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ORIGINAL ARTICLE

Rac1 is crucial for Ras-dependent skin tumor formation by controlling Pak1-Mek-Erk hyperactivation and hyperproliferation *in vivo*

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Rac1 has a role in proliferation and survival of tumor cells *in vitro*. The exact effects of Rac1 on growth, apoptosis and corresponding signaling pathways during tumorigenesis *in vivo*, however, have not been explored yet. Using mice with a keratinocyte-restricted deletion of the *Rac1* gene, we found that Rac1 is essential for DMBA/TPA-induced skin tumor formation. This corresponded to a decreased keratinocyte hyperproliferation, although apoptosis was not detectably altered. Activated Rac1 promoted Erk-dependent hyperproliferation by Pak1-mediated Mek activation independent of Mek1 phosporylation at serine 298. Rac1 was furthermore required for Pak2-dependent hyperactivation of Akt, which under *in vivo* condition was restricted to the suprabasal cell layers corresponding to a suprabasal-specific expression of Pak2. It is surprising that none of these signaling pathways was altered in untreated Rac1-deficient skin, indicating a hyperproliferation-specific function of Rac1 *in vivo*. These data suggest that blocking of Rac1 function might allow tumor-specific growth repression, as Rac1 is not required for normal growth and growth signaling controlling pathways in skin *in vivo*.

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Introduction

Skin cancer is the most common form of cancer with more than one million cases diagnosed annually in the United States. About one-third of these skin cancers carry an activating mutation of H-Ras, which stimulates various signaling pathways including the Raf-Mek-Erk cascade and PI3K, suggested to be crucial for growth and survival of tumor cells. Many *in vitro* evidences suggested the small Rho GTPase Rac1 to be important for transformation, growth and survival of tumor cells, at least partially by controlling Erk and PI3K activation downstream of Ras (Sahai and Marshall, 2002; Karlsson *et al*, 2009). However, different pathways have been suggested for Rac1-dependent regulation of the Raf-Mek-Erk cascade. Rac1 was reported to promote Raf-Mek-Erk signaling by Pak-mediated phosphorylation of Raf at serine 338 (King *et al*., 1998), or of Mek1 at serine 298 (Slack-Davis *et al*., 2003), by promoting interaction between Mek and Erk (Eblen *et al*., 2002), by direct interaction of Rac1 with Erk (Sundberg-Smith *et al*., 2005) and by controlling the nuclear translocation of Erk (Hirsch *et al*., 2002). With respect to PI3K signaling, Rac1 was shown to be upstream (Keely *et al*., 1997) and downstream of PI3K signaling (Hawkins *et al*., 1995), and the Rac1 effector, Pak1, was shown to activate Akt by direct interaction, independent of the kinase function of Pak (Higuchi *et al*., 2008). However, although Rac1 activation was found to promote cell survival by activation of Akt, Erk and NF-κB, it can also induce apoptosis by stimulation of p38 and JNK (Xia *et al*., 1995).

In human patients, Rac1 expression was increased in testicular, gastric, breast and oral squamous cell carcinoma (Karlsson *et al*., 2009). In a colorectal carcinoma model involving orthotopic injection of adenocarcinoma cells into mice, Rac1 overexpression promoted, and Rac1 knockdown decreased tumor progression (Espina *et al*., 2008). More recently, activation of Rac proteins was described to be crucial for the development of chronic myelogenous leukemia (Thomas *et al*., 2007) and K-Ras-induced lung cancer (Kissil *et al*., 2007). Indirect evidence also suggested a role for Rac1 in skin tumor formation, as mice lacking the Rac1-specific GEF Tiam1, which display about 50% reduction in GTP-bound Rac1, are rather resistant to 7,12-dimethylbenz(α)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumor formation (Malliri *et al*., 2002).

The manner how Rac1 affects tumor formation mechanistically *in vivo*, however, has not been investigated, and it is currently not clear. We and other groups recently generated mice with a keratinocyte-restricted deletion of the *Rac1* gene (Benitah *et al*., 2005; Chrostek *et al*., 2006; Castilho *et al*. 2007). Our Rac1-mutant mice lose all hairs at around 3 weeks of age, but show normal differentiation and ultrastructure of the interfollicular epidermis. In this study, we analyzed the formation of

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papillomas and corresponding Rac1 signaling under *in vivo* condition by applying the DMBA/TPA skin tumor model (Perez-Losada and Balmain, 2003). Our data demonstrate an essential role for Rac1 in the formation of DMBA/TPA-induced skin tumor and indicate that Rac1 controls keratinocyte hyperproliferation *in vivo* by regulating hyperactivation of Mek1/2 through Pak1-dependent phosphorylation of Mek1/2 at serine 217/221, independent of phosphorylation of c-Raf at serine 338 and Mek1 at serine 298.

Results

Rac1 is crucial for skin tumor formation

To investigate the role of Rac1 in skin tumor formation, we treated adult mice carrying a keratinocyte-restricted deletion of the *Rac1* gene and control littermate once with the mutagen DMBA and repeatedly with the growth-promoting substance TPA. This protocol causes papillomas derived from the interfollicular epidermis and, to a very low percentage, squamous cell carcinoma derived from progenitors in the bulge region of the hair follicle (Perez-Losada and Balmain, 2003). As Rac1-deficient mice lost the lower parts of the hair follicles and might have no or impaired bulge progenitors (Chrostek *et al*., 2006; Castilho *et al*., 2007), we were only expecting papilloma, but no carcinoma in the Rac1-mutant mice.

In control mice, the first papilloma was observed 7 weeks after the DMBA treatment, and after 21 weeks, all 12 control mice had developed papilloma (Figures 1a–c). The number of papillomas per mouse steadily increased reaching about six tumors per mouse after 30 weeks (Figure 1c). Littermates with a keratinocyte-restricted deletion of Rac1, however, were rather resistant to skin tumor formation (Figures 1a–c). Only 1 of 10 mice developed a single papilloma after 22 weeks.

These data show that Rac1 function in keratinocytes is highly important for the formation of skin tumors.

Rac1 controls TPA-induced hyperproliferation in vivo

To understand the mechanism of the tumor-promoting function of Rac1, we analyzed epidermal thickening, proliferation and apoptosis in the back skin of DMBA/TPA-treated and -untreated mice.

In untreated mice, loss of Rac1 had no effect on epidermal thickness, proliferation and apoptosis, confirming earlier observations (Figures 1d and e; Chrostek *et al*., 2006). However, DMBA/TPA-induced hyperproliferation as determined by incorporation of 5-bromodeoxyuridine (BrdU) into cycling cells was significantly reduced in mice lacking Rac1 (Figures 1d and e). Most BrdU+ keratinocytes were observed in the basal keratinocyte layer, both in control and Rac1-deficient mice. Treatment of back skin with DMBA/TPA induced similar epidermal thickening and increase of keratin 6 expression in control and Rac1-mutant mice, indicating that these process are independent of Rac1 (Figures 1d and e). Rac1, therefore, is essential for DMBA/TPA-induced keratinocyte hyperproliferation *in vivo*, although it is not required for proliferation under physiological conditions.

Studies with mice lacking the Rac1-activating molecule Tiam1 in keratinocytes showed that reduced Rac1 activity promotes keratinocyte apoptosis in serum-free conditions (Mertens *et al*., 2005). In DMBA/TPA-treated epidermis, we detected an increase of cleaved caspase 3+ apoptotic cells, but no difference between control and Rac1-deficient skin (Figures 1d and e). Analyzing programmed cell death by TdT-mediated dUTP nick end-labeling (TUNEL) staining confirmed this observation (Figure 1d).

These data indicate that Rac1 is crucial for the DMBA/TPA-induced proliferation of keratinocytes *in vivo*, although it does not detectably alter programmed cell death or epidermal thickening in response to DMBA/TPA.

Rac1 is important for Erk hyperactivation following DMBA/TPA treatment

Many cell culture studies demonstrated that Rac1 controls Erk activation *in vitro*. The suggested mechanisms, however, varied dramatically ranging from regulation of Raf activation by Pak-mediated phosphorylation of serine 338 (King *et al*., 1998) to Rac1-controlled transport of phosphorylated Erk into the nucleus (Hirsch *et al*., 2002). To assess, which of the suggested mechanisms is important for the DMBA/TPA-induced hyperproliferation in keratinocytes *in vivo*, we analyzed phosphorylation of c-Raf (Ser 338), b-Raf (Ser 445), Mek1/2 (Mek1: Ser 298; Mek1: Thr292; Mek 1/2: Ser 217/221) and Erk (Thr 202/Tyr 204) by immunofluorescence and by western blot analysis of epidermal lysates (Figures 2a and b).

In untreated skin, none of these phosphorylation sites displayed altered phosphorylation in the absence of Rac1, indicating that Rac1 is not required for physiological activation of the Ras-Erk cascade (Figure 2b). Treatment with DMBA/TPA strongly increased phosphorylation of c-Raf, b-Raf, Mek and Erk at all sites tested in control mice. Immunofluorescent staining furthermore revealed phosphorylated c-Raf (Ser 338), Mek1/2 (Ser 217/221) and Erk (Thr 202/Tyr 207) in all epidermal layers (Figure 2a).

In the absence of Rac1, phosphorylations of c-Raf (Ser 338), b-Raf (Ser 445), and of Mek1 at serine 298 and threonine 292, an inhibitory phosphorylation site, were similarly increased as in control mice (Figure 2b). However, phosphorylation of Mek1/2 at serine 217/221, which is known to correlate with Mek activity, as well as phosphorylation of Erk (Thr 202/Tyr 204) were not increased in Rac1-mutant epidermis after DMBA/TPA treatment. Immunofluorescent stainings of cryosections of back skin confirmed normal phosphorylation of c-Raf (Ser 338), and impaired phosphporylation of Mek1/2 (Ser 217/221) and Erk (Thr 202/Tyr 204) in Rac1-mutant epidermis after DMBA/TPA treatment (Figure 2a).

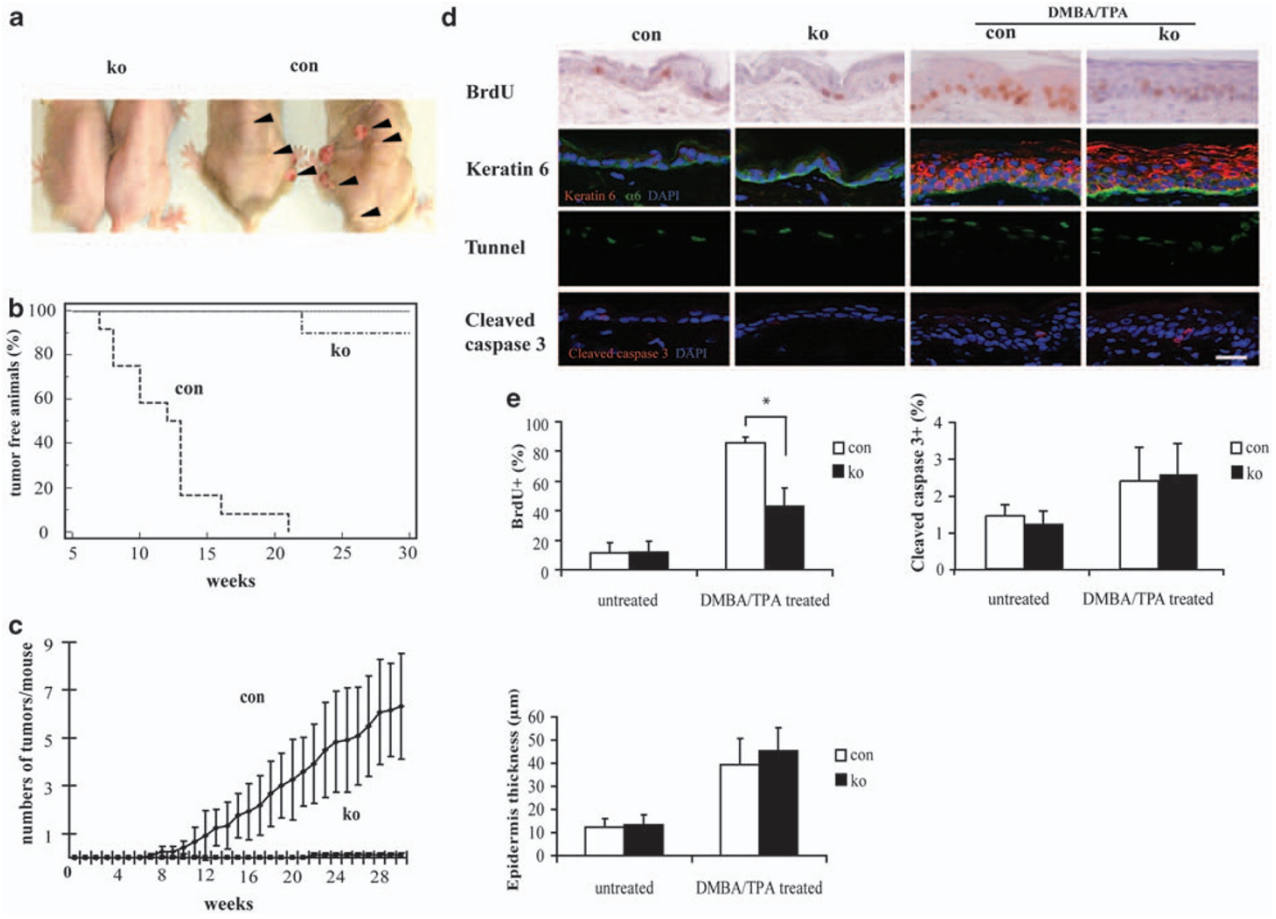

Figure 1 Rac1 is crucial for skin tumor formation. Control (con) and Rac1 knockout (ko) litter mates were subjected to DMBA/TPA-induced skin carcinogenesis as described in experimental procedures (n:12/10). (**a**) Representative image of ko and con mice after 30 week of treatment. Arrows point to typical papillomas. (**b**) Percentage of tumor-free mice. (**c**) Average number of tumors per mouse. (**d**) Staining for BrdU incorporation, keratin 6/α6 integrin (red/green), TUNEL and cleaved caspase 3 (red). DAPI (blue) counterstaining indicates nuclei. Scale bar: 20 μm. (**e**) Quantification of percentage of BrdU+ and cleaved caspase 3+ keratinocytes in basal layer. Epidermal thickness was calculated by Cell^A FIVE program (Olympus, Tokyo, Japan) ($n=4$; $^*P<0.01$).

These data show that Rac1 mediates DMBA/TPA-induced hyperactivation of Erk *in vivo* by facilitating Mek activation. Phosporylation of serine 298, however, is not sufficient for Mek1 hyperactivation *in vivo*.

Decreased hyperactivation of Pak1/2in Rac1-mutant mice after DMBA/TPA treatment

As none of the described phosphorylation sites for the Rac1 effector Pak1/2 in the Ras-Erk cascade (c-Raf (Ser 338), Mek1 (Ser 298)) showed a changed phosphorylation in the absence of Rac1, we tested whether Pak1/2 activation is at all altered in Rac1-deficient epidermis. In untreated mice, phosphorylation of Pak1/2 at serine 144/141, corresponding to Pak1/2 activation, and at threonine 423/402, corresponding to increased activation, were not changed in the absence of Rac1 (Figure 3b). DMBA/TPA treatment, however, caused a twofold increase of both phosphorylations in control, but not in Rac1-deficient mice, indicating that hyperactivation of Pak1/2 is dependent on Rac1, whereas basal Pak1/2 activation is Rac1 independent.

Loss of Rac1 affects the survival-regulating Akt and p38 pathways

In vitro, Rac1 activation was shown to activate the survival and proliferation through Akt, but also to inhibit it through p38 and JNK. In untreated epidermis, none of these pathways is affected by the loss of Rac1 (Figure 3a and b). Treatment with DMBA/TPA increased activation of Akt, p38 and, slightly, of JNK in control epidermis (Figures 3a and b). It is interesting that immunofluorescent staining detected phosphorylated Akt (Ser 473) only in suprabasal layers, but not in basal keratinocytes (Figure 4a). This suggests that the increased proliferation of basal keratinocytes following DMBA/TPA treatment is not related to a change in Akt activity.

In the absence of Rac1, DMBA/TPA-induced hyperactivation of p38 and Akt were largely decreased, although hyperactivation of JNK was not significantly affected (Figures 3a and b). It is interesting that some suprabasal cells showed immunofluorescence for phosphorylated Akt at the cell membrane in the absence of Rac1, suggesting that Rac1 is not essential for membrane translocation of Akt to the cell membrane.

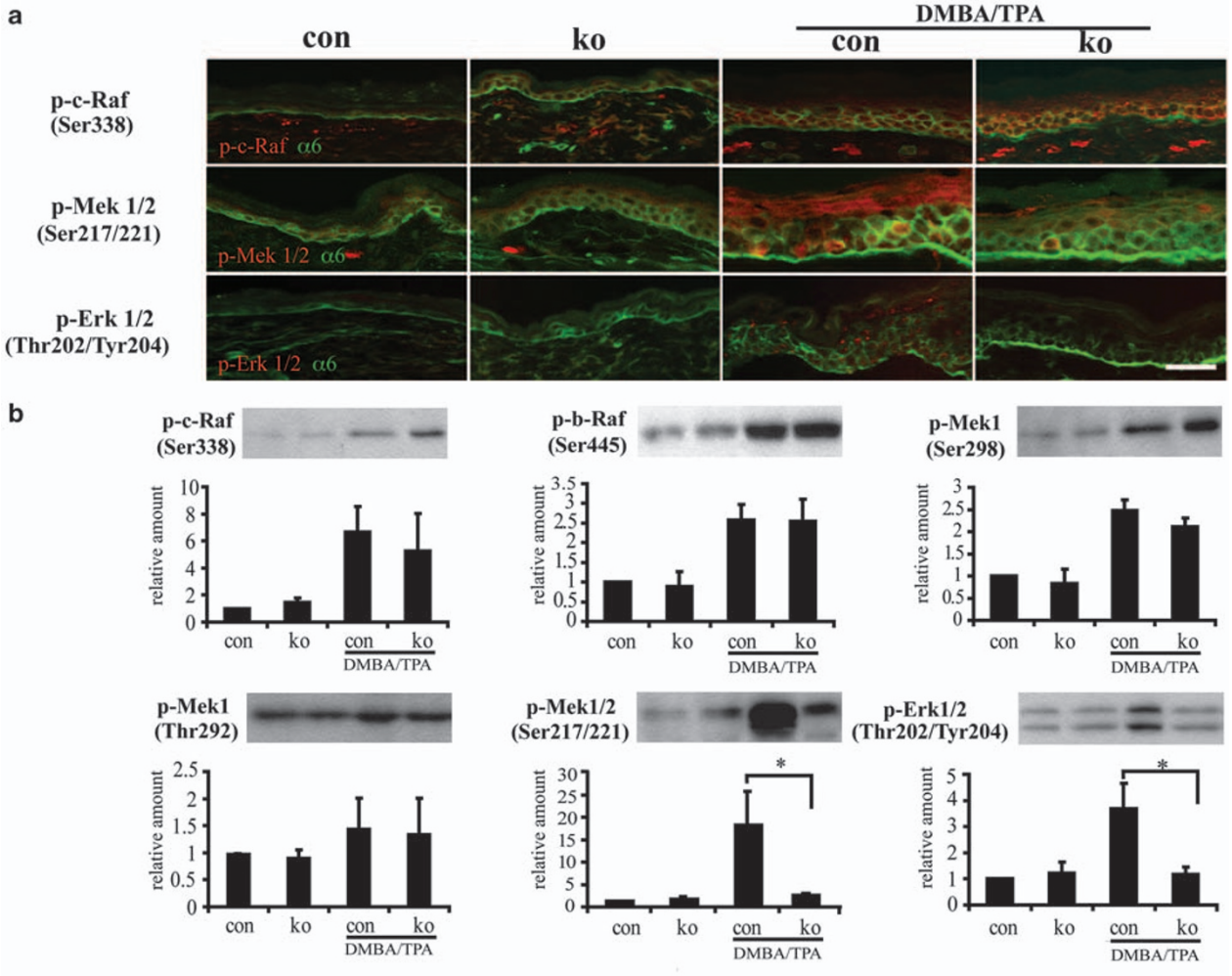

Figure 2 Rac1 is crucial for Erk hyperactivation after DMBA/TPA treatment. (**a**) Immunofluorescent analysis of back skin sections of control (con) and Rac1 knockout (ko) mice, untreated or treated with DMBA/TPA, for p-c-Raf (Ser 338), p-Mek1/2 (Ser 217/221) and p-Erk1/2 (Thr 202/Tyr 204) (all in red) in the epidermis. Counterstaining for α6 integrin (α6; green) indicates the dermal–epidermal junction. Scale bar: 20 μm. (**b**) Western blot analysis of epidermal lysates of control (con) and Rac1 ko mice, untreated or treated with DMBA/TPA, for p-c-Raf (Ser 338), p-b-Raf (Ser 445), p-Mek1 (Ser 298), p-Mek1 (Thr 292), p-Mek1/2 (Ser 217/221) and p-Erk1/2 (Thr 202/Tyr 204) ($n=4$; $^*P<0.01$).

These data indicate that Rac1 controls survival-regulating pathways in the skin *in vivo*. Although we could not observe an influence of Rac1 on apoptosis of DMBA/TPA-treated, hyperproliferative epidermis, this pathway might be important in malignant squamous cell carcinoma.

Pak-regulated Erk activation controls proliferation of primary and RasV12-transformed keratinocytes in vitro

The *in vivo* analysis showed that Rac1 is important for hyperproliferation of normal cells, but did not test whether Rac1 has a similar role in established tumors. To address this question at least *in vitro*, we generated RasV12- and RasV12/E1A-transformed keratinocyte cell lines and tested the importance of specific Rac1-dependent signaling pathways for cell growth using inhibitors. The RasV12-transformed cell lines showed increased activation of Mek1/2, Erk, Pak1/2 and Akt compared with primary keratinocytes (Figures 4a and b), mimicking the increased activation of these pathways in DMBA/TPA-treated epidermis *in vivo*. Proliferation of these cells, however, was similar to primary mouse keratinocytes (Figure 4c 'con'), indicating that standard growth conditions *in vitro* are strongly promoting hyperproliferation of normal keratinocytes. To check the contribution of Rac1-dependent signaling pathways to the proliferation of RasV12-transformed keratinocytes, we applied cell-permable inhibitors to Mek (PD98059), PI3K (Ly294002 and wortmannin) and PAK1/2 (PAK18) to normal and RasV12-transformed cells.

Inhibition of Mek by PD98059 resulted in reduced proliferation (Figure 4c) and reduced phosphorylation of Mek1/2 (Ser 217/221) and Erk (Thr 202/Tyr 204), whereas phosphorylation of Mek1 at serine 298 was not altered (Figures 5a and b; Supplementary Figures 1a and b).

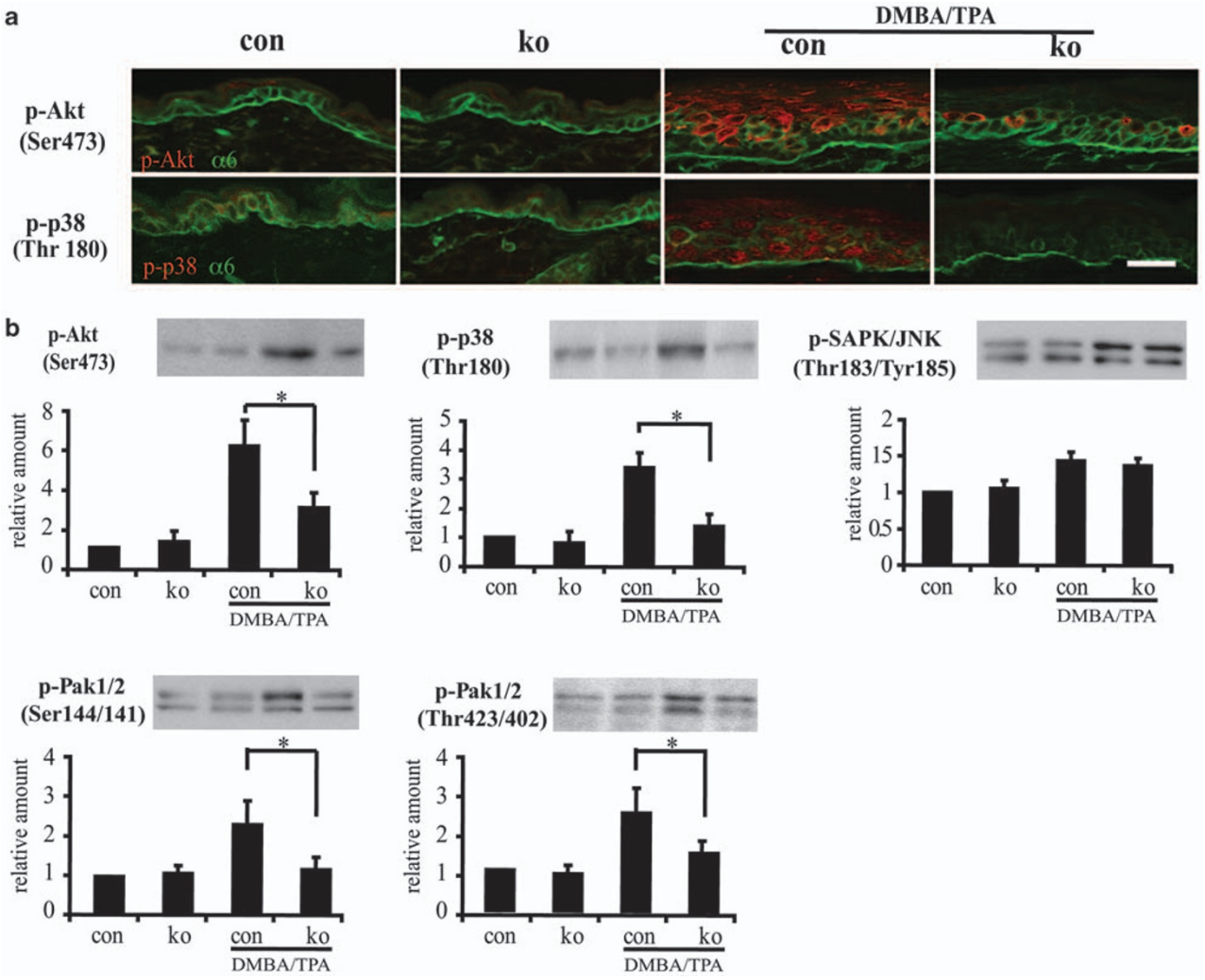

Figure 3 Rac1 is required for activation of Pak1/2, Akt and p38 after DMBA/TPA treatment. (**a**) Immunofluorescent analysis of back skin sections of control (con) and Rac1 knockout (ko) mice, untreated or treated with DMBA/TPA, for pAkt (Ser 478), p-p38 (Thr 180), pPKCε (Ser 729) (all in red). Counterstaining for α6 integrin (α6; green) indicates the dermal–epidermal junction. Scale bar: 20 μm. (**b**) Representative blot and quantification of western blot analysis of epidermal lysates of control (con) and Rac1 knockout (ko) mice, untreated or treated with DMBA/TPA for p-Akt (Ser 473), p-p38 (Thr 180), p-SAPK/JNK (Thr 183/Tyr 185), p-Pak1/2 (Ser 144/141) and p-Pak1/2 (Thr 423/402) ($n=4$; $^*P<0.01$).

The PI3K inhibitors, LY294002 and wortmannin, severely decreased the proliferation (Figure 4c) and efficiently inhibited activation of Akt (Figures 5a and b; Supplementary Figures 1a and b). In addition, they blocked Mek and Erk activation, similar to the Mek inhibitor PD98059. However, they did not affect Pak phosphorylation (Ser 144/141; Thr 423/402). These data indicate that Erk activation is crucial for proliferation of primary and transformed keratinocytes. PI3K signaling is required for Erk activation in a Pak-independent manner.

The importance of the Rac1 effector, Pak, for Erk activation and proliferation was tested by the inhibitor PAK18, which blocks the interaction of Pak with Pix (Maruta *et al*., 2002)

This inhibitor reduced Pak1/2 phosphorylation at serine 144/141, but had no effect on the Pak1/2 phosphorylation at threonine 423/402, suggesting only a partial inhibition of Pak1/2 activation (Figures 5a and b; Supplementary Figures 1a and b). Incubation with PAK18 partially reduced proliferation (Figure 4c) and decreased phosphorylation of Mek1/2 at serine 144/141 and of Erk (Thr 202/Tyr 204), whereas Mek1 phosphorylation of Mek at serine 298 was not altered (Figures 5a and b; Supplementary Figure 1a and b). c-Raf, Akt and p38 activation were not altered.

These data suggest that Pak regulates keratinocyte proliferation by controlling Mek activation independent of Mek (serine 298) phosphorylation. Inhibitors of Rac1-dependent signaling pathways similarly affected the growth of primary and RasV12-transformed keratinocytes *in vitro*, suggesting that Rac1 signaling is equally important for proliferation of normal and transformed keratinocytes (Figure 4c).

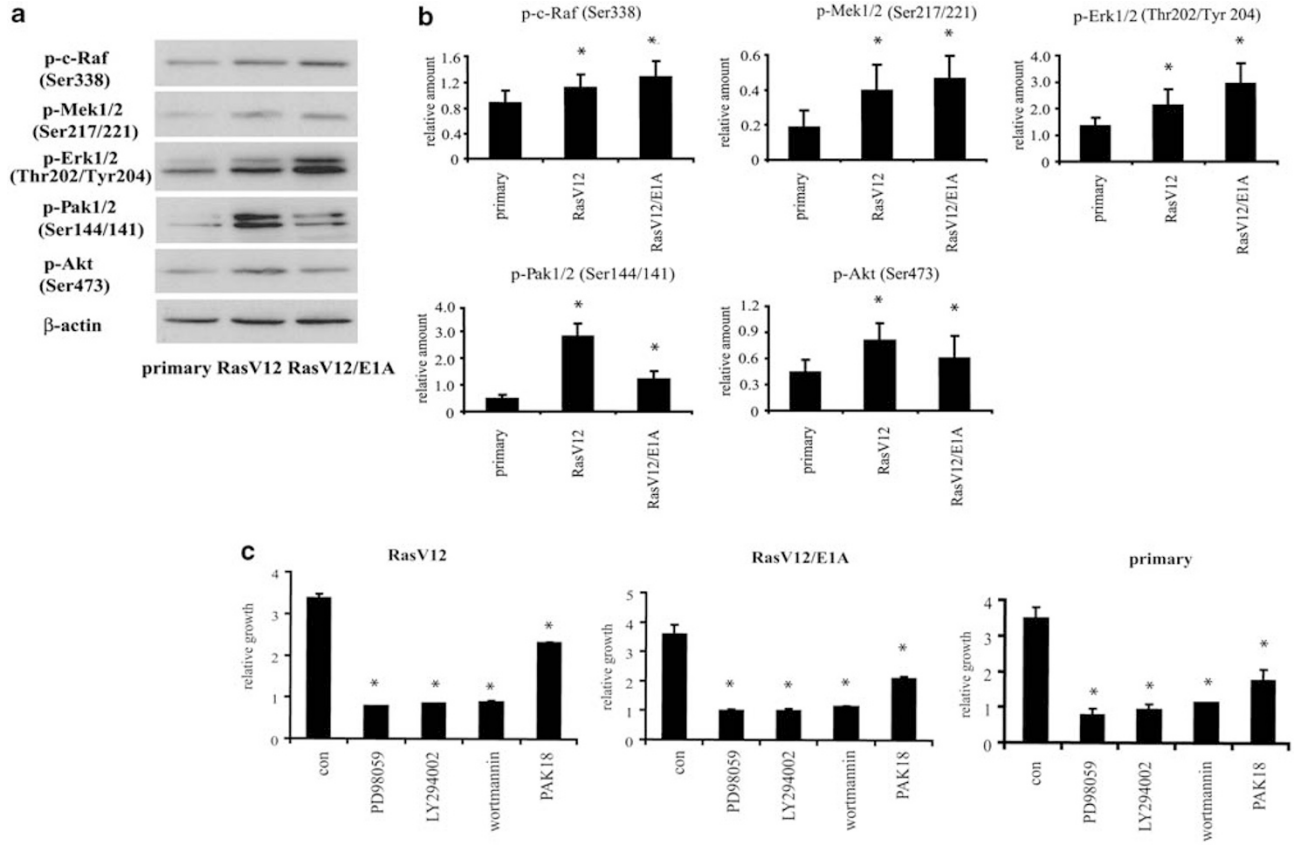

Figure 4 Proliferation and activation of Erk pathway, Pak and Akt in RasV12- and RasV12/E1A-transformed keratinocytes. (**a**) Western blot analysis of cell lysates shows amount of phosphorylation of c-Raf at Ser 338, Mek1/2 at Ser217/221, Erk1/2 at Thr 202/Tyr 204, Pak1/2 at Ser144/141 and Akt at Ser473. Representative blots are from three independent experiments. (**b**) Quantification of western blot analysis ($n=3$; $^*P<0.01$). (**c**) Primary, RasV12- and RasV12/E1A-transformed keratinocytes were treated with Mek inhibitor PD985402 (50 μM), the PI3K inhibitors LY294002 (50 μM) and wortmannin (50 nM), and Pak inhibitor PAK18 (50 μM) and analyzed for growth by crystal violet staining 2 days later ($n=4$; $^*P<0.01$).

Specific roles for Pak1 and Pak2 in the regulation of Erk and Akt activation

As PAK18 inhibited only one of two Pak1/2 phosphorylation sites and did not distinguish between Pak1 and Pak2, we performed knockdown of Pak1 and Pak2 in primary keratinocytes. Pak1 knockdown cells showed normal phosphorylation of c-Raf (Ser 338) and Mek1 (Ser 298), but reduced phosphorylation of Mek1/2 at serine 217/221 and of Erk (Thr 202/Tyr 204) (Figures 6a and b).

Knockdown of Pak2, on the other hand, resulted in reduced phosphorylation of Mek1 (Ser 298) and Akt, whereas phosphoryation levels of c-Raf (Ser 338), Mek1/2 (Ser 217/221) and Erk were unchanged (Figures 6a and b). Cells transfected with an unrelated knockdown construct showed normal phosphorylation levels (Figures 6a and b).

These data suggest preferential functions for Pak1 and Pak2. Pak1 regulates Erk activation by controlling phosphorylation of Mek1/2 (Ser217/221), whereas Akt signaling is mainly regulated by Pak2. Pak2-dependent phosphorylation of Mek1 (Ser298) is neither required nor sufficient for Erk activation *in vitro*.

Suprabasal Pak2 expression correlates with suprabasal Akt activation

As the knockdown experiments *in vitro* indicated specific functions for Pak1 and Pak2, we wanted to test whether their expression *in vivo* is regulated by DMBA/TPA treatment and whether Pak1 and Pak2 are differentially distributed in the epidermis.

Analyzing expression of Pak1, Pak2 and Akt in DMBA/TPA-treated and -untreated control and Rac1-null epidermis, we found similar expression levels in all samples (Figure 7a), indicating that DMBA/TPA- and Rac1-dependent effects on hyperproliferation, Pak and Akt activation are not mediated by changes in gene expression.

We then assessed the localization of Pak1 and Pak2 in the epidermis. As immunofluorescent staining for Pak1 and Pak2 did not result in detectable signals, we sorted primary keratinocytes of untreated skin of wild-type mice into a basal (α6 integrin high) and a suprabasal (α6 integrin low) fraction using fluorescence-activated cell sorting and analyzed the expression of Pak1, Pak2, Akt and pAkt (serine 473) by western blot.

Although Pak1 and Akt were similarly expressed in both fractions, Pak2 was specifically expressed in the

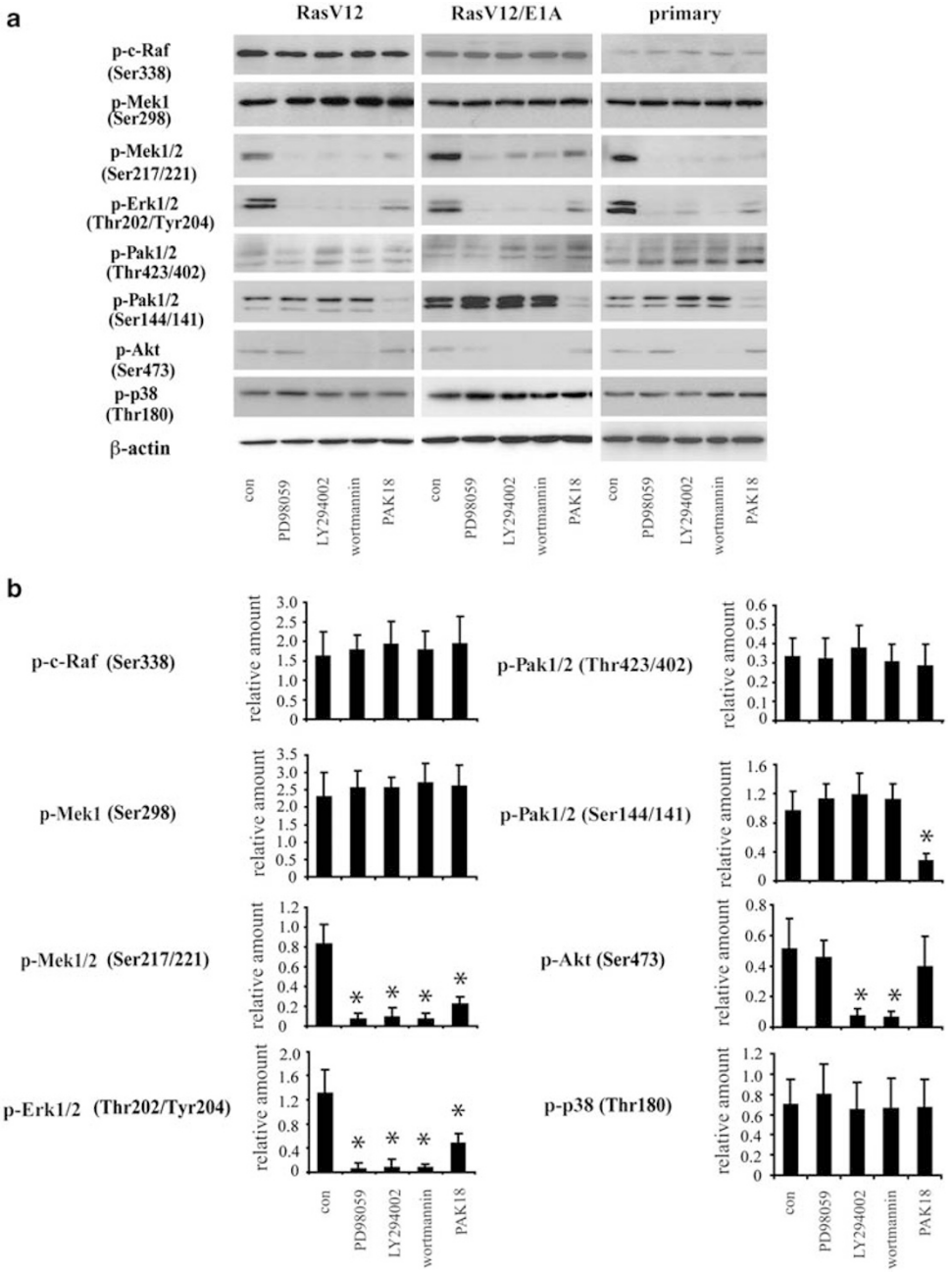

Figure 5 Growth signaling pathways of primary and Ras V12-transformed keratinocytes. Primary and RasV12-transformed keratinocytes were treated with the indicated inhibitors (refer Figure 4) for 45 min and then lysed. (**a**) Western blot analysis of cell lysates shows amount of phosphorylation of c-Raf (Ser 338), b-Raf (Ser 445), Mek1 (Ser 298), Mek1/2 (Ser 217/221), Erk1/2 (Thr 202/Tyr 204), Pak1/2 (Ser 144/141), Pak1/2 (Thr423/402), Akt (Ser 338) and p38 (Thr180). Representative blots from three independent experiments are shown. (**b**) Quantification of western blot analysis from RasV12-transformed keratinocytes ($n=3$; $^*P<0.01$).

suprabasal fraction (Figure 7b). Moreover, Akt was significantly more activated in the suprabasal cells as determined by pAkt (serine 473) levels, suggesting that Pak2 expression is important for Akt activation *in vivo*.

Finally, we investigated whether the suprabasal-specific activation of Akt is only observed in hyperproliferative normal epidermis, or whether this feature is also present in tumor tissue. Indeed, we observed that also in DMBA/TPA-induced papillomas of control mice, active Akt (pAkt (serine 473)) is restricted to the suprabasal, α6 integrin low-keratinocyte layers (Figure 7c), indicating that Pak2-mediated Rac1 signaling is crucial for Akt activation in tumors.

Discussion

This study shows a crucial role of the small GTPase Rac1 for epidermal hyperplasia and skin tumor

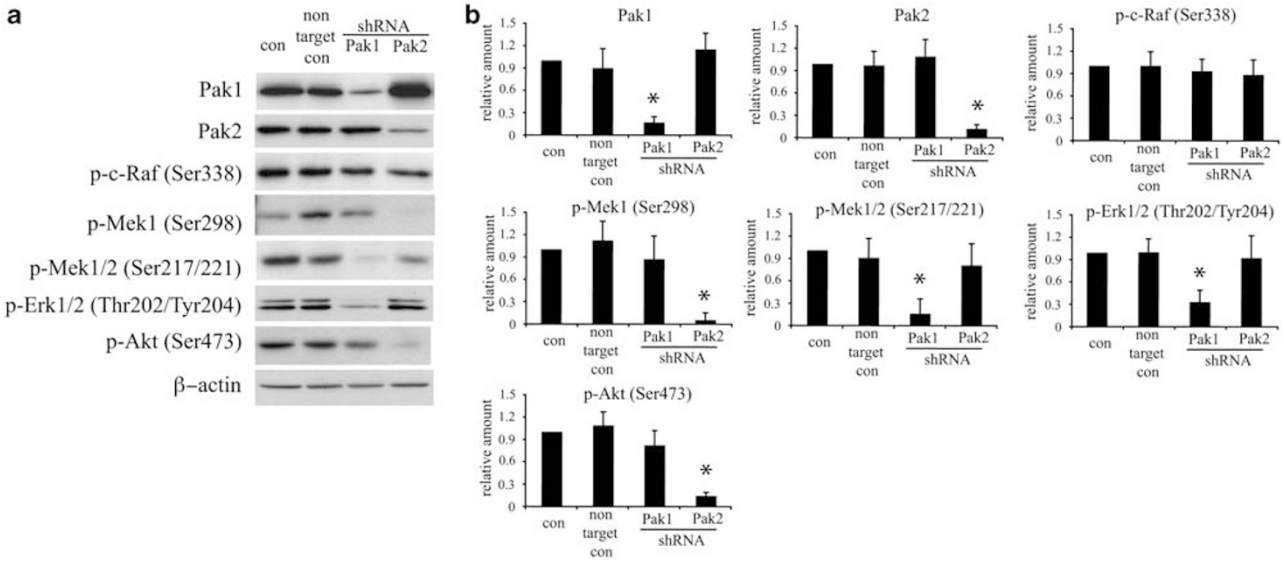

Figure 6 Pak1 and Pak2 differentially regulate Erk and Akt activation. Primary keratinoytes were knocked down for Pak1, Pak2, a nontarget control (nontarget con) or left unmodified (con) and then lysed. (**a**) Western blot analysis of cell lysates for p-Pak1/2 (Ser 144/141), p-Pak1/2 (Thr 423/402), p-c-Raf (Ser 338), p-Mek1 (Ser 298), p-Mek1 (Thr 292), p-Mek1/2 (Ser 217/221) and p-Erk1/2 (Thr 202/Tyr 204), and p-Akt (Ser 473). Representative blots from three independent experiments are shown. (**b**) Quantification of western blot analysis ($n=3$; $^*P<0.01$).

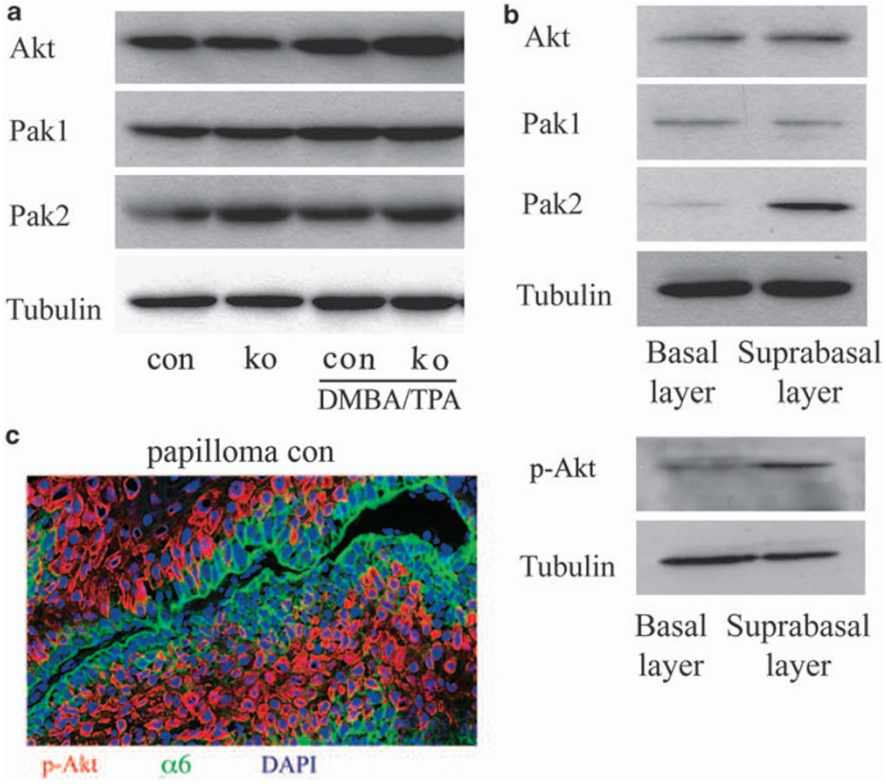

Figure 7 Suprabasal Pak2 expression correlates with suprabasal Akt activation. (**a**) Epidermal lysates from untreated and DMBA/TPA-treated control and Rac1-mutant mice were tested in western blot for expression of Pak1, Pak2 and Akt. Representative blots from three independent experiments are shown. (**b**) Lysates from basal (α6 integrin high) and suprabasal (α6 integrin low) primary keratinocytes from wild-type mice were tested in western blot for expression of Pak1, Pak2, Akt and pAkt (serine 473). Representative blots from two independent experiments are shown. (**c**) Cryosection of DMBA/TPA-induced papilloma of control mice stained for α6 integrin (α6; green) and pAkt (serine 473) (red). DAPI (blue) counterstaining indicates the nuclei.

