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Activated Mutant NRas^{Q61K} Drives Aberrant Melanocyte Signaling, Survival, and Invasiveness via a Rac1-Dependent Mechanism

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Around a fifth of melanomas exhibit an activating mutation in the oncogene NRas that confers constitutive signaling to proliferation and promotes tumor initiation. NRas signals downstream of the major melanocyte tyrosine kinase receptor c-kit and activated NRas results in increased signaling via the extracellular signal–regulated kinase (ERK)/MAPK/ERK kinase/mitogen-activated protein kinase (MAPK) pathways to enhance proliferation. The Ras oncogene also activates signaling via the related Rho GTPase Rac1, which can mediate growth, survival, and motility signaling. We tested the effects of activated NRas^{Q61K} on the proliferation, motility, and invasiveness of melanoblasts and melanocytes in the developing mouse and *ex vivo* explant culture as well as in a melanoma transplant model. We find an important role for Rac1 downstream of NRas^{Q61K} in mediating dermal melanocyte survival *in vivo* in mouse, but surprisingly NRas^{Q61K} does not appear to affect melanoblast motility or proliferation during mouse embryogenesis. We also show that genetic deletion or pharmacological inhibition of Rac1 in NRas^{Q61K} induced melanoma suppresses tumor growth, lymph node spread, and tumor cell invasiveness, suggesting a potential value for Rac1 as a therapeutic target for activated NRas-driven tumor growth and invasiveness.

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INTRODUCTION

Mutations in the Ras proto-oncogene family (HRas, NRas, and KRas) are among the most frequently mutated genes in human cancers, occurring in 20–30% of human tumors (Downward, 2003). Residue G12 in the phosphate-binding loop and the catalytic residue Q61 are most commonly mutated (Malumbres and Barbacid, 2003). Constitutively active NRas^{Q61K} is frequently found in nevi and early-stage melanomas (Platz *et al.*, 2008). Expression of human NRas^{Q61K} in the mouse melanocyte lineage results in hyperpigmented skin (Ackermann *et al.*, 2005), which can progress to cutaneous melanoma on an INK4a-deficient background.

The mitogen-activated protein kinase pathway, modulated by Raf directly interacting with Ras, is a key downstream effector in Ras signaling (Cook and McCormick, 1994; Marshall, 1995; Gray-Schopfer *et al.*, 2007). However, the recruitment of other Ras targets is indispensable to elicit a full Ras biological response. Among these Ras-dependent, Raf-independent pathways are those connecting Ras to the Rho subfamily of small GTPases. There are no published reports of activating Rho-family mutations in human tumors, but Rho-family proteins often exhibit upregulation of their activity in tumors (Sahai and Marshall, 2002).

The small GTPase Rac1 is required for cell cycle regulation and Ras-induced transformation *in vitro* (Qiu *et al.*, 1995), but relatively little is known about its role in cancer *in vivo*. Rac1 is required for KRas^{G12D}-driven formation of lung tumors (Kissil *et al.*, 2007) and oral papillomas (Samuel *et al.*, 2011). However, activated Ras isoforms can exert profoundly different effects in different forms of cancer (Hancock, 2003; Whitwam *et al.*, 2007; Karreth and Tuveson, 2009). Here, we show that Rac1 activity is required for NRas^{Q61K}-induced dermal melanocyte survival *in vivo* and for increased invasiveness conferred by NRas^{Q61K} on primary melanocytes *in vitro and in vivo*. In addition, genetic deletion or pharmacological inhibition of Rac1 in NRas^{Q61K}-expressing melanoma tumors suppressed tumor growth and lymph node spread. Thus, many of the crucial downstream effects of NRas^{Q61K} in melanoma are likely to be mediated by Rac1 and implicate Rac1 and its downstream partners as potential key targets for melanoma therapy.

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Abbreviations: AIG, anchorage-independent growth; OHT, 4-hydroxytamoxifen

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RESULTS

Rac1 is required for anchorage-independent growth (AIG) of $NRas^{Q61K}$-expressing primary murine melanocytes in culture

In our recent study (Li *et al.*, 2011), we generated two independent immortalized Rac1 f/f $Ink4a^{-/-}$ Tyr::$CreERt2^{+/o}$ melanocyte cell lines #3 and #4, which were unable to grow in soft agar (Figure 1c–e). To test the importance of Rac1 downstream of $NRas^{Q61K}$ in oncogenic transformation for melanocytes, we generated melanocyte cell lines from 1-day-old Tyr::$NRas^{Q61K+/o}$ Rac1 f/f $Ink4^{-/-}$ Tyr::$CreERt2^{+/o}$ (#10 and #11) and Tyr::$NRas^{Q61K+/o}$ Rac1 f/f $Ink4a^{-/-}$ Tyr::$\ominus$ $CreERt2^{o/o}$ (#5) littermate mice (also littermate of #3 and #4). As expected, $NRas^{Q61K+/o}$-expressing melanocytes (#5, #10 and #11) have elevated activation of mitogen-activated protein kinase signaling compared with Rac1 f/f $Ink4a^{-/-}$ Tyr::$CreERt2^{+/o}$ melanocyte cell lines #3 and #4 (Figure 1a; Ackermann *et al.*, 2005; Whitwam *et al.*, 2007; Li *et al.*, 2011), but Rac1 expression level was not affected by expression of $NRas^{Q61K}$ (Figure 1a). Rac1 deletion was induced with tamoxifen analog 4-hydroxytamoxifