RhoA is dispensable for skin development, but crucial for contraction and directed migration of keratinocytes

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ABSTRACT **RhoA is a small guanosine-5′-triphosphatase (GTPase) suggested to be essential for cytokinesis, stress fiber formation, and epithelial cell–cell contacts. In skin, loss of RhoA was suggested to underlie pemphigus skin blistering. To analyze RhoA function in vivo, we generated mice with a keratinocyte-restricted deletion of the RhoA gene. Despite a severe reduction of cofilin and myosin light chain (MLC) phosphorylation, these mice showed normal skin development. Primary RhoA-null keratinocytes, however, displayed an increased percentage of multinucleated cells, defective maturation of cell–cell contacts. Furthermore we observed increased cell spreading due to impaired RhoA-ROCK (Rho-associated protein kinase)-MLC phosphatase-MLC–mediated cell contraction, independent of Rac1. Rho-inhibiting toxins further increased multinucleation of RhoA-null cells but had no significant effect on spreading, suggesting that RhoB and RhoC have partially overlapping functions with RhoA. Loss of RhoA decreased directed cell migration in vitro caused by reduced migration speed and directional persistence. These defects were not related to the decreased cell contraction and were independent of ROCK, as ROCK inhibition by Y27632 increased directed migration of both control and RhoA-null keratinocytes. Our data indicate a crucial role for RhoA and contraction in regulating cell spreading and a contraction-independent function of RhoA in keratinocyte migration. In addition, our data show that RhoA is dispensable for skin development.**

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INTRODUCTION

RhoA is a ubiquitously expressed small guanosine 5′-triphosphatase (GTPase) of the Rho family that is present in either an active GTP-bound state or an inactive guanosine diphosphate (GDP)-bound state. Only in its active form does RhoA interact with different effector molecules such as Rho-associated protein kinase (ROCK) or mDia1, which mediate its biological functions (Bustelo *et al.*, 2007). RhoA is highly similar to RhoB and RhoC, which bind to the same effectors, although with different affinities (Wheeler and Ridley, 2004). Whereas RhoA and RhoC are found in the cytoplasm and at the cell membrane, RhoB is mainly found on endosomal membranes, suggesting a different biological role for the latter. RhoA function has mainly been studied by overexpression of dominant negative RhoA (dnRhoA) or expression of the bacterial inhibitor C3 toxin. The interpretation of these studies, however, is not always easy, because dnRhoA and C3 toxin also block RhoB and RhoC. Furthermore, there might be only partial inhibition in case of low expression of the inhibitors and inhibition of Rho GTPases other

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Abbreviations used: DEJ, dermal-epidermal junction; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; FIAU, fialuridine; GAP; GTPase activating protein; GEF, guanine nucleotide exchange factor; ko, RhoA (fl/fl) K5 cre; MLC, myosin light chain; PBS, phosphate-buffered saline; ROCK, Rho-associated protein kinase.

than RhoA, B, and C in case of high expression (Rottner *et al.*, 1999; Czuchra *et al.*, 2005).

RhoA was suggested to be essential for cytokinesis by regulating cortical contractility and furrow formation (Piekny *et al.*, 2005). It was further shown to be crucial for maturation of focal adhesions (Small *et al.*, 2002) and proposed to control stress fiber formation by stimulating mDia1-dependent actin nucleation and ROCK-dependent cell contraction (Pellegrin and Mellor, 2007).

The role of RhoA in migration is less clear and might be cell type dependent. Strong inhibition of RhoA was suggested to cause cell detachment in fibroblasts (Nobes and Hall, 1999). In monocytes, in contrast, inhibition of RhoA/ROCK was reported to increase integrin-mediated adhesion (Worthylake and Burridge, 2003). Rho/ROCK-dependent cell contraction was supposed to be important for cell translocation and for rear detachment in mesenchymally migrating cells (Ridley *et al.*, 2003). ROCK inhibition by Y27632, however, sometimes inhibited (Worthylake and Burridge, 2003; Goulimari *et al.*, 2005), sometimes promoted (Nobes and Hall, 1999; Magdalena *et al.*, 2003; Totsukawa *et al.*, 2004), and sometimes had no effect on cell migration (Arthur and Burridge, 2001; Wojciak-Stothard and Ridley, 2003; Yamana *et al.*, 2006). Whereas several studies suggested that inhibition of RhoA at the leading edge is promoting directional persistence of migrating cells (Arthur and Burridge, 2001; Wang *et al.*, 2003; Wojciak-Stothard and Ridley, 2003; Tomar *et al.*, 2009), other studies proposed the opposite (Worthylake and Burridge, 2003; Goulimari *et al.*, 2005; Yamana *et al.*, 2006). Finally, RhoA might affect cell migration by inhibition of Rac1 activation (Narumiya *et al.*, 2009).

Several studies reported an important role of RhoA in the formation and maintenance of cell–cell contacts. First, inhibition of RhoA in keratinocytes in vitro impaired mature as well as newly established E-cadherin–dependent cell–cell contacts (Braga *et al.*, 1997). Second, RhoA/ROCK-dependent cell contractility was required for the expansion of spontaneously formed cell–cell contacts between MDCK cells, although ROCK-mediated contraction was dispensable for the maintenance of epithelial cell–cell contacts (Yamada and Nelson, 2007). Finally, RhoA was revealed to be crucial for the maintenance of desmosomes, and inhibition of RhoA activity was shown to cause pemphigus skin blistering in skin explants (Waschke *et al.*, 2006).

Not much is known about the in vivo function of RhoA. In chicken embryos, loss of basally localized RhoA activation resulted in basement membrane disassembly and epithelial-mesenchymal transition (Nakaya *et al.*, 2008). Knockout mice for RhoB (Liu *et al.*, 2001) and RhoC (Hakem *et al.*, 2005) did not display any developmental phenotype and showed no altered function of primary cells. This finding might indicate either that RhoA is the only crucial member of the Rho subfamily or that RhoA, RhoB, and RhoC have highly redundant functions. Whereas overexpression of RhoA and RhoC correlated with the progression of many human cancers, RhoB expression seems to negatively correlate with tumor progression, suggesting that RhoA and RhoB might have partially antagonistic functions (Karlsson *et al.*, 2009). Knockouts of ROCKI (Shimizu *et al.*, 2005) and ROCKII (Thumkeo *et al.*, 2003) led to embryonic defects in closure of eyelid and ventral body. mDia1 knockout mice showed normal skin development (Peng *et al.*, 2007).

To investigate the specific function of RhoA in keratinocytes in vivo and in vitro, we generated and analyzed mice with a keratinocyte-restricted deletion of the RhoA gene. Whereas skin development and maintenance were normal, we found reduced contraction and ROCK-independent impairment of directed migration of primary RhoA null keratinocytes.

RESULTS

RhoA is dispensable for development of the epidermis

To study the specific role of RhoA in skin, we generated mice with a conditional knockout of the RhoA gene by using the Cre-loxP system (Figure 1A). Homologously recombined clones were identified by Southern blot with an external probe (Figure 1A; floxed with neo). After transient transfection with Cre recombinase to remove the neo-TK cassette and selection for fialuridine (FIAU)-resistant cells that had lost the TK gene, floxed clones were detected by Southern blot with an external probe (Figure 1A; floxed). RhoA fl/fl mice were generated by blastocyst injection of floxed ES cells and subsequent crossing with K5 Cre mice (Ramirez *et al.*, 2004) to obtain mice with a keratinocyte-restricted deletion of the RhoA gene. Offspring were routinely genotyped by genomic PCR (Figure 1B).

RhoA (fl/fl) K5 Cre (ko) mice were born at the expected Mendelian ratio, suggesting no embryonic lethality, and did not display any obvious phenotypic difference to RhoA (fl/+) K5 Cre (control) littermates (Figure 1B). Western blot analysis of epidermal lysates isolated from ko mice showed an efficient loss of RhoA protein in RhoA ko keratinocytes (Figure 1C). In the absence of RhoA, RhoB protein amounts were increased fourfold (Figure 1C), whereas RhoC could not be detected using two different commercial antibodies in either control or RhoA ko mice (unpublished data).

Microarray gene expression analysis revealed no change in the expression of GEFs, GAPs, and RhoA effectors in freshly isolated keratinocytes in the absence of RhoA (Supplemental Table 1). RhoB mRNA levels were not increased (Supplemental Table 2), indicating that the increased amounts of RhoB protein in RhoA null epidermis are caused by altered posttranscriptional regulation. Raw values for RhoC were low, suggesting endogenously low expression of RhoC in the epidermis (Supplemental Table 2).

Histological investigation of the dorsal skin of adult mice by hematoxylin and eosin staining revealed no obvious morphological differences between epidermis and hair follicles of control and ko mice and no blistering (Figure 1D). Analysis of keratinocyte differentiation by immunofluorescence staining revealed a normal pattern of differentiation. Keratin 14, a marker of basal keratinocytes, was similarly distributed in ko and control skin (Figure 2, a and a′). $\alpha 6$ integrin, a subunit of the laminin receptor $\alpha_6\beta_4$ integrin, was detected primarily at the basal side of the basal keratinocytes in control and ko epidermis, suggesting normal polarization and attachment to the basement membrane in the absence of RhoA (Figure 2). Also, keratin 10, a marker for suprabasal keratinocytes, and loricrin, a protein associated with cornified cell envelope, displayed normal distribution in RhoA ko mice (Figure 2, b, b′, c, and c′). Finally, staining for keratin 6, a marker of hyperproliferation and stress in the interfollicular epidermis, was not detectable in control and RhoA ko interfollicular epidermis (Figure 2, d and d′).

RhoA is not required for the maintenance of cell–cell junctions in vivo

RhoA activation has previously been described to be essential for the formation and maintenance of adherens and tight junctions (Braga *et al.*, 1997; Yamada and Nelson, 2007). In addition, it was reported that inhibition of RhoA activity causes pemphigus skin blistering due to desmosome disintegration (Waschke *et al.*, 2006). We therefore analyzed whether these junctional structures are altered in RhoA-deficient skin.

Immunofluorescence staining for the adherens junction protein E-cadherin (Figure 2, e and e′), the tight and adherens junction protein ZO-1 (Figure 2, f and f′), and the desmosomal component

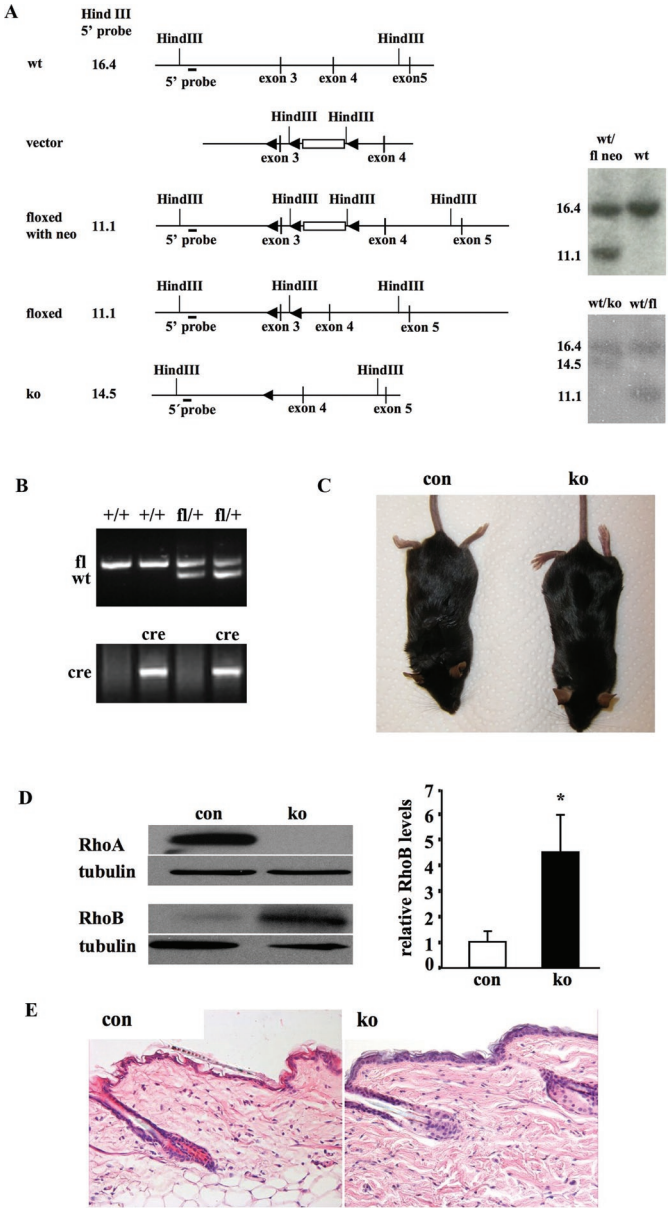

FIGURE 1: Generation of mice with a keratinocyte-restricted deletion of the RhoA gene. (A) Targeting scheme showing the wild-type RhoA gene (wt), the targeting construct (vector), the recombined RhoA gene with floxed neo-TK cassette (floxed with neo; white box), the floxed RhoA gene (floxed), and the knockout allele (ko) after homozygous recombination of the targeting construct and removal of the floxed exon 3. The respective alleles were distinguished by *Hin*dIII digestion and Southern blot hybridization with an external 5′ probe, resulting in the detection of the indicated fragment sizes (examples for the Southern blot hybridization are shown at the right side of the scheme). (B) Mice with a keratinocyte-restricted deletion of the RhoA gene (ko) show no obvious phenotype. (C) Western blot analysis reveals efficient loss of RhoA but compensatory increase of RhoB protein in the epidermis of RhoA-deficient mice (ko), compared to controls (con; n = 3; *p < 0.05). (D) Hematoxylin and eosin–stained paraffin sections of back skin of 5-mo-old RhoA fl/fl K5 Cre (ko) and control (con) mice indicate normal skin structure.

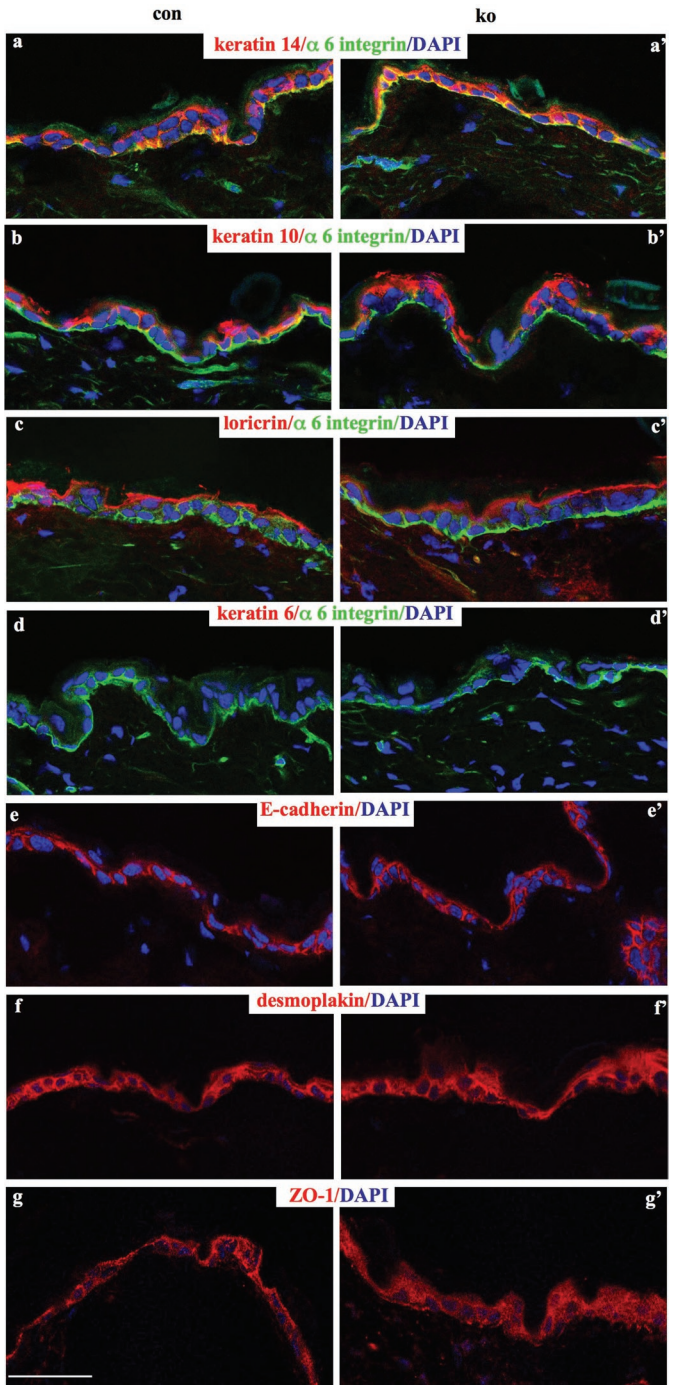

FIGURE 2: Normal differentiation and cell–cell junctions in RhoA-null epidermis. Cryosections of back skin of 5-mo-old mice were analyzed for the presence of keratin 14 (a, a′), keratin 10 (b, b′), loricrin (c, c′), keratin 6 (d, d′), E-cadherin (e, e′), desmoplakin (f, f′), and ZO-1 (g, g′). Counterstaining for α6 integrin indicates the DEJs (a-d, a′- d′). Nuclei are visualized by DAPI. (Bar = 50 μm.)

desmoplakin (Figure 2, g and g′) were indistinguishable in RhoA ko and control mice, indicating no defect at light microscopic level in the absence of RhoA. We then carried out ultrastructural analysis to reveal more subtle morphological impairments. Electron microscopic analysis of interfollicular epidermis did not reveal any substantial pathological alterations due to the loss of RhoA. Physiological keratinocyte arrangement as well as desmosomes, adherens junctions, hemidesmosomes, and basement membrane of normal ultrastructural appearance were observed both in control and RhoA ko mice (Figure 3). Integrity of the dermal-epidermal junction (DEJ)

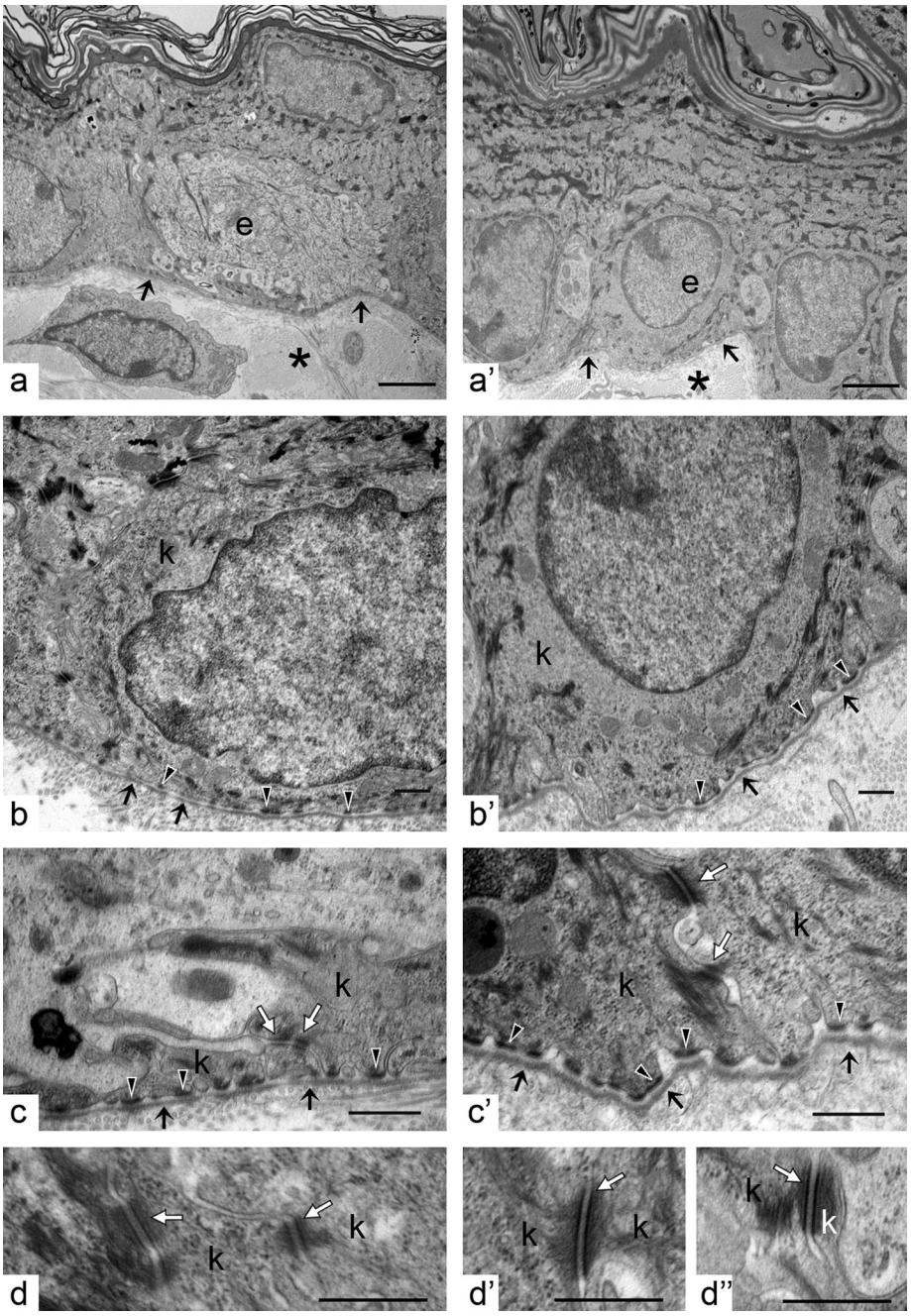

FIGURE 3: Normal ultrastructure of RhoA-null epidermis. Ultrastructure of control (a-d) and RhoA deficient (a′–d′, d″) epidermis of 2-mo-old mice examined by transmission electron microscopy. (a, a′) At low magnification, the epidermis (e) is recognizable as a layer well demarcated from the underlying dermis (asterisk) in control (a) and in absence of Rho A (a′). Scale bars = 2 μm. (b, b′) The images depict keratinocytes (k) closely apposed to a structured basement membrane (black arrows) and connected to it via hemidesmosomes (arrowheads) in both control (b) and RhoA-deficient (b′) epidermis. Scale bars = 500 nm. (c, c′) Higher magnification of the DEJ. Normal structure of the basement membrane (black arrows) and hemidesmosomes (arrowheads) is visible in control (c) and RhoA deficient (c′) epidermis. Black/white arrows = desmosomes. Scale bars = 500 nm. (d, d′, d″) Normally structured desmosomes (black/white arrows) are recognizable in controls (d) and in RhoA deficient (d′, d″) epidermis. Scale bars = 500 nm.

was then evaluated quantitatively. An ultrastructurally defined basement membrane with a distinct lamina densa and lamina rara was similarly present in control and mutant mice (control: 82.40% ± 3.89; ko: 90.87% ± 2.61; p = 0.109; n = 5). Frequency of hemidesmosomes (control: 16.92 ± 1.10; ko: 16.59 ± 1.11 hemidesmosomes/10 μm of DEJ; p = 0.834; n = 5) as well as percentage of the DEJ covered by hemidesmosomes (control: 23.16% ± 1.32; ko: 21.28% ± 1.58; p = 0.387; n = 5) were comparable in control and RhoA mutant mice. Taken together, our data indicate that RhoA is not necessary for normal skin development and maintenance under physiological conditions.

RhoA is crucial for the phosphorylation of myosin light chain and cofilin in the epidermis

The normal development of RhoA-deficient epidermis could be due to functional compensation by RhoB, resulting in normal activation of RhoA effectors. An important effector molecule of RhoA, B, and C is ROCK, which, together with other kinases, contributes to the phosphorylation of myosin light chain (MLC) and cofilin, which are involved in cell contraction and remodeling of the actin cytoskeleton, respectively. To test whether loss of RhoA in keratinocytes affects phosphorylation of MLC and cofilin, we performed Western blot analysis of epidermal lysates. In the absence of RhoA, phosphorylation of MLC is reduced by approximately 70%, whereas total amounts of MLC were unchanged (Figure 4, A and B). Also the amount of inactive, phosphorylated cofilin was decreased by approximately 70%. Total cofilin was decreased by only approximately 30%, indicating an increase in active, filamentous actin severing cofilin in RhoA-null keratinocytes.

These data suggest that RhoA activation is crucial for controlling ROCK activation and phosphorylation of MLC and cofilin in keratinocytes and that even fourfold increased RhoB levels are not able to sufficiently compensate for the lack of RhoA with respect to ROCK-dependent phosphorylation.

RhoA controls occludin expression and formation of mature cell–cell junctions in vitro

Although Cdc42 is crucial for the de novo formation of mature tight junctions (Wu *et al.*, 2007; Du *et al.*, 2009), it is not an immediate requirement for their maintenance in the epidermis in vivo (Wu *et al.*, 2006). To investigate whether RhoA is more important for the formation of cell–cell junctions than for their maintenance in epidermis, we isolated primary keratinocytes from control and RhoA mutant mice and induced the formation of adherens and tight junctions in vitro by high calcium treatment. After 12 h, continuous cell–cell contacts had been formed in control keratinocytes, resulting in continuous staining of the cell borders for

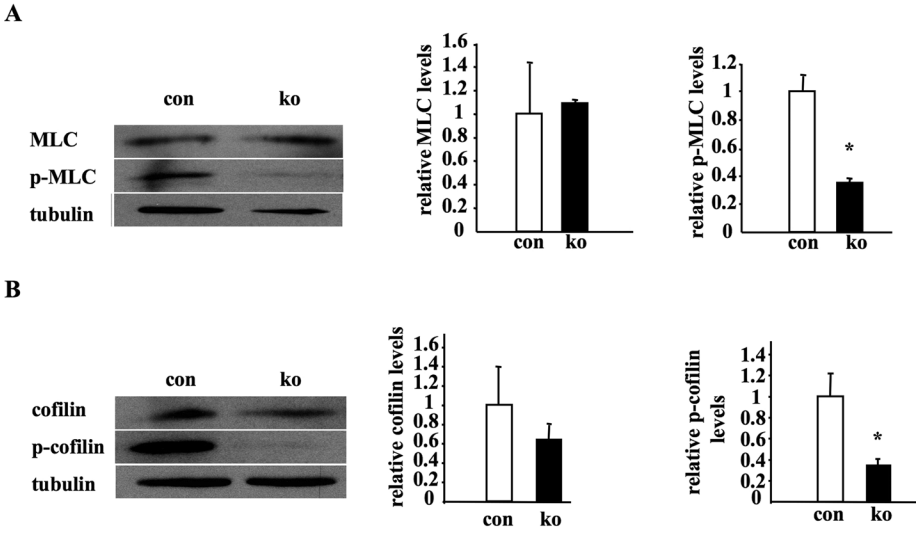

FIGURE 4: Reduced phosphorylation of MLC and cofilin in RhoA-null epidermis. RhoA-null and control epidermis was assessed by Western blot for (A) MLC and pMLC and (B) cofilin and p-cofilin (n = 3, *p < 0.05).

ZO-1, present in adherens and tight junctions, and occludin, present only in tight junctions (Figure 5, A and B). RhoA-deficient keratinocytes, however, displayed a discontinuous staining for ZO-1 and occludin (Figure 5A). Western blot analysis revealed reduced amounts of occludin protein and normal amounts of ZO-1 in RhoA-null keratinocytes (Figure 5B). Microarray gene expression analysis indicated reduced occludin expression in cultured keratinocytes, suggesting that RhoA regulates occludin expression in vitro at the level of mRNA (Supplemental Figure 3). Staining for F-actin revealed focal patches colocalizing with occludin at the cell–cell contacts of differentiated RhoA-null keratinocytes (Figure 5C). Parallel bundles of F-actin inserted at these foci and protruded into the cell. In control cells, F-actin was continuous at the cell–cell junctions, and no obvious bundles of F-actin were observed. Knockout induction in vitro by transfection of RhoA fl/fl keratinocytes with Cre followed by high calcium treatment reproduced the defective ZO-1 distribution observed with primary RhoA null keratinocytes, indicating that this impairment is a cell-autonomous defect (Figure 5D).

These data suggest that the impaired junctional maturation in vitro might be related to decreased amount of occludin. In vivo, occludin mRNA was only slightly reduced in the absence of RhoA, which might explain the absence of cell–cell contact defects in unstressed RhoA-null skin in vivo (Supplemental Table 3).

RhoA is important for cytokinesis

Rho-mediated activation of ROCK, citron kinase, and mDia were reported to control cytokinesis (Piekny *et al.*, 2005). The specific role of RhoA, however, was not studied. The normal skin maintenance of RhoA ko mice suggested that RhoA is not essential for cytokinesis in keratinocytes. Moreover, analysis of skin sections by staining of the nuclei with 4′,6′-diamidino-2-phenylindole (DAPI) and of the cell borders with E-cadherin did not indicate any obvious multinucleated cells in RhoA-null epidermis (Figure 2, e and e′). We then investigated the role of RhoA in cytokinesis in primary keratinocytes in vitro by staining subconfluent cells with DAPI (Figure 6A). In cultures obtained from control mice, the percentage of multinucleated cells was 6.4 ± 2.2%. RhoA ko cells showed a significant increase in multinucleated cells to 13.2 ± 4.2%. To rescue this defect, we transfected RhoA-null keratinocytes with an enhanced green fluorescent protein (EGFP)-RhoA fusion protein or an activated form of RhoA (RhoA F30L) coexpressing EGFP. Whereas the EGFP-RhoA fusion partially rescued multinucleation, the activated RhoA fully rescued the phenotype, indicating that the cytokinesis defect in RhoA ko cells is caused by a loss of active RhoA (Figure 6A). Knockout induction in vitro by transfection of RhoA fl/fl keratinocytes with Cre reproduced the multinucleation defect (Figure 6A), indicating that it is a cell autonomous defect. These data indicate that RhoA contributes to cytokinesis, without being essential.

To analyze whether RhoB and RhoC contribute to cytokinesis, we first assessed expression and activation of RhoB and RhoC in cultured RhoA-null keratinocytes. Protein amount and activation of

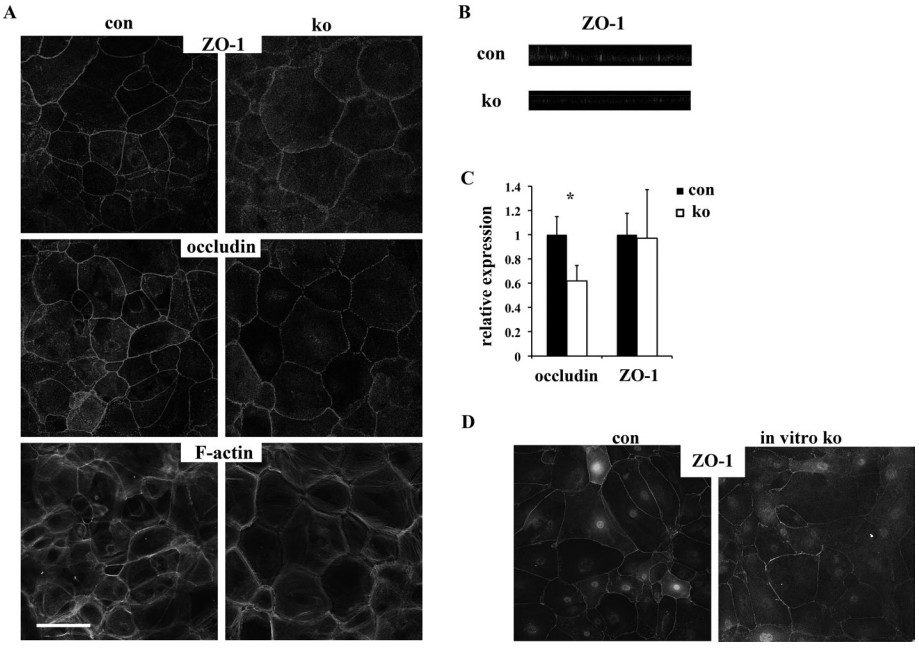

FIGURE 5: Defective formation of mature cell–cell contacts in vitro in the absence of RhoA. Formation of mature cell–cell contacts was induced in confluent monolayers by 12-h incubation with high-calcium growth medium. Cells were stained by immunofluorescence for ZO-1, occludin, and F-actin (occludin and F-actin are double stainings). Shown are confocal pictures (A) and for ZO-1sections through z-stacks of confocal pictures (B). Western blot analysis revealed decreased amounts of occludin protein and unchanged levels of ZO-1 in cultured RhoA-null keratinocytes (C; n = 6/6; p < 0.05). (C) Immunofluorescence for ZO-1 after RhoA knockout induction in vitro and high calcium treatment.

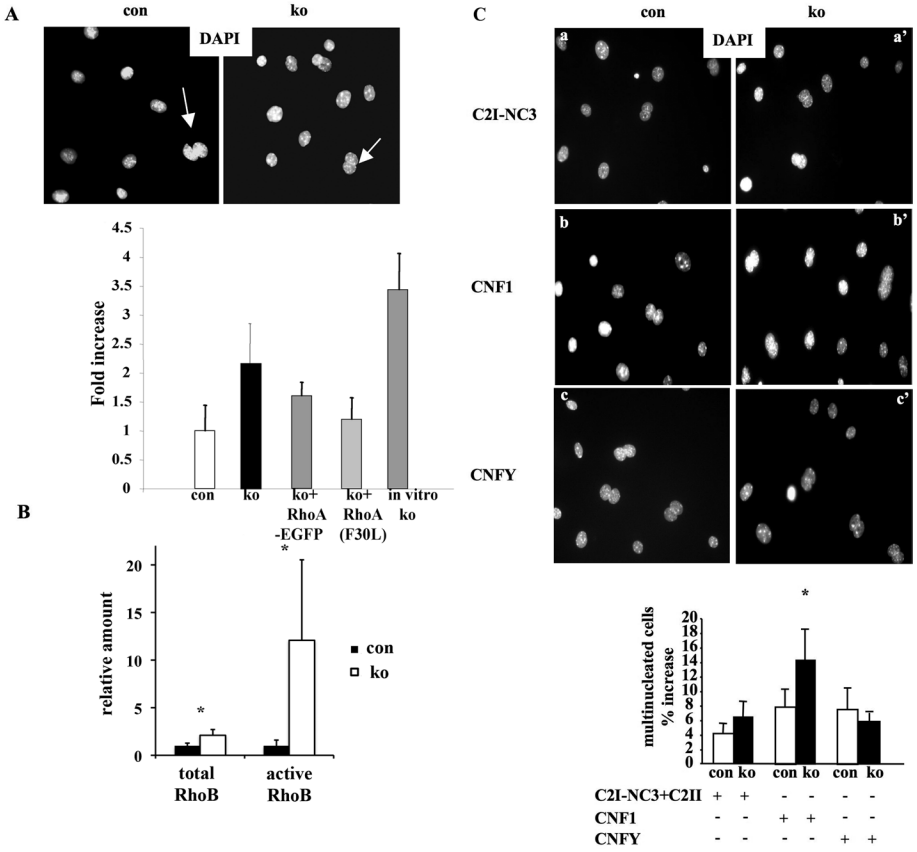

FIGURE 6: Increased number of multinucleated cells in the absence of RhoA. (A) Primary keratinocytes were cultured in vitro and fixed, and nuclei were stained with DAPI. RhoA-null keratinocytes (ko; n = 11) showed an increased number of multinucleated cells (arrows) compared to control cells (con; n = 13). Transfection of an EGFP-RhoA fusion protein partially rescued (ko + EGFP-RhoA; n = 2), and a high-cycling mutant form of RhoA (F30L) fully rescued the phenotype (ko + RhoA (F30L); n = 3). RhoA knockout induction in vitro reproduced the multinucleation phenotype (ko in vitro; n = 2). (B) Increased levels of total and GTP-bound active RhoB in cultured RhoA-null keratinocytes (n = 3/3). (C) Treatment for 3 h with the Rho inhibitor C2I-NC3 increased multinucleation in both control and ko keratinocytes, whereas the activating toxins CNF1 and CNFY could not rescue the phenotype.

RhoB were significantly increased in RhoA-deficient keratinocytes (Figure 6B), whereas RhoC could not be detected by Western blot (unpublished data). On mRNA level, microarray gene expression suggested a slight increase in RhoB and RhoC mRNA in the absence of RhoA, although the RhoC raw values remained low (Supplemental Figure 2)

To investigate the functional importance of RhoB and RhoC for cytokinesis, we used different toxins to inhibit or activate RhoGTPases. Cultured keratinocytes treated for 3 h with the fusion toxin C2I-NC3 (which inactivates RhoA, B, and C) showed a similar increase in the number of multinucleated cells from the respective basal level for both control and RhoA ko cells. (Figure 6C displays the difference in the percentage of multinucleated cells between toxin treated and untreated keratinocytes.) This finding suggests that, in addition to RhoA, RhoB and RhoC contribute to the regulation of cytokinesis.

We then tried to rescue the multinucleation phenotype of RhoA ko cells by treatment with toxins that activate Rho, Rac, and Cdc42 (CNF1) or RhoA, RhoB, and RhoC (CNFY), which we hoped might decrease the number of multinucleated cells in the RhoA ko relative to the control by increasing activation of RhoB. CNFY enhanced the number of multinucleated cells in RhoA ko and control keratinocytes to a similar extent, however (Figure 6C; note that it displays the difference in percentage of multinucleated cells between toxin treated and untreated keratinocytes), indicating that excessive activation of RhoB impairs cytokinesis. CNF1 induced even more multinucleation in RhoA-deficient keratinocytes than in controls (Figure 6C), suggesting that RhoA functions to protect cytokinesis against stress from excessive activation of Rac and Cdc42.

RhoA regulates cell spreading in keratinocytes

Many studies suggested an essential function for RhoA in the formation of actin-myosin stress fibers and focal adhesions. Furthermore, RhoA was described in different reports to either inhibit or promote cell adhesion (Nobes and Hall, 1999; Arthur and Burridge, 2001; Wang *et al.*, 2003; Wojciak-Stothard and Ridley, 2003; Worthylake and Burridge, 2003; Goulimari *et al.*, 2005; Yamana *et al.*, 2006; Tomar *et al.*, 2009).

Cell adhesion to fibronectin was measured and found to be increased in RhoA-null keratinocytes by 21% ± 3.7% compared to control cells (n = 3; $p < 0.05$), indicating that high RhoA activity counteracts cell adhesion or facilitates detachment.

To study whether stress fibers are able to form in the absence of RhoA, we isolated keratinocytes from control and RhoA ko mice and cultured them on glass at a low density to avoid cell–cell contact. Cells were fixed 2 d after plating and stained for actin filaments and the focal adhesion protein paxillin. Surprisingly, thick actin bundles anchored in paxillin containing focal adhesions at the cell membrane were found both in control and RhoA-null keratinocytes, indicating that RhoA is not essential for the formation of actin stress fibers and focal adhesions (Figure 7), consistent with the finding that their assembly is not only inducible by constitutively active RhoA, but also by RhoB and RhoC (Aspenström *et al.*, 2004).

During this analysis we noted that keratinocytes lacking RhoA appeared more spread. Quantifying the relative cell area of keratinocytes in culture, we observed an increased number of widely spread cells in RhoA-deficient keratinocytes. On average, the cell area of RhoA ko cells showed a twofold increase compared to control keratinocytes (Figure 8A). To rescue this defect, we transfected RhoA-null keratinocytes with an EGFP-RhoA fusion protein or an activated form of RhoA (RhoA F30L) coexpressing EGFP. The increased area phenotype was rescued by the EGFP-RhoA fusion and the activated RhoA, indicating that the altered spreading of RhoA ko cells is caused by a loss of active RhoA (Figure 8C). Knockout induction in vitro by transfection of RhoA fl/fl keratinocytes with Cre reproduced the increased spreading (Figure 8C), indicating that it is a cell-autonomous defect.

Control keratinocytes treated with the Rho inhibitor C2I-N-C3 showed an increased cell area comparable to the size of the RhoA ko cells (Figure 8B). No further increase in size was noticed with C2I-N-C3–treated ko keratinocytes, suggesting that RhoA, but not RhoB

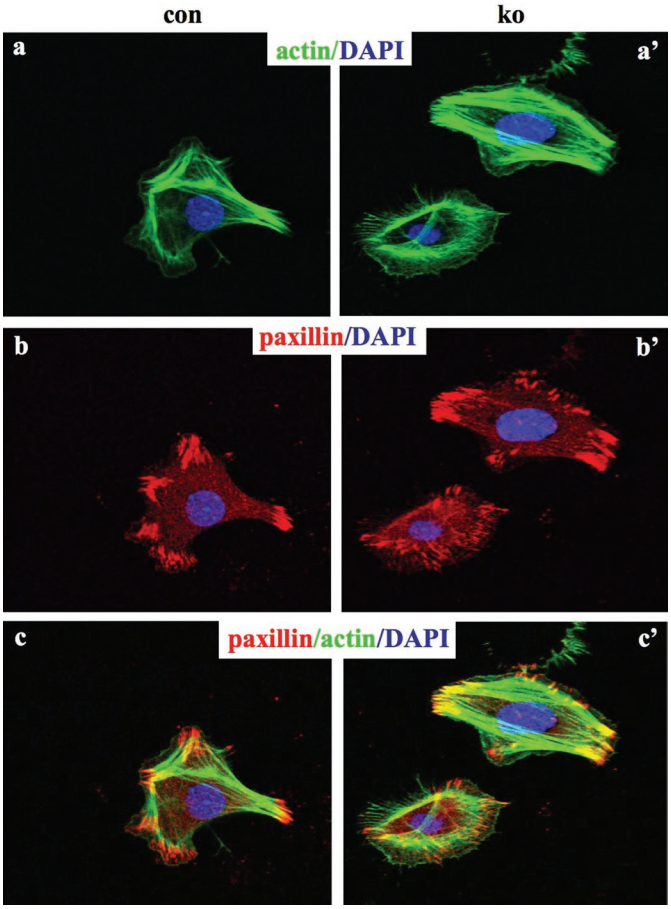

FIGURE 7: Stress fibers and focal adhesion form in the absence of RhoA. RhoA-null (ko) and control (con) keratinocytes were seeded on glass and stained for filamentous actin (a, a′, c, c′) and paxillin (b, b′, c, c′).

or RhoC, is crucial for the regulation of cell size. This conclusion was confirmed by the finding that neither the Rho activator CNFY nor the Rho, Rac, Cdc42 activator CNF1 were able to rescue the phenotype (Figure 8B).

RhoA regulates spreading via ROCK and MLCP, independently of Rac1

Our previous data showed that loss of RhoA in keratinocytes resulted in reduced phosphorylation of MLC, suggesting a decrease in cell contraction. Inhibition of contractility can effect at least transient cell protrusion (Köstler *et al.*, 2008), which could explain the increase in spreading area. To investigate this possibility, we treated control keratinocytes with the ROCK inhibitor Y27632. Indeed, incubation with Y27632 induced a significant increase in cell area in control cells, but it had no effect on the cell area of RhoA-null keratinocytes, corroborating that RhoA regulates cell area of keratinocytes via ROCK (Figure 8C).

ROCK was shown to promote MLC phosphorylation via direct phosphorylation of MLC and indirectly via inhibiting MLCP, thus reducing the dephosphorylation of MLC. To test whether altered activation of MLCP is important for the RhoA-ROCK action on MLC, we tried to rescue the RhoA ko phenotype by inhibition of MLCP using the inhibitor calyculin A. Treatment of keratinocytes for 24 h with 2 nM calyculin A resulted in a dramatic decrease in cell size in both control and RhoA knockout cells, indicating that RhoA regulation of MLCP is of crucial importance for the control of cell area (Figure 8C).

In epithelial cells, inhibition of RhoA can lead to activation of Rac1, which promotes cell spreading by lamellipodia formation (Narumiya *et al.*, 2009). We therefore tested whether loss of RhoA in keratinocytes results in increased activation of Rac1. Pull-down assays, however, indicated decreased levels of GTP-bound Rac1 in freshly isolated or cultured RhoA-null keratinocytes, whereas total levels of Rac1 protein were not altered (Figure 8D).

To further test whether RhoA-ROCK–dependent regulation of cell area in RhoA-deficient keratinocytes is independent of Rac1, we plated Rac1-null keratinocytes, which are unable to spread under normal culture conditions (Chrostek *et al.*, 2006), in the presence of the ROCK inhibitor Y27632. Under these conditions, Rac1-null cells could spread and displayed a similar cell area to Y27632-treated control cells (Figure 8E). These data suggest that keratinocytes can spread in the absence of Rac1 if the RhoA-ROCK–mediated contractility is inhibited.

RhoA is required for directed keratinocyte migration in vitro

Keratinocytes have a mesenchymal mode of migration, which requires protrusion formation, adhesion of protrusions to the surrounding extracellular matrix, cell contraction, rear detachment, and cell polarization. As RhoA-null cells showed a severe reduction in MLC phosphorylation and cell contraction, we hypothesized that this defect will cause a reduced migration speed and an impaired rear detachment, which is supposed to be partially dependent on cell contraction.

To test this hypothesis experimentally, we assessed directed migration of RhoA-null keratinocytes by an in vitro wound-closure assay monitored by time-lapse microscopy. As expected, RhoA-deficient keratinocytes showed a reduced wound-closure speed and frequently displayed elongated cells with delayed rear detachment (Figure 9A; Supplemental Movies 1 and 2). To show that this impairment is due to a reduction in cell contractility, we tried to reproduce the wound-closure defect by treating control cells with the ROCK inhibitor Y27632. As expected, ROCK inhibitor–treated control keratinocytes showed defective rear detachment, similar to the RhoA-null cells (Figure 9A; Supplemental Movies 3 and 4). Surprisingly, however, ROCK inhibition accelerated wound closure both in control and in RhoA-null keratinocytes. Even in the presence of ROCK inhibitor, RhoA-null cells showed significantly slower wound closure than did control keratinocytes (Figure 9A, compare "ko+Y27632" with "con+Y27632"). These data suggest that strong cell contraction is not required for efficient keratinocyte migration. Importantly, RhoA regulates directed cell migration via mechanisms independent of ROCK.

To better understand how RhoA affects directed migration of keratinocytes in vitro, we made a single cell analysis of the migration paths to determine migration speed and directional persistence. This analysis revealed a reduced migration speed and an increased tortuosity of RhoA-deficient keratinocytes (Figure 9B). Treatment with the ROCK inhibitor Y27632 increased migration speed and decreased tortuosity, both in control and in RhoA-null keratinocytes.

The reduced migration speed of RhoA-null keratinocytes could correspond to the reduced activation of Rac1 observed in RhoA-null keratinocytes in vitro (Figure 8D). To assess whether the reduced directionality of RhoA-deficient keratinocytes might relate to a reduced level of GTP-bound Cdc42, pull-down assays were performed. Cdc42-GTP levels were indeed decreased in RhoA-null keratinocytes (Figure 9C), suggesting that reduced Cdc42 activation might contribute to the decreased directionality of RhoA-deficient keratinocytes. Total levels of Cdc42 protein were not changed in the absence of RhoA. Loss of RhoA did not alter expression of GEFs, GAPs, or RhoA effectors in cultured keratinocytes, besides a twofold

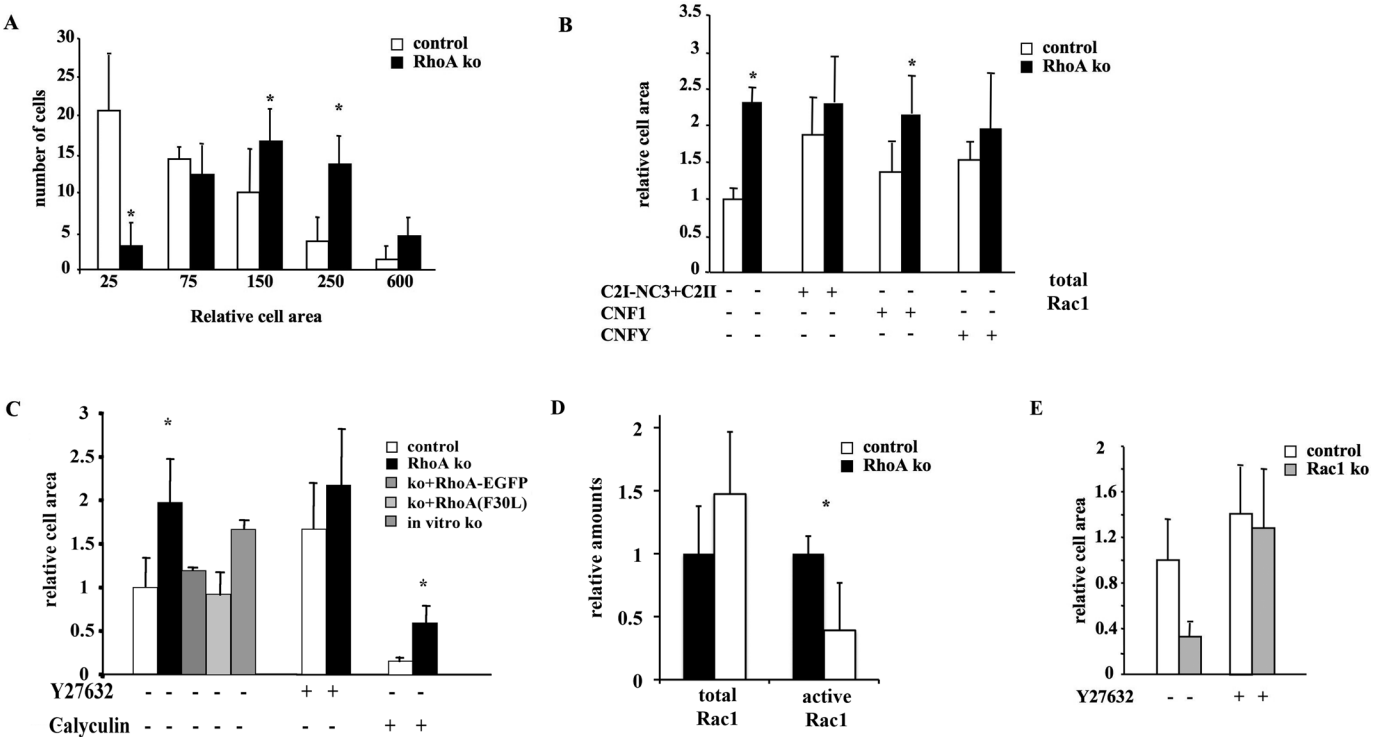

FIGURE 8: RhoA and ROCK-mediated contraction counteract spreading in the presence and absence of Rac1. Area of primary RhoA-null (ko) and control (con) keratinocytes was measured (A) in the absence of inhibitors, (B) in the presence of the Rho inhibitor C2I-NC3 (the activating toxins CNFY and CNF1), and (C) in the presence of the ROCK inhibitor Y27632 and the MLCP inhibitor calyculin. Transfection of RhoA-null keratinocytes with an EGFP-RhoA fusion (ko + EGFP-RhoA) or a high-cycling mutant form of RhoA (F30L) rescued the increased spreading phenotype (ko + RhoA [F30L]), whereas RhoA knockout induction in vitro reproduced defect (ko in vitro). (D) Reduced levels of GTP-bound active Rac1 in cultured RhoA-null keratinocytes (n = 3/3; $p < 0.05$). (E) Spreading-defective Rac1-null keratinocytes treated with Y27632 are able to spread.

decrease of RhoGTPase activating protein 8 (ARHGAP8), indicating that the reduction of GTP-bound Rac1 and Cdc42 was not caused by alteration in the expression of GEFs and GAPs (Supplemental Table 1). These data propose that RhoA affects keratinocyte migration in vitro by cross-regulation of Rac1 and Cdc42, but not by regulation of cell contraction.

RhoA is dispensable for wound healing in vivo

The impairment of migration of RhoA-null keratinocytes in vitro suggested that RhoA function might contribute to skin wound healing in vivo. We therefore tested skin wound healing in RhoA mutant and control mice by making two full-thickness wounds in the back skin of each mouse. Three and five days after wounding mice were sacrificed and the wound gap was measured on hematoxylin and eosin–stained skin sections. In 3 d wounds, no difference was observed between control and mutant mice (Figure 10A). Five days after wounding, wounds were completely closed in both control and RhoA mutant mice (unpublished data).

To investigate the reason for the different migration behavior of RhoA-null keratinocytes in vitro and in vivo, we measured the activation of Rac1 and Cdc42 in freshly isolated keratinocytes that were not cultured in vitro. In these keratinocytes, loss of RhoA did not alter the activation of Rac1 and Cdc42 (Figure 10B).

These data suggest that the in vitro migration defect of RhoA-null keratinocytes is due to the in vitro–specific decrease of GTP-bound Rac1 and Cdc42. The reduced cofilin and MLC phosphorylation observed in RhoA-null epidermis (Figure 4) is apparently not sufficient to cause significant alterations in skin wound healing.

DISCUSSION

In this study on the specific function of RhoA in keratinocytes, we report three major new findings. First, we find, surprisingly, that RhoA is dispensable for skin development and maintenance. Second, we show that the RhoA/ROCK/MLCP/MLC pathway is a major regulator of cell contraction in keratinocytes and that inhibition of contraction promotes spreading by Rac1-independent pathways. Finally, we demonstrate that RhoA contributes to directional persistence and migration speed in a ROCK- and contraction-independent manner.

RhoA in skin development and maintenance

Constitutive knockouts of RhoB and RhoC did not result in any developmental phenotype. It was therefore speculated that most of the severe effects observed in vitro by treatment with C3 toxin or expression of dnRhoA were due to inhibition of RhoA and that RhoA is crucial for development. At least in epidermis this is not the case, because no developmental phenotype could be observed in mice with a keratinocyte-specific deletion of the RhoA gene. We restricted the deletion of RhoA gene to the epidermis, because it was reported previously that a partial reduction of RhoA activation in keratinocytes causes pemphigus skin blistering and loss of cell–cell contacts in keratinocyte monolayers (Braga *et al.*, 1997; Waschke *et al.*, 2006). Although we could detect a mild defect in the formation of mature cell–cell contacts between RhoA-null keratinocytes in vitro, we were unable to see any obvious alterations in adherens junctions, tight junctions, or desmosomes in RhoA-null epidermis in vivo. This impairment correlated with a reduction of occludin

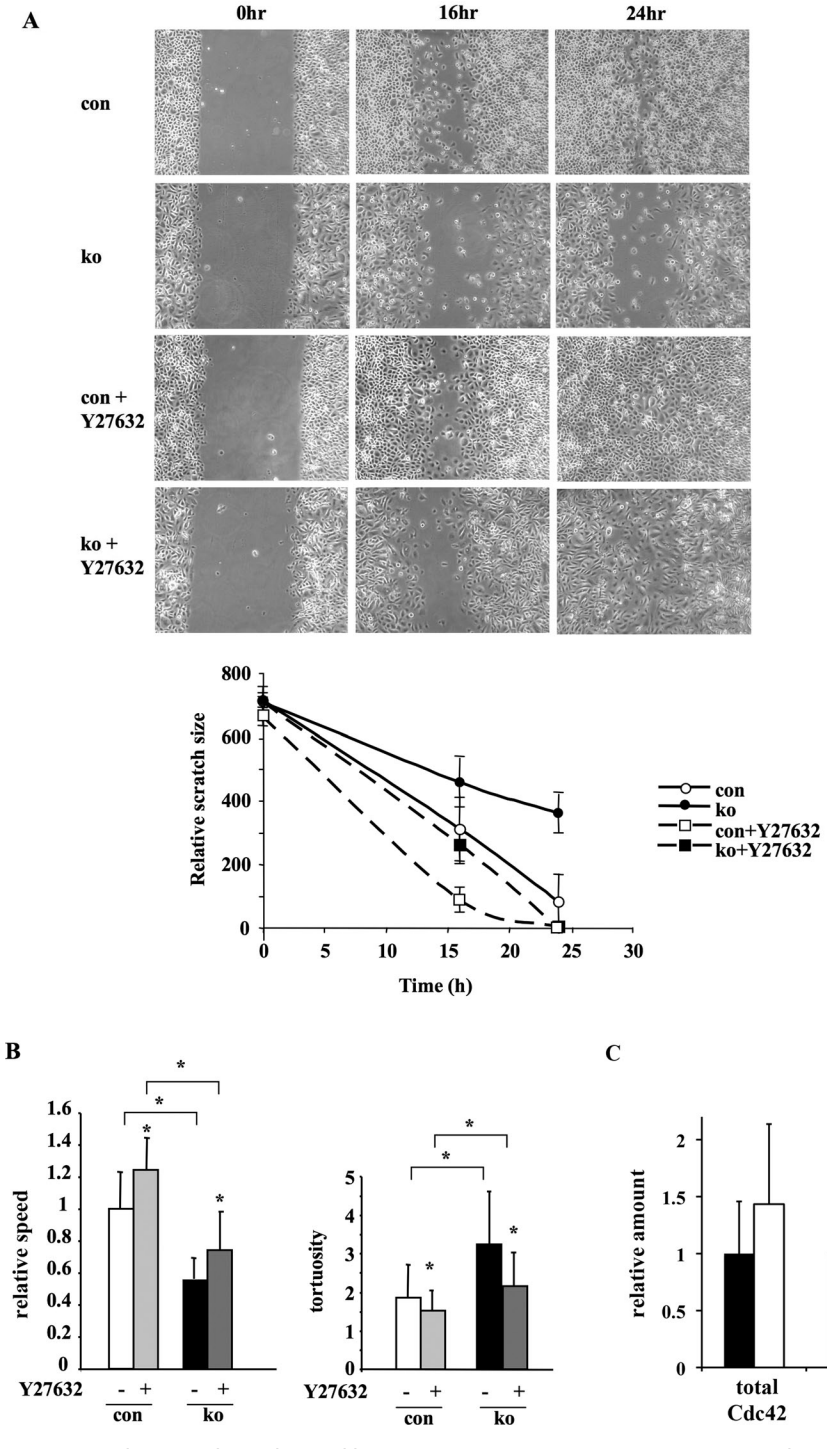

FIGURE 9: RhoA regulates directed keratinocyte migration in vitro. In vitro scratch assays carried out on confluent monolayers in the presence or absence of 25 μM Y27632, as described in *Materials and Methods*, indicated impaired directed migration of RhoA-null keratinocytes. (A) Shown are representative pictures of the gap closure and the kinetics of gap closure over time (n = 3). (B) Movies from gap-closure experiments were analyzed by tracking single migrating cells and calculating migration speed and tortuosity of migration (n > 100). (C) Reduced levels of GTP-bound active Cdc42 in cultured RhoA-null keratinocytes (n = 3/3).

expression in vitro, but not in vivo. Another phenotype expected for the RhoA mutant mice was the dissolution of the basement membrane at the DEJ and epithelial-mesenchymal transition of the RhoA-null keratinocytes (Nakaya *et al.*, 2008). We could not find any indication, however, for such impairment in RhoA-deficient skin. One explanation for the absence of these expected effects could be the presence of RhoB, which might have overlapping functions with RhoA. Another explanation could be off-target effects of the Rho inhibitors, which are absent in our RhoA knockout system. RhoA/RhoB double knockouts will be helpful to further investigate this point. Another important task for the future will be to apply different stress models to the RhoA mutant mice, to test whether RhoA-deficient epidermis will react differently under pathophysiological conditions.

RhoA and cytoskeleton

RhoA is the crucial regulator of cell contraction in keratinocytes, as inhibition of RhoB via C2I-N-C3 did not lead to an increase in cell area of RhoA-null keratinocytes. RhoA exerts its effect via ROCK-mediated phosphorylation of MLCP, resulting in inhibition of MLCP and increased phosphorylation of MLC. Direct phosphorylation of MLC by ROCK seems to be less important, since inhibition of MLCP by calyculin was able to rescue the impaired contraction. Inhibition of RhoA/ROCK uncovered a Rac1-independent mechanism of keratinocyte spreading, which even allows culturing of Rac1-deficient keratinocytes. The molecular details of this pathway deserve further investigation.

Despite the severe reduction of MLC phosphorylation in the absence of RhoA and the increase in cell spreading caused by decreased contraction, other contraction-dependent processes, such as the formation of stress fibers and focal adhesions or the contraction of the cleavage furrow during cell division, seemed to be less strongly affected, suggesting maybe less dependence on contractility.

In addition, cofilin phosphorylation in keratinocytes is largely dependent on RhoA. However, total levels of cofilin are slightly lowered in the absence of RhoA. Taken together, the amounts of unphosphorylated, active cofilin are increased in RhoA-null keratinocytes compared to control cells. The cellular consequences of this increased cofilin activity are not quite clear, because cofilin on the one hand was shown to decrease lamellipodia formation (Hotulainen *et al.*, 2005; Iwasa and Mullins, 2007; Lai *et al.*, 2008), but on the other hand is suggested to promote actin polymerization by increasing the number of barbed ends (van Rheenen *et al.*, 2009).

In conclusion, our data suggest that RhoA-mediated inactivation of cofilin by phosphorylation constitutes a major pathway of regulation of actin dynamics and turnover in keratinocytes.

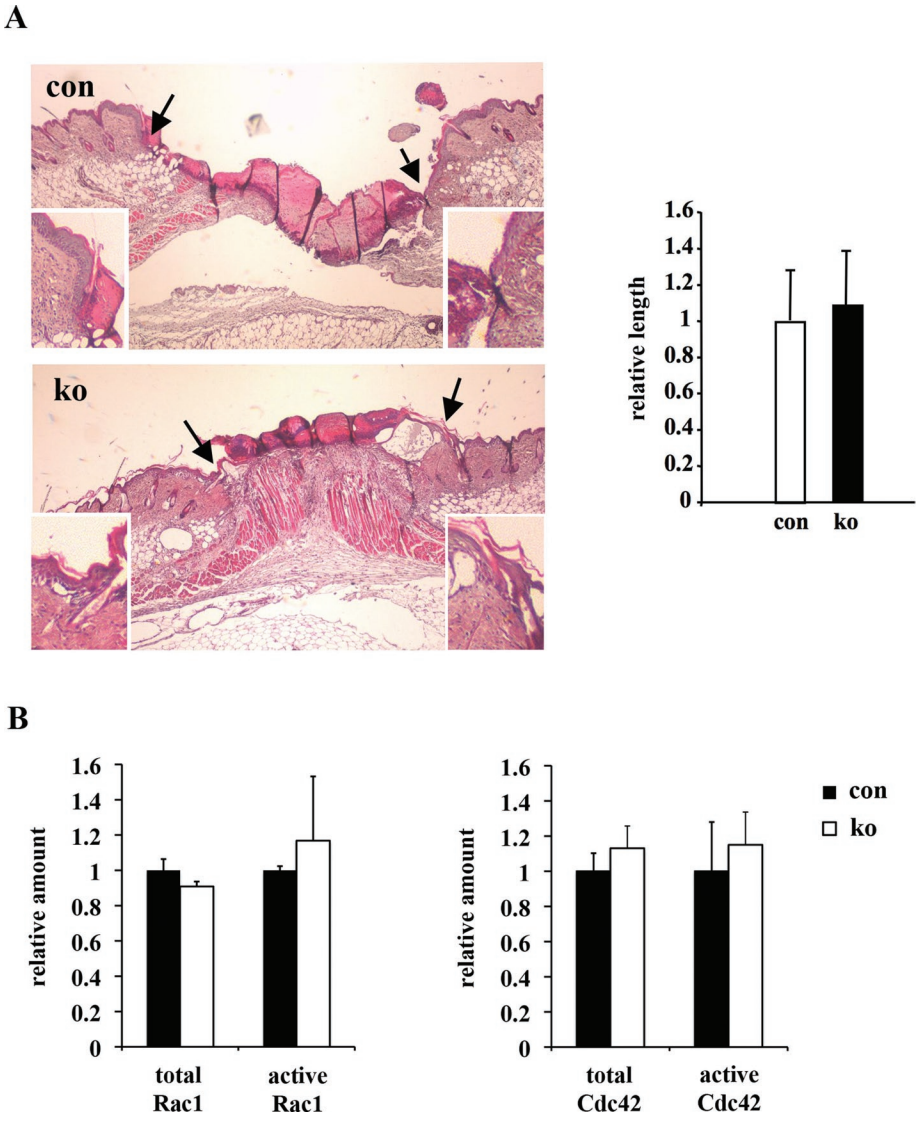

FIGURE 10: RhoA is not crucial for skin wound closure in vivo. In vivo wound closure was analyzed by full thickness wounds in the back skin of control (con) and RhoA fl/fl K5 Cre (ko) mice. Hematoxylin and eosin–staining was used to identify the wound borders in bisectioned wounds (indicated by arrows). Three days after wounding, wound diameters were not significantly different in control and mutant mice (six wounds, three mice). Insets show magnifications of the wound edges. (B) Normal levels of GTP-bound active Rac1and Cdc42 in freshly isolated RhoA-null keratinocytes (n = 3/3).

RhoA and migration

Strong Rho inhibition by C3 toxin was suggested to cause fibroblast detachment (Nobes and Hall, 1999). In keratinocytes, however, loss of RhoA alone increased adhesion, confirming an anti-adhesive function of RhoA, which could contribute to tail detachment during mesenchymal cell migration. Maybe off-target inhibition of Rac1 by high concentrations of C3 (Rottner *et al.*, 1999) could explain the observations in fibroblasts.

Rear detachment was impaired both in RhoA-null and ROCK inhibitor–treated keratinocytes, confirming the importance of the RhoA/ROCK pathway for detachment of the trailing edge. This defect, however, does not seem to be relevant for efficient migration of keratinocytes, as treatment with the ROCK inhibitor increased rather than decreased the speed of wild-type keratinocytes. Therefore, the decreased migration speed of RhoA-null keratinocytes must be independent of ROCK and cell contraction. Furthermore, the spreading increase in RhoA-null keratinocytes and its phenocopy by ROCK inhibition also appear unrelated to the decreased migration speed caused by the loss of RhoA function.

In addition to the reduced speed, RhoA-null keratinocytes displayed a clear defect in directional persistence, which was independent of ROCK and cell contraction. In fact, treatment with ROCK inhibitor Y27632 considerably increased the directional persistence of RhoA-deficient keratinocytes, even though prior to treatment these cells already had low levels of ROCK activity as revealed by severely reduced MLC phosphorylation. Also, control keratinocytes showed a slight increase of directional persistence when treated with the ROCK inhibitor Y27632. These findings are similar to those in a previous report where Y27632 increased directional migration of gerbil fibroma cells (Totsukawa *et al.*, 2004) and might be due to a ROCK-independent off-target effect of Y27632. The impaired migration of RhoA-null keratinocytes in vitro, however, corresponded to decreased levels of GTP-bound Rac1 and Cdc42, which possibly reduce migration speed and directionality, respectively.

Reduction of cofilin can lead to reduced turning frequency in adenocarcinoma cell lines (Sidani *et al.*, 2007). The altered amounts of active and total cofilin in the RhoA-null keratinocytes might therefore have an effect on their directional persistence during migration. A RhoA effector shown to promote directional migration is mDia1 (Goulimari *et al.*, 2005; Yamana *et al.*, 2006). Reduced activation of mDia1 might be another mechanism contributing to the impaired directional migration of RhoA-null keratinocytes.

In vivo, RhoA was not required for wound healing, which corresponded well to the unchanged levels of active Rac1 and Cdc42, indicating that environmental factors strongly affect the consequences of RhoA gene deletion in keratinocytes. What could be the molecular mechanisms mediating the environmental effects? Switching wild-type keratinocytes from in vivo to in vitro conditions resulted in more than twofold up- or down-regulation in the expression of approximately half of the GEFs GAPs, and Rho effectors listed in Supplemental Table 1. Also, Rho GTPase expression is altered (Supplemental Table 2). These differences most likely affect the function of RhoA and its cross-talk to other Rho GTPases.