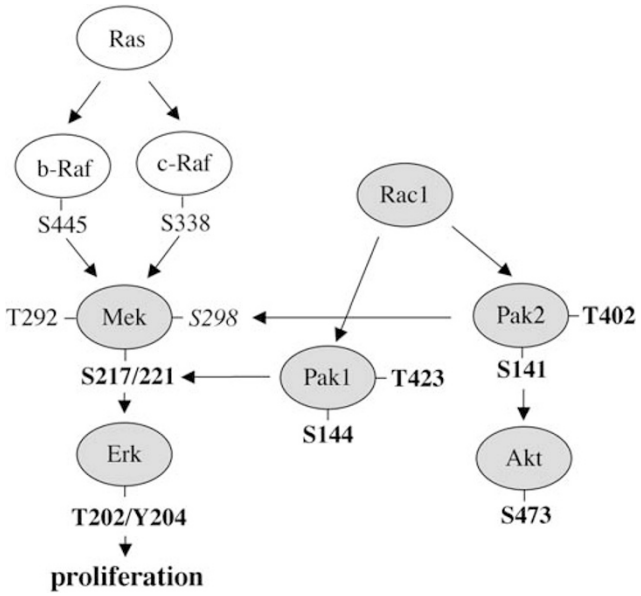

Figure 8 Schematic presentation of Rac1 signaling in hyperproliferative keratinocytes. Rac1 is crucial for keratinocyte hyperproliferation by regulation of Erk signaling through Pak1-mediated control of Mek1/2 (serine 217/221) phosphorylation. Rac1 is important for Akt hyperactivation by controlling Pak2 activation. Pak2-mediated phosphorylation of Mek1 (serine 298) is neither required nor sufficient for Mek activation and was not Rac1 dependent *in vivo* (gray filling: Rac1-dependent signaling; bold: Rac1-dependent phosphorylation sites; italics: Pak2 dependent *in vitro*, Rac1 independent *in vivo*).

formation in mice. This hyperproliferation-promoting effect of Rac1 is dependent on the hyperactivation of the Pak1-Mek-Erk cascade, and independent of the Pak2-mediated phosphorylation of Mek1 at serine 298 (Figure 8). Pak2, on the other hand, is important for Akt activation restricted to suprabasal layers.

Rac1 in skin tumors

Rac1 is overexpressed in several human tumors including oral squamous cell carcinoma, suggesting it to be important for formation and progression of solid tumors. Indeed, altering Rac1 activation in a colon cancer cell line regulated tumor formation in mice (Espina *et al*., 2008) and deletion of the *Rac1* gene in lung epithelium inhibited K-Ras-induced lung tumors (Kissil *et al*., 2007). The manner how Rac1 affects proliferation and apoptosis during tumorigenesis *in vivo*, however, was not studied. We show now that Rac1 is crucial for DMBA/TPA-induced keratinocyte hyperproliferation *in vivo*, although it is dispensable for normal proliferation (Chrostek *et al*., 2006). Our data therefore indicate that Rac1 activation contributes to skin tumor formation by controlling keratinocyte hyperproliferation *in vivo*. Previously, wound-induced keratinocyte hyperproliferation was shown to be dependent on Rac1 (Tscharntke *et al*., 2007), and it is therefore possible that Rac1 is important for keratinocyte hyperproliferation in general.

It was reported earlier that inhibition of Rac1 increased apoptosis of primary keratinocytes under starving conditions *in vitro* (Mertens *et al*., 2005). *In vivo*, however, we did not detect increased apoptosis in Rac1-deficient keratinocytes, neither in DMBA/TPA-treated nor in untreated mice. These data do not support a role for Rac1-mediated cell survival in tumor formation, although this could be relevant during tumor progression.

Rac1 signaling during hyperproliferation in vivo

Many *in vitro* studies have implicated Rac1 as an important regulator of proliferation-regulating pathways such as the Ras-Erk cascade, PI3K, p38 and JNK. However, as these investigations most often used transformed cells in cultures lacking normal three-dimensional organization, extracellular matrix, and interactions with other cells, used exogenous overexpression, and starving/stimulation schemes, and frequently described transient effects, the physiological significance of these results is not clear. We tested now in keratinocytes *in vivo* which signaling pathways are affected by the loss of Rac1. Most surprisingly, we found no significant alteration in the activation of the Ras-Erk cascade, Akt, Pak, JNK and p38 in untreated Rac1-null skin, indicating that Rac1 is dispensable for all these pathways under physiological conditions, contrary to the expectations. One explanation for this could be that other molecules compensate for the loss of Rac1, although no obvious upregulation of other Rho GTPases was observed in microarray gene expression analysis. This lack of apparent signaling defects fitted well to the unchanged proliferation and apoptosis of Rac1-null keratinocytes in untreated skin.

The situation drastically changed when we stimulated the Ras-Erk cascade, Akt, Pak1/2, JNK and p38 by treatment of the skin with DMBA/TPA. Under these conditions, increased activation of Erk, Akt, Pak1/2 and p38 was strongly dependent on Rac1, whereas hyperactivation of JNK was not altered in the absence of Rac1. As p38 is known to be antiproliferative and proapoptotic, decreased activation of p38 is unlikely to explain the reduced proliferation of Rac1-null keratinocytes *in vivo*. In addition, reduced Akt activation was unlikely to contribute to decreased proliferation of Rac1-deficient basal keratinocytes, as DMBA/TPA-induced Akt hyperactivation was confined primarily to the suprabasal keratinocyte layers. Erk activation is known to promote cell proliferation and, indeed, inhibition of Erk activation blocked growth of primary and H-RasV12-transformed keratinocytes *in vitro*, suggesting that Rac1 facilitates hyperproliferation *in vivo* by enabling hyperactivation of Erk.

Our study furthermore elucidates the manner how Rac1 regulates the Ras-Erk cascade in keratinocytes *in vivo*. In skin, loss of Rac1 blocked the DMBA/TPA-induced hyperphosphorylation of Mek1/2 (Ser 217/221) and Erk, but had no effect on phosphorylation of c-Raf (Ser 338), b-Raf (Ser 445), Mek1 (Ser 298) and Mek 1 (Thr 292). *in vitro*, Pak inhibitor PAK18 or knockdown

of Pak1 inhibited Erk activation *in vitro* by regulating Mek1/2 (Ser 217/221) phosphorylation without affecting phosphorylation of Mek1 (Ser 298) or c-Raf (Ser 338).

These data suggest that neither Rac1-dependent regulation of c-Raf (Ser 338) (King *et al*., 1998), Mek1 (Ser 298) (Slack-Davis *et al*., 2003), complex formation between Mek and Erk (Eblen *et al*., 2002), complex formation between Pak and Erk (Sundberg-Smith *et al*., 2005), nor regulation of nuclear translocation of activated Erk (Hirsch *et al*., 2002) are the rate-limiting steps of Rac1 regulation of activation *in vivo*, but rather control of Mek1/2 (Ser217/221) hyperphosphorylation. Rac1-controlled Pak1 activation seems to function as a gatekeeper allowing activated Raf to interact with Mek1/2. One possibility is that Rac1 is recruiting Mek through Pak1 to the membrane, thus facilitating phosphorylation by Raf, which is membrane associated through Ras. Alternatively, Pak1 might phosphorylate Raf or Mek at a yet unknown site, which could promote interaction.

