramah et al., 2006).

Irf6 belongs to the IRF family of transcription factors, which mediate the interferon response after viral infection (Honda and Taniguchi, 2006). In contrast to most IRFs, Irf6 is essential during embryogenesis. Mice lacking Irf6 exhibit perinatal lethality, as well as limb, craniofacial and epidermal anomalies (Ingraham et al., 2006; Richardson et al., 2006). In humans, mutations in Irf6 cause Van der Woude syndrome (VWS) and pterygium syndrome, two orofacial clefting disorders (Kondo et al., 2002). Interestingly, patients with VWS were more likely to have wound complications following corrective cleft surgery than patients with non-syndromic cleft (Jones et al., 2010), consistent with a role for Irf6 in wound healing. Although Irf6 is expressed in suprabasal keratinocytes of the epidermis and plays a crucial role in epidermal differentiation in vivo and in vitro (Biggs et al., 2012; Ingraham et al., 2006), its function in keratinocyte migration is currently unknown.

Cellular migration is a highly coordinated biological process that includes the assembly of cell–cell and cell–matrix contacts followed by the disassembly of older ones (for a review, see Vicente-Manzanares et al., 2005). The coordination of migration and the force required to achieve it is driven by the reorganization of the actin cytoskeleton (for a review, see Le Clainche and Carlier, 2008). The actin cytoskeleton normally provides dynamic structure and organization to the cell but, in the event of migration, this cellular scaffold reorganizes the cellular contents, drives the formation of lamellipodia and filopodia (two cellular protrusions defining the leading edge of a cell), and disassembles to retract the tail of the cell (Le Clainche and Carlier, 2008; Vicente-Manzanares et al., 2005). Migratory cues are diverse, and sensors of such cues include clusters of integrins, located on cellular protrusions, that assemble and disassemble to allow migration (Zaidel-Bar and Geiger, 2010). Simultaneously, E-cadherin-mediated cell–cell adhesions form initial contacts with adjacent cells that subsequently evolve into linear adhesions (Vasioukhin et al., 2000). The cytoskeleton requires integrins and cadherins for information about the environment, and, in turn, the contraction of actin is necessary for the assembly of the adhesions that these proteins form with the substrate and with other cells (Vasioukhin et al., 2000).

Members of the Rho family of small GTPases are the central regulators of actin cytoskeleton dynamics. GTPases cycle between GTP-bound (active) and GDP-bound (inactive) forms through the control of guanine nucleotide exchange factors (GEFs, activating) and GTPase activating proteins (GAPs, inactivating) (Guilluy et al., 2011; Heasman and Ridley, 2008; Van Aelst and D’Souza-Schorey, 1997). Of particular interest is RhoA, the main small GTPase responsible for assembling stress fibers that are anchored at adhesion complexes and that support the cell contraction necessary for translocation (Ridley and Hall, 1992). In vitro studies demonstrate a role for RhoA GTPase in keratinocyte differentiation (Grossi et al., 2005; McMullan et al., 2003). In vivo, however, RhoA has been found to be dispensable for epidermal differentiation but necessary for directed keratinocyte migration (Jackson et al., 2011). RhoA activation is also required for the formation of TGFβ3-induced stress fibers and for mediating TGFβ3 signaling during palatogenesis (Kaartinen et al., 2002). Additionally, we identified Arhgap29,
a GEF with high affinity for RhoA, as a novel cleft candidate gene downstream of Irf6 (Leslie et al., 2012; Saras et al., 1997). Because Irf6 is required for proper palatogenesis (Ingraham et al., 2006; Knight et al., 2006) and is a downstream effector of TGFβ3 (Knight et al., 2006; Xu et al., 2006), we hypothesize that Irf6 regulates the actin cytoskeleton in keratinocytes and alters cellular migration.

In this study, we established a role for Irf6 in epithelialization during the healing of embryonic wounds. By using culture of primary keratinocytes from wild-type and Irf6-deficient embryos, in combination with scratch-wound and adhesion assays, we demonstrated that Irf6 is required for the proper migration of keratinocytes. This Irf6-dependent process is mediated by RhoA.

RESULTS

Irf6 is present at the wound edge and in the neoformed epidermis following excisional wounding

We used our excisional murine wound-healing model (Le et al., 2012) to evaluate the expression of Irf6 in adult mice. We observed the presence of Irf6 in keratinocytes of unwounded adult mice (Fig. 1A), at the wound edge at 1 day after injury (Fig. 1B) and in cells that had just completed epithelialization (Fig. 1C). Irf6 expression decreased in the neoformed epidermis, before returning to the normal expression level at 11 days following injury (data not shown). In the open wound area (Fig. 1B), we noted the presence of a strong Irf6 signal that likely reflected background staining from necrotic or inflammatory cells.

Irf6 is required for proper embryonic wound healing

Irf6-deficient mice die perinatally from orofacial and epidermal anomalies (Ingraham et al., 2006), limiting wound-healing studies in Irf6-deficient animals to the embryo. Well-established embryonic wound-healing models have been described that follow epidermal closure after hindlimb resection at embryonic day (e)11.5 (McCluskey and Martin, 1995). Because of the severe hindlimb phenotype in the late-stage Irf6-deficient embryos, we modified the classic protocol and removed the forelimb in e10.5 animals. At this developmental time-point, wild-type and Irf6-deficient embryos were indistinguishable (Fig. 1D,E), and the size of their wounds immediately following limb removal were identical (Fig. 1D–F). After 24 h, the wounds of wild-type embryos were closed (Fig. 1G,I), whereas the wounds were still significantly open in embryos deficient for Irf6 (Fig. 1H,I). These data demonstrate that Irf6 is required for proper embryonic wound healing.

Irf6-deficient keratinocytes are delayed in closing an in vitro scratch wound

The closure of embryonic wounds mainly consists of keratinocyte migration across the wound. To test the hypothesis that Irf6 contributes to epidermal migration, we generated a scratch wound in confluent monolayers of wild-type and Irf6-deficient keratinocytes. Static images were taken at 0, 6, 8 and 24 h post-scratch (Fig. 2A–F; data not shown). Despite similar initial wound sizes, Irf6-deficient keratinocytes were significantly delayed in closing the scratch wound (59% versus 95% of the wound area was closed at 24 h for Irf6-deficient and wild-type keratinocytes, respectively) (Fig. 2E–G).

In order to further investigate the migratory defect of Irf6-deficient keratinocytes, we used time-lapse video microscopy to perform live imaging of the in vitro scratch assay. Images taken every 5 min over an 18 h period revealed that the Irf6-deficient keratinocytes seemed to adhere to one another or to the substrate, whereas wild-type keratinocytes moved as individual cells (supplementary material Movies 1, 2). This behavior could be noted over time in the centroid tracks and stacked perimeter plots generated by using a two-dimensional dynamic image analysis system (2D-DIAS). The data are presented in Fig. 2H,I at 10-min intervals. It can be seen from the centroid tracks that the majority of wild-type keratinocytes (Fig. 2H) oriented and moved...

Fig. 2. Impaired closure of a scratch wound because of reduced speed and directionality of Irf6-deficient keratinocytes. (A–F) Still recording of in vitro scratch wounds in confluent monolayers of wild-type (A,C,E) and Irf6-deficient (B,D,F) keratinocytes. Cells were grown to confluency (A,B), then scratched with a yellow tip (C,D). By 24 h, wild-type keratinocytes had closed the scratch (E), whereas Irf6-deficient cells had not (F). Scale bar: 100 μm. (G) Quantification of the percentage of wound closure over time. Data show the mean±s.e.m. (H,I) 2D-DIAS-generated centroid tracks and stacked perimeter plots of representative wild-type (H) and Irf6-deficient (I) keratinocytes. The large arrow at the bottom of each panel indicates the direction of the scratch wound, and small arrows indicate cellular direction of travel. The final cell perimeter in each perimeter plot is shown in gray. (J–N) Analysis of video recording of in vitro scratch-wounds. Horizontal black bars show the mean; *P<0.05; ***P<0.001 (Student’s t-test). (O) Method for calculating net path length, total path length and direction change.

Persistently (small arrows) in the direction of the scratch wound (large arrow). Perimeter plots revealed that these cells made net progress by the preferential extension of lamellipodia towards the wound. Irf6-deficient keratinocytes (Fig. 2I), by contrast, frequently extended lamellipodia in random directions (small arrows), resulting in reduced persistent crawling, decreased net progress towards the wound and less-persistent tracks. To quantify and statistically analyze these defects, net path length, total path length and direction change were computed from centroid positions over time, as illustrated in Fig. 2O and described in the Materials and Methods. The net path (distance from A to B, Fig. 2J) and the total path (distance a+b+c+d, Fig. 2K) were both significantly decreased (P<0.001) in cells deficient for Irf6 compared with those of wild-type keratinocytes. In addition, the average direction change (Fig. 2O, angles α, β and γ) was significantly increased in the absence of Irf6 compared with that of control cells (Fig. 2L). The instantaneous velocity of Irf6-deficient keratinocytes was 0.39 μm/min (±0.023; ±s.e.m.), approximately half that of wild-type cells (0.73±0.054), and the difference was highly significant (P<0.001, Fig. 2M).

Consequently, the persistence of Irf6-deficient keratinocytes was three times lower than that of wild-type cells (Fig. 2N, P<0.001). Collectively, our results indicate a deficient directional migration and reduced speed in cells lacking Irf6, suggesting that Irf6 is necessary for the efficient healing of keratinocyte scratch wounds in vitro.

Irf6-dependent cellular size is independent of the extracellular matrix

In order to further our understanding of the role of Irf6 in keratinocyte migration, we investigated cellular adhesion to the substrate at 1 h after plating. Our data showed no difference in the number of adhered keratinocytes between wild-type and Irf6-deficient keratinocytes (Fig. 3A). We further investigated whether this outcome was dependent on the type of extracellular matrix. Despite an increase in the number of adherent cells on laminin-332 compared with that on other substrates (fibronectin, collagen IV and plastic), we did not detect differences in the number of adherent cells between wild-type and Irf6-deficient keratinocytes (Fig. 3B), suggesting that initial cellular adhesion
to the extracellular matrix is independent of Irf6. However, at 1 h after plating, we consistently observed that Irf6-deficient cells were larger than wild-type cells (Fig. 3C versus 3D). We quantified this observation by measuring the cellular area of keratinocytes after 1 h of plating on plastic, fibronectin, collagen IV and laminin-332. With the exception of cells grown on fibronectin, keratinocytes deficient for Irf6 were significantly more spread than their wild-type counterparts (Fig. 3E). Interestingly, cells were more spread when plated on laminin-332 compared with cells plated on any other substrate, but the difference between wild-type and mutant was not changed. The plating of Irf6-deficient keratinocytes on preformed extracellular matrix produced by wild-type keratinocytes did not change the number or the size of adherent Irf6-deficient cells compared with cells plated on collagen IV (data not shown), supporting the hypothesis that the larger cell size is independent of the extracellular matrix, but is intrinsic to Irf6-deficient keratinocytes.

Focal adhesions are cellular structures involved in cell–matrix adhesion and cellular spreading, which connect the actin cytoskeleton to the point of contact between the cell and the extracellular matrix. Immunostaining for vinculin, a component of the focal adhesion complex, did not show differences between wild-type and Irf6-deficient keratinocytes (Fig. 3C,D). However, stress fibers, as identified by phalloidin staining, appeared more prominent at 1 h after plating in the absence of Irf6. Thus, our data suggest that Irf6 acts to restrict the spreading of keratinocytes in a substrate-independent fashion by potentially regulating the actin cytoskeleton.

Irf6 regulates the actin cytoskeleton and the amount of active RhoA

Data presented in Fig. 3 showed an increase in cellular size at 1 h after plating. We have previously reported that, when grown for several days, cultures of Irf6-deficient keratinocytes contain larger cells than wild-type cultures, and this is not due to the occurrence of epithelial to mesenchymal transition (Biggs et al., 2012). We confirmed this increase in cellular size in Irf6-deficient cells during scratch closure (Fig. 4A), and we found that it was accompanied by an increase in cell roundness (Fig. 4B), leading us to hypothesize that Irf6 regulates the actin cytoskeleton.

We first investigated the pattern of actin stress fiber arrangement by staining with phalloidin, a marker of polymerized actin (Wehland et al., 1977). We observed more prominent stress fibers in the Irf6-deficient keratinocytes compared with those of wild-type cells (Fig. 4C,D). The percentage of cells with prominent stress fibers varied from 24.1% to 50.6% across six independent experiments. The combined data showed that cultures of Irf6-deficient keratinocytes had 1.77 times more cells with prominent stress fibers than cultures of wild-type cells. A premature assembly of cytoplasmic stress fibers has been previously associated with an elevation in the activity of RhoA-GTPase, leading to an inhibition of cellular migration (Arthur and Burridge, 2001). In order to test the hypothesis that Irf6 regulates the activity of RhoA, we performed an affinity precipitation assay for GTP-bound RhoA, the active form of RhoA (Fig. 4E). We observed an increase in the amount of active RhoA in Irf6-deficient keratinocytes compared with that of wild-type cells. These results confirm a role for Irf6 in negatively regulating stress fibers through RhoA.

To determine whether the increased prominence of stress fibers and the delays in scratch-wound healing observed in Irf6-deficient cells were dependent on RhoA, we blocked ROCK, a Rho-associated protein kinase and downstream effector of RhoA (Leung et al., 1996). Both wild-type and Irf6-deficient keratinocytes were treated with Y27632. Irf6-deficient keratinocyte cultures exhibited a greater reduction in the prominence of stress fibers, yet a reduction was observed in both cultures (Fig. 4F,G). These data suggest that blocking ROCK partially rescued the phenotypic characteristics of Irf6-deficient keratinocytes, furthering our hypothesis that Irf6 regulates RhoA. To test whether this partial rescue of stress fibers had functional consequences, we scratched wild-type and Irf6-deficient confluent monolayers of keratinocytes in the presence of Y27632. After 18 h, both wild-type and Irf6-deficient scratches were 80% closed, with no statistically significant difference between the two groups (Fig. 4H). Taken together, these data indicate that Irf6 regulates the balance between active and inactive RhoA, thus controlling stress fiber formation and keratinocyte migration.

In order to further investigate the effect of Y27632, we analyzed time-lapse video microscopy data from wild-type and Irf6-deficient keratinocytes treated with the ROCK inhibitor, as described for Fig. 2. Our results showed that Y27632 had no significant effect on cellular size and shape (Fig. 4A,B). However, the presence of the ROCK inhibitor rescued the net path (Fig. 4I), the total path (Fig. 4J), the instantaneous velocity (Fig. 4L) and, consequently, the persistence (Fig. 4K) of Irf6-deficient keratinocytes.
keratinocytes. Collectively, our results indicate that ROCK inhibitor rescues deficient directional migration and reduced speed in cells lacking Irf6, but does not rescue cellular size and shape.

**Irf6 regulates Arhgap29 to modulate RhoA activity**

Two classes of proteins regulate RhoA activity – GAPs and GEFs, inactivating and activating RhoA, respectively (Cherfils and Zeghouf, 2013). To identify how Irf6 regulates RhoA levels, we searched our microarray data, which compares wild-type to Irf6-deficient embryonic skin, for either decreased GAP expression or increased GEF expression in the Irf6-deficient samples relative to the wild-type ones (Ingraham et al., 2006). We identified Arhgap29 as a candidate because it was expressed at higher levels in the skin than the other GAPs and it showed a non-significant but reduced expression in the absence of Irf6. We confirmed the presence and the decrease in Arhgap29 expression at the protein level in cutaneous tissues from e17.5 Irf6-deficient embryos compared with wild-type tissues by immunostaining (Fig. 5A,B) and western blot analysis (Fig. 5C). *In vivo*, Arhgap29 was mainly expressed throughout the epidermis, with some expression in the dermal compartment. In cells in culture, Arhgap29 was detected in murine embryonic keratinocytes (Fig. 5D,E) and fibroblasts (data not shown). The protein appeared to be perinuclear within the cytoplasm of the cells, displaying a punctate pattern, with no apparent alteration of localization between wild-type and Irf6-deficient keratinocytes. However, levels of Arhgap29 expression were reduced in the absence of Irf6 (Fig. 5D,E). These data provide evidence that Arhgap29, a key regulator of RhoA activity, lies downstream of Irf6 in keratinocytes.

**DISCUSSION**

Using our Irf6-deficient murine model (Biggs et al., 2012; Ingraham et al., 2006), we demonstrate that Irf6 acts as a regulator of keratinocyte migration. We show that Irf6 inhibits the...
activity of the small GTPase RhoA by regulating the level of Arhgap29, a RhoA inactivator. These molecular changes result in increased formation of actin stress fibers, increased cellular area and slower migration. This provides a potential molecular rationale for the observed increased likelihood of post-surgical complications in patients with mutations in IRF6 compared with those without (Jones et al., 2010).

Our data show delays in wound closure using both an ex vivo murine embryo culture wound-healing assay and an in vitro keratinocyte scratch assay. A potential migratory defect of epithelial cells lacking Irf6 was previously postulated in the zebrafish. Zebrafish embryos injected with a dominant-negative form of irf6 failed to undergo proper epiboly (Sabel et al., 2009), a process during which the epithelial enveloping layer moves as a coherent layer to cover the yolk cell. The absence of irf6 in the fish led to the rupture of the embryo at late gastrula stage. The enveloping layer contains an actin cytoskeleton and cadherins at the cell–cell junctions (Zalik et al., 1999), reminiscent of mammalian epithelial cells, suggesting a potential evolutionarily conserved role for Irf6 in epithelial cell migration.

The defect in keratinocyte migration in the absence of Irf6 is reminiscent of a few murine models. Mice that lack grainy-head like 3 (Grhl3) are particularly relevant because, like Irf6, Grhl3 encodes a transcription factor that is required to regulate epidermal proliferation and differentiation (Yu et al., 2006). In humans, mutations in IRF6 and GRHL3 have both been identified in VWS (Kondo et al., 2002; Peyrard-Janvid et al., 2014). In addition, embryos lacking Grhl3 fail to close an ex vivo wound, and Grhl3-deficient keratinocytes were delayed in closing an in vitro scratch wound (Caddy et al., 2010; Hislop et al., 2008). Finally, GRHL3 was identified as a direct target for IRF6 in human keratinocytes (Botti et al., 2011) and in the zebrafish periderm (de la Garza et al., 2013), suggesting that GRHL3 and IRF6 function in a common pathway in regulating epidermal migration. In support of this hypothesis, keratinocytes that lack either of these genes showed altered levels of stress fibers and Rho activity. However, whereas Irf6-deficient keratinocytes display an increase in the prominence of stress fibers and Rho activity (this study), Grhl3-deficient keratinocytes display a decrease in stress fiber formation and Rho activity (Caddy et al., 2010). Thus, although both genes share a common function in regulating keratinocyte migration during wound healing, they appear to act in different pathways that converge at regulating the activity of RhoA. Specifically, Irf6 was shown to regulate Arhgap29 (Leslie et al., 2012), but Grhl3 was shown to regulate RhofGEF19 (also known as Arhgef19) (Caddy et al., 2010). Future studies will be needed to understand the complex gene regulatory network between these two transcription factors during keratinocyte migration and wound healing.

Irf6-deficient keratinocytes were more spread than wild-type cells, already observable at 1 h after plating and persisting throughout the course of the scratch-wound assay. Concomitantly, time-lapse recording analysis indicated that Irf6-deficient keratinocytes were slower cells that traveled less distance. As stress fibers are more prominent in stationary cells (Couchman and Rees, 1979) and inhibit cell migration (Burridge, 1981), we were not surprised to find the presence of prominent stress fibers in Irf6-deficient keratinocytes (1.77 times more cells with prominent stress fibers in the Irf6-deficient group compared with the wild-type group, data not shown), accompanied by an increase in active RhoA. The addition of a ROCK inhibitor rescued the migratory phenotype of Irf6-deficient keratinocytes, thus providing further evidence that the extensive fibers in mutant cells contribute to their slower migration, as reported previously (Arthur and Burridge, 2001). In separate studies, the addition of the same ROCK inhibitor led to the immortalization of human keratinocytes and increased proliferation (Champion et al., 2010; McMullan et al., 2003), but this effect was dependent on culture with human fibroblasts (Champion et al., 2010). Our culture system does not contain fibroblasts, and the number of cells dividing during the scratch assay was not significantly different between the two groups (data not shown), suggesting that proliferation is unlikely to contribute to the rescued migratory phenotype.

Irf6 promotes epidermal differentiation, both in vivo and in vitro (Biggs et al., 2012; Ingraham et al., 2006). If Irf6 is upstream of RhoA–ROCK, we would hypothesize that increasing levels of RhoA or blocking ROCK would promote epidermal differentiation. However, the keratinocyte-specific RhoA knockout mouse exhibits a normal epidermis (Jackson et al., 2011). Depletion of ROCK inhibits keratinocyte terminal differentiation in vitro and in vivo (Lock and Hotchin, 2009; Shimizu et al., 2005; Thumkeo et al., 2005), yet it has no effect on full-thickness wound healing. Interestingly, ROCK-I and ROCK-II knockout animals exhibit an ‘open-eye’ phenotype – a classic periderm defect – and an open ventral body wall (Shimizu et al., 2005; Thumkeo et al., 2005). Irf6-deficient mice also exhibit a mild defect in the ventral body wall (M.D. and B.C.S., data not shown) and a periderm defect (this study; Peyrard-Janvid et al., 2014; Richardson et al., 2009), yet do not exhibit the open-eye phenotype. Redundancy in the Rho family members, and diversity in targets in these pathways, is likely to contribute to the observed similarities and discrepancies in phenotypes.

Cell–cell and cell–matrix adhesions are crucial components of cellular migration. Our results show no defect in cell–matrix adhesion at 1 h after plating in the absence of Irf6. This was rather surprising, based on the increase in both the levels of active RhoA and the prominence of stress fibers, which have typically been associated with increased cell–matrix adhesion (Arthur and Burridge, 2001), and the increased levels of integrin α2 (Ingraham et al., 2006) and integrin α3 (Botti et al., 2011) reported for Irf6-deficient embryonic skin and adult human keratinocytes with knockdown of IRF6, respectively. However, the migratory phenotype was observed during the healing of a scratch wound, which occurs after cells have reached confluence and therefore have been in culture for at least 48 h. Using vinculin as an indicator of focal adhesions, we did not detect...
Two 6-mm punch biopsies were performed on the back of 8–12-week-old mice at the University of Iowa. Two distinct forms at the University of Iowa. Two distinct forms were performed as described previously (Biggs et al., 2012; Ingraham et al., 2006). The Irf6gt1 allele and the Irf6del1 allele was performed as described previously (Biggs et al., 2012; Ingraham et al., 2006). The presence of a copulatory plug was designated as embryonic day (e0.5).

In vivo excisional wound healing
Two 6-mm punch biopsies were performed on the back of 8–12-week-old wild-type animals, as described previously (Le et al., 2012). Animals (n = 4–6 per group) were euthanized at 1, 4, 7 and 11 days post-wounding, and wounds were fixed in 4% paraformaldehyde. Serial sections and immunostaining were performed as described previously (Biggs et al., 2012; Le et al., 2012).

Ex vivo embryo culture
Embryonic wound healing was performed as described previously (McCluskey and Martin, 1995; New and Cockroft, 1979), with modifications as follows. Embryos were removed from pregnant females at e10.5. They were dissected out of their amniotic sacs and left connected to their placentas. To generate the embryonic wound, the left forelimb bud was amputated using scissors with 4 mm blades. The embryos were then placed in a conical tube containing 4 mL of filter-sterilized medium [3 parts 0.9% (w/v) NaCl supplemented with 1% penicillin-streptomycin, 1 part fresh rat serum]. The vials were gassed at the beginning of the experiment and every 12 h with 95% O2/5% CO2 and placed in a Belco Autoblot Micro Hybridization oven at 37°C with rotation. After 24 h in culture, embryos with a strong heartbeat and good circulation were processed for scanning electron microscopy. At the end of the culture, a piece of the tail was removed for genotyping.

Keratinocyte culture
Skin from e17.5 embryos was incubated with 5 U/ml Dispase II (Roche Diagnostics, Indianapolis, IN) at 4°C for 4 h. The epidermis was peeled from the dermis and incubated in 0.25% trypsin (Gibco Invitrogen, Carlsbad, CA) for 20 min at 37°C. Keratinocytes were grown in N-Medium (Hager et al., 1999), which contains 0.06 mM CaCl2, and were used after their first or second passage.

In vitro scratch assay
Scratches were generated with a P200 tip in confluent monolayers of both wild-type and Irf6-deficient keratinocytes, and static images were recorded at regular intervals over a 24-h period. Static images were obtained using a Nikon Eclipse Ti microscope with Nikon Digital Sight CCD camera and NIS-Elements D 3.0 software (Melville, NY). The open area was traced using ImageJ. Movies of scratches generated with a P10 tip were captured using a Zeiss Axioview 200M Mat microscope with heated and humidified chamber, acquired with a Hamamatsu Orca ER CCD camera (Bridgewater, NJ) and AxioVision Rel 4.7 software (Zeiss, Thornwood, NY) and converted to QuickTime format. Analysis of cell behavior was performed from the QuickTime movies using 2D-DIAS software, as described elsewhere in detail (Soll and Voss, 1998; Wessels et al., 2009). Briefly, accurate cell outlines from phase-contrast images were obtained using the manual trace feature and were converted to beta-spline replacement images. Total path length, net path length, instantaneous velocity, persistence and direction change were computed from the cell centroid position at 10-min intervals over an 18-h period. Instantaneous velocity was computed by drawing a line from the centroid in frame n−1 to the centroid in frame n+1 and then dividing the length of that line by twice the time interval between frames (Soll and Voss, 1998). Directional change was computed as the direction in the interval (n−1, n) minus the direction in the interval (n, n+1). Directional change values greater than 180° were subtracted from 360°, resulting in a positive value between 0° and 180°. Persistence was essentially computed by dividing the speed by direction change, the latter given in radians rather than degrees, and adding one to the denominator to prevent division by 0. Area and roundness were computed from contours of the beta-spline replacement images (Soll and Voss, 1998; Wessels et al., 2004).

Cell-substrate adhesion assay
Keratinocytes (passage 2, 21,500 cells/cm2) were plated in 24-well plates on plastic, collagen-IV- or fibronectin-coated wells (1 μg/cm2, BD Biosciences, Bedford, MA). Laminin-332-coated plates were prepared from human keratinocyte cultures. Briefly, confluent keratinocytes were removed from their matrix by successive incubation first in 1% Triton X-100 in PBS for 10 min, then in 30 mM Tris-HCl pH 8, 2 M urea in 1 M NaCl for 10 min and finally in 30 mM Tris-HCl pH 8 in 8 M urea for 10 min. All the buffers contained 0.1% inhibitor cocktail (set III, Novagen 539134) and 1 mM EDTA. Finally, the coated plates were washed with 0.1% inhibitor cocktail and 1 mM EDTA in PBS and used immediately or stored at −80°C (Kirtschig et al., 1995).

After 60 min, the medium was aspirated and the wells were washed with PBS. Remaining cells were then stained with Giemsa and fixed with
Giordano buffer, as described previously (Biggs et al., 2012). Two images per well were taken, and the cells were counted and averaged. Three experiments were performed in duplicate. Similarly, cells were plated at a density of 21,500 cells/cm² onto collagen-V-coated glass coverslips and fixed in methanol:acetone (75:25, v/v) 60 min later. The coverslips were then stained with vinculin and phallolidin.

**Active RhoA pulldown**

Rho assays were performed as described previously (Ren et al., 1999). Briefly, the RhoA-binding domain of rhoetkin was immobilized on glutathione-S-transferase-conjugated beads. Cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 100 mM MgCl₂ and 12 μg/ml each of PMSF, leupeptin and aprotonin), and equal amounts of cell lysate were incubated with beads with 30 μg of GST-bound rhoetkin for 30 min at 4°C with rotation. Total lysate and bead-conjugated lysate were prepared for western blotting and run on 10% Bis-Tris gels (Invitrogen). The membranes were then probed with RhoA-specific antibody. Three independent experiments were performed. ROCK inhibitor Y27632 was obtained from Sigma (St Louis, MO) and used at a concentration of 10 μM. Cells were incubated in Y27632 or DMSO control for 24 h and fixed in 70% ethanol.

**Antibodies**

Mouse monoclonal antibodies against vinculin and Rhodamine-conjugated phallolidin were obtained from Sigma. Rabbit polyclonal antibody against Arhgap29 was obtained from Novus Biologicals (Littleton, CO). Mouse monoclonal antibody against RhoA (clone 26C4) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse-IgG and anti-rabbit-IgG horseradish-peroxidase-conjugated secondary IgG antibodies, antigen detection was performed with the chemiluminescent detection system ECL (GE Healthcare, Littleton, CO). Mouse monoclonal antibodies against vinculin and Rhodamine-conjugated phallolidin were obtained from GE Healthcare (Piscataway, NJ) and Santa Cruz Biotechnology, respectively.

**Protein analysis**

Radios immunoprecipitation assay (RIPA) extraction buffer was used for protein preparation. Equal amounts of protein were separated on 10% Bis-Tris (Invitrogen) SDS-PAGE gels under denaturing conditions. Proteins were transferred onto polyvinylidene fluoride membranes (Bio Rad Laboratories, Hercules, CA), blocked in 10% nonfat dried milk and incubated with primary antibodies. After incubation with horseradish-peroxidase-conjugated secondary IgG antibodies, antigen detection was performed with the chemiluminescent detection system ECL (GE Healthcare).

**Microscopy**

Keratinocytes at passage 1 were grown on collagen-IV-coated coverslips and fixed as described previously (Michel et al., 1996). After blocking with 3% goat serum (Vector Laboratories, Burlingame, CA), cells were incubated with primary antibodies, washed in PBS and incubated with secondary antibodies. 4,6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain. Images were viewed with a Nikon Eclipse E800 (Melville, NY) and acquired with a SPOT RT slider CCD camera using Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI). Black and white images were pseudocolored and merged. For confocal microscopy, images were acquired using a Zeiss LSM 710 microscope (Thornwood, NY) and ZEN 2009 software (Thornwood, NY).

**Statistics**

Data are the means of at least three biological replicates. Statistical analysis was performed with appropriate tests for each study, as indicated in the figure legends.

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**Competing interests**

The authors declare no competing interests.

**Author contributions**


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**Supplementary material**

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.139246/-/DC1

**References**


Movie 1: Wild type keratinocytes closing a scratch. Images were recorded every 10 min, and movie generated using quicktime.

Movie 2: Irf6-deficient keratinocytes closing a scratch. Images were recorded every 10 min, and movie generated using quicktime.