Our data suggest that alteration of the physiological interactions of keratinocytes with soluble factors, other cells, and the extracellular matrix (as, e.g., in cancer) might have a huge impact on the role of RhoA in cell migration. It will therefore be interesting to test whether the impaired migration of RhoA-null keratinocytes in vitro translates to a defective migration of RhoA-null cancer cells in vivo.

MATERIALS AND METHODS

Mice

Mice with a keratinocyte-restricted deletion of the RhoA gene were generated using the Cre-loxP system. In short, a genomic 129Sv mouse PAC library was screened using the complete mouse cDNA of RhoA as a probe. A 10-kb fragment of the RhoA gene containing exons 3 and 4 was subcloned by Red/ET recombination (Gene Bridges, Heidelberg, Germany). A single loxP site was introduced 0.55 kB upstream of exon 3 and a floxed neo-TK cassette 1.1 kB downstream of exon 3. After electroporation, stable neomycin-resistant clones were selected, and homologous recombinants were identified by *Hin*dIII digest of genomic DNA and Southern blot with a 5′ external probe (wild type 16.4 kB, floxed with neo 11.1 kB). The third loxP site was detected by PCR using the primers JVH11 (agccagcctcttgaccgatta) and JVH15 (tgtgggataccgtttgagcat) giving rise to a 297-bp product in case of wild type and a 393-bp product in case of a floxed allele. Southern blot with an internal probe demonstrated a single integration of the targeting construct. The neo-TK cassette was removed by transient Cre expression in vitro, and unrecombined clones (floxed with neo) were eliminated by FIAU treatment. Removal was confirmed by *Hin*dIII digestion of genomic DNA and Southern blot with a 5′ external probe (wild type 16.4 kB, floxed 11.1 kB, ko 14.5). Embryonic stem (ES) cell culture and blastocyst injection were carried out as described (Talts *et al.*, 1999). Keratinocyte-specific deletion of the RhoA gene was achieved by intercrossing mice expressing Cre recombinase under the control of a K5 promoter (Ramirez *et al.*, 2004). Mice were genotyped for RhoA by genomic PCR from tail DNA using the primers JVH11 and JVH15 and for the presence of Cre using the primers cre3′ (ttcggatcatcagctacacc) and cre5′ (aacatgcttcatcgtcgg).

Histology, immunohistochemistry, and electron microscopy

Histology and immunofluorescence staining were performed as described previously (Chrostek *et al.*, 2006). Proliferation of keratinocytes in the epidermis was analyzed by bromodeoxyuridine incorporation (Chrostek *et al.*, 2006). Images of histochemical stainings were taken at room temperature (RT) using an Olympus BX51 microscope (Olympus, Ballerup, Denmark) equipped with 10× PlanC N (NA 0.25), 20× PlanC N (NA 0.40), and 40× UPlanSApo (NA 0.90) objectives and a digital camera (Colorview IIIu; Olympus) controlled by Cell^A software.

Olympus primary antibodies were used: keratin 14, keratin 10, keratin 6, loricrin (all Covance, Princeton, NJ); paxillin, fluorescein isothiocyanate–conjugated CD49f (integrin α6 chain; all BD Biosciences, Brøndby, Denmark); phalloidin-Alexa 488/568 (Molecular Probes, Carlsbad, CA); desmoplakin (Research Diagnostics/RDI, Concord, MA); E-cadherin, occludin, and ZO-1 (all Zymed Laboratories, San Francisco, CA). As secondary reagent, Cy3-conjugated goat anti–rabbit immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) was used. Nuclei were stained with DAPI (Sigma, St. Louis, MO). Immunofluorescence was analyzed at RT by confocal microscopy using a Leica TCS SP2 system with a DM RXA2 microscope, equipped with 20× HC PL Apo (NA 0.70), 40× HCX PL APO (NA 1.25–0.75) and 63× HCX PL APO (NA 1.40–0.60) objectives, controlled by Leica Microsystems confocal software (version 2.61 Build 1537; all Leica Microsystems, Bannockburn, IL).

For electron microscopy, small samples of back skin were fixed in 4% paraformaldehyde, 2% glutaraldehyde, in 0.1 M Na-cacodylate buffer, pH 7.4, supplemented with 2 mM $CaCl_2$. Following fixation, samples were washed with 0.1 M Na-cacodylate buffer, pH 7.4, supplemented with 2 mM $CaCl_2$ and secondarily fixed with 1% osmium tetroxide in 0.1 M Na-cacodylate buffer, pH 7.4, supplemented with 2 mM $CaCl_2$ for 2 h. Samples were then dehydrated in a graded ethanol series and incubated in propylene oxide for 1 h. Afterward, samples were infiltrated with a mix of agar low-viscosity resin/propylene oxide at increasing resin concentrations for 5 h (50%/50%) and overnight (75%/25%). After an 8-h infiltration with pure resin, samples were embedded in agar low-viscosity resin. Semithin sections (1 µm) were cut for orientation purposes and stained with toluidine blue. Ultrathin sections (90–100 nm) were cut with a Reichert–Jung ultramicrotome, collected on 250-mesh copper grids, and stained with uranyl acetate for 35 min and lead citrate for 20 min in a Leica EM AC20 stainer. Sections were examined with a Hitachi H-7000 (Tokyo, Japan) electron microscope. Statistical evaluation of the integrity of the DEJ was carried out on tissue samples from 2- to 7-mo-old mice. Three samples were taken per mouse, and from each sample 10 nonoverlapping images of the DEJ (20,000× magnification) were used. Measurements were taken using Image-J software (National Institutes of Health, Bethesda, MD), and significance was calculated by using Student's *t* test.

Biochemical analysis

Western blotting was performed according to standard protocols and quantified using TotalLab TL100 software (Nonlinear Dynamics, Durham, NC). Tubulin was used to normalize for different protein amounts. Pull-down assays for Cdc42 and Rac1 were performed as previously described (Zondag *et al.*, 2000).

The following primary antibodies were used: RhoA, RhoB, RhoC (all Santa Cruz Biotechnology, Santa Cruz, CA); RhoC (Cell Signaling Technology, Danvers, MA); Rac1 (BD Biosciences); Cdc42 (Cytoskeleton, Denver, CO); cofilin, phospho-cofilin, MLC, phospho-MLC (all Cell Signaling Technology); occludin, ZO-1 (all Zymed Laboratories); and tubulin (Y/L1/2; provided by J. Wehland, Braunschweig, Germany).

Primary keratinocyte culture, transfection, and microarray gene expression analysis

Isolation of primary keratinocytes from adult mice and subsequent in vitro culture was carried out as described previously (Chrostek *et al.*, 2006).

For cell spreading and cytokinesis experiments, cells were treated for 24 h in growth medium containing 25 µM Y27632 or 2 nM calyculin A (all Calbiochem/EMD Chemicals, Gibbstown, NJ). The toxins C2I-N-C3 (200 ng/ml), C2II (400 ng/ml), GST-CNF1 (300 ng/ml) and CNFY (300 ng/ml) (Hoffmann *et al.*, 2004) were added to subconfluent cells for 3 h.

Keratinocytes were transfected with vectors expressing an EGFP-RhoA fusion protein, coexpressing RhoA F30L and EGFP, coexpressing Cre and EGFP or expressing EGFP alone, using the *Trans*IT-Keratinocyte Transfection Reagent (Mirus, Madison, WI). EGFP+ cells were isolated 2 d later by preparative flow cytometry, and RhoA expression was tested by Western blot.

For microarray analysis, RNA was isolated from keratinocytes according to standard protocols. RNA was prepared from both freshly isolated (in vivo) or cultured (in vitro) primary keratinocytes. Hybridization to GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) was carried out at the Copenhagen University Hospital Microarray Center using standard protocols.

Adhesion assay

For adhesion assay, 96-well plates were coated for 2 h at 37°C with collagen type I at 30 µg/ml (Inamed Biomaterials, Freemont, CA) and fibronectin at 10 µg/ml (Biological Industries, Israel) in phosphate-buffered saline (PBS), and blocked with 1% bovine serum albumin in PBS for 90 min at 37°C. Keratinocytes were seeded and

allowed to attach to the wells for 1 h at 34°C. Nonadherent cells were washed away with PBS. Attached cells were fixed with 70% ethanol and stained with crystal violet at 5 mg/ml in 20% methanol. After washing away excess dye and air drying, 1% SDS in PBS was added to the wells, and absorbance was measured at 590 nm with a plate reader (Bio-Tek Instruments, Winooski, VT).

Keratinocyte migration assays

For in vitro wound-healing assays, cells were seeded at high density on six-well plates in growth medium (8% fetal calf serum [FCS]) and wounded 1 d later by scraping across the confluent monolayers. 8 h before scratching, medium was exchanged to keratinocyte growth medium containing 4% FCS, with or without Y27632. Phase contrast and fluorescence movies were recorded on an Axiovert 200 automatic microscope system equipped with closed heating and CO_2 perfusion devices (Carl Zeiss, Jena, Germany) essentially as previously described (Lai *et al.*, 2009). Movies were analyzed using Image J software, and speed and tortuosity were calculated.

Wound-healing assay

Full-thickness wounds (two per mouse) were created on the back skin of mice using a 4-mm biopsy punch. Three or five days after wounding, mice were killed and wounds were excised, bisected, and fixed in 4% paraformaldehyde overnight. Hematoxylin and eosin–stained paraffin sections of the wounds were then analyzed for wound width.

Statistical analysis

Data are expressed as means ± SD, with error bars representing SD. Statistical significance was determined by two-tailed Student's *t* test, and significant differences ($p < 0.05$) are indicated by asterisks.

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