Rac1 mediates Akt activation during hyperpoliferation in vivo

Akt signaling promotes cell proliferation and cell survival by different mechanisms. In mice, Akt activation promotes tumor formation (Segrelles *et al*., 2002; Skeen *et al*., 2006) and, in humans, Akt activation by loss of PTEN corresponds to increased formation of skin lesions including benign tumors (Cowden's disease). Using immunofluorescent staining of cryosections, we could detect increased Akt activation following DMBA/TPA treatment in suprabasal, but not in basal keratinocytes. These findings support a role for Akt in the survival and proliferation of squamous, but not of basal cells. Akt hyperactivation was dependent on Rac1, suggesting that Rac1 function might be important in squamous cell survival in skin carcinoma.

in vitro data suggested that Rac1 controls Akt activation primarily through Pak2, as knockdown of Pak2 in keratinocytes abolished Akt activation, whereas knockdown of Pak1 displayed no obvious effect. Partial inhibition of Pak activity by PAK18 did not alter Akt activation, which may be due to a kinase-independent mode of Akt activation by Pak as reported recently (Higuchi *et al*., 2008). The preferential control of Mek activation by Pak1 and of Akt activation by Pak2 suggests that cell type-specific expression of Pak1 and Pak2 will result in cell type-specific effects of Rac1 on Mek1 and Akt, respectively. *in vivo*, suprabasal-specific Pak2 expression correlated with suprabasal-specific Akt activation, indicating an important role for Pak2 in controlling Akt activation *in vivo*.

In vitro, inhibitor and knockdown experiments indicated that PI3K contributes to Erk activation in a Pak- and Akt-independent manner. This mechanism might have a role in the proliferation of suprabasal keratinocytes *in vivo*.

With respect to Rac1 function, our data indicate a crucial role for Rac1 in Pak2-mediated Akt activation during hyperplastic situations.

Conclusion

This study demonstrates the necessity of analyzing signaling pathways *in vivo* to understand the relative importance of the different effector mechanisms of multifunctional proteins like Rac1 in complex diseases such as tumor formation.

Our data suggest that Rac1 is an excellent drug target for inhibiting skin hyperproliferation by blocking hyperactivation of Erk and Akt. Blocking kinase activity of the Rac1 effector, Pak1, will specifically reduce Erk-mediated hyperproliferation, whereas inhibition of Pak2 interferes with Akt activity. As complete loss of Rac1 does not alter normal proliferation and apoptosis in the epidermis, Rac1 is an ideal drug target for skin tumor and conceivably also other Ras-dependent cancers.

Materials and methods

Skin tumor induction

Mice with a keratinocyte-restricted deletion of Rac1 (Rac1 fl/fl K5 cre) and littermate controls expressing Rac1 with a 129Sv/C57BL/6 outbred background (Chrostek *et al*., 2006) were treated with DMBA and TPA to induce skin tumors (Perez-Losada and Balmain, 2003). In brief, the backs of 8-week-old mice were shaved and 24 h later 50 μg DMBA (Sigma, St Louis, MO, USA) in 200 μl acetone was applied topically on the shaved area of the dorsal skin. After a week, the back skin of the mice was treated twice a week with 5 μg TPA (Sigma) in 200 μl acetone for 30 weeks. 'Untreated' mice received acetone, but neither DMBA nor TPA. Tumors were counted twice a week.

Histology, BrdU, TUNEL and immunofluorescent staining

Samples of back skin were collected and processed as previously described (Chrostek *et al*., 2006). Hematoxylin/eosin staining of 6 μm thick paraffin sections was performed according to standard protocols. Proliferation and apoptosis of keratinocytes in the epidermis was analyzed by BrdU and TUNEL staining, respectively, as described earlier (Chrostek *et al*., 2006).

Immunofluorescent staining was performed on 10 μm thick cryosections of back skin according to standard protocols. In brief, slides were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 2% BSA before staining with primary antibodies. The following primary antibodies were used: p-c-Raf (Ser 338), p-Mek1/2 (Ser 217/221), p-Erk1/2 (Thr 202/Tyr204), p-Akt (Ser 473), cleaved caspase-3 (Asp 175) (all from Cell Signaling Technology, Danvers, MA, USA), p-p38 (Thr180) (BD Transduction Laboratories, San Jose, CA, USA) and CD49f (integrin α6 chain) directly coupled to fluorescein isothiocyanate (BD Pharmingen, San Jose, CA, USA). Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used as a secondary reagent. Nuclear DNA was visualized with DAPI (Sigma). Images were collected with a confocal microscope (Leica Microsystems, Wetzlar, Germany) using Leica Microsystems confocal software (version 2.61 Build 1537).

Preparation and analysis of epidermal lysates

Skin was dissected from killed, shaved mice, and dermis and fat were removed with a scalpel on ice. Epidermis was lysed in ice-cold lysis buffer (150 mm NaCl, 1% Triton X-100, 1 mm EDTA, 1 mM NaF, 1 mM Na_3VO_4, 50 mM Tris, pH 7.6)

containing protease inhibitor cocktail tablets (Complete Mini, EDTA free; Roche, Basel, Switzerland). Western blotting was performed according to standard protocols. The following primary antibodies were used: p-c-Raf (Ser 338), p-b-Raf (Ser445), p-Mek1 (Ser 298), p-Mek1/2 (Ser 217/221), p-Erk1/2 (Thr 202/Tyr 204), p-Akt (Ser 473), p-Pak1/2 (Ser 144/141), p-Pak1/2 (Thr 423/402), p-SAPK/JNK (Thr 183/Tyr185), (all from Cell Signaling Technology), p-p38 (Thr 180) (BD Transduction Laboratories), p-Mek1 (Thr 292) (Sigma) and β-actin (Abcam, Cambridge, MA, USA). Secondary reagents used were horseradish peroxidase-coupled goat anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch Laboratories). Western blot results were quantified using TotalLab TL100 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) and β-actin was used to normalize for different protein amounts.

Keratinocyte culture
Primary keratinocytes were isolated from adult mice and cultured as described previously (Chrostek *et al*., 2006). Primary keratinocytes from Rac1 (fl/fl) mice were retrovirally transformed with either H-*Ras* V12 or H-*Ras* V12/E1A (vectors kindly obtained from Dr Anders Lund, BRIC, Copenhagen) and selected with 1 μg/ml puromycin (Sigma) for 14 days.

To test the effect of different inhibitors on signaling, keratinocytes were treated for 45 min with 50 μM PD98059, 50 μM LY294002, 50 nM wortmannin and 50 μM PAK18 (all from Calbiochem, Gibbsatown, NJ, USA). Afterward, cells were lysed and analyzed as described above for epidermal lysates.

To test the effect of different inhibitors on proliferation, cells were seeded in 96-well plates incubated with inhibitors for 2 days. Cell numbers were then determined by crystal violet staining. In brief, cells were fixed with 70% ethanol in phosphate-buffered saline and stained with 0.5% crystal violet in 20% methanol for 15 min at room temperature. Excess dye was washed away and cell-bound crystal violet was extracted with 10% SDS. Absorbance at 570 nm was measured using a microplate reader.

To fractionate basal and suprabasal cells, freshly prepared primary keratinocytes were stained with fluorescein isothiocyanate-conjugated anti-mouse α6 integrin antibody (BD Pharmingen) and then sorted for α6 integrin high and low populations on a FACSaria flow cytometer.

Immunoprecipitation
NIH 3T3 cells were retrovirally infected with myc-tagged Rac1. Cell were lysed, immunoprecipitated with anti-myc antibody (Upstate, Billerica, MA, USA) or a control antibody (TCR, eBioscience, San Diego, CA, USA), and analyzed by western blotting as described above.

Knockdown
Primary keratinocytes were transfected with lentiviral expression vectors containing nontarget control short-hairpin RNA (shRNA) or shRNA targeting Pak1 (NM-011035.1-1108S1C1) or Pak2 (NM-177326.1-686S1C1; all from Sigma-Aldrich). Transfection of shRNA was performed using the TransIT Keratinocyte Transfection Reagent (Mirus, Madison, WI, USA) according to the manufacturer's instructions. At 2 days after transfection, keratinocytes were selected with 1 μg/ml puromycin for 14 days. Knockdown was confirmed by western blotting for each experiment.

Statistics
Averages are shown with s.d. Significance was calculated by Student's *t*-test.

Conflict of interest

The authors declare no conflict of interest.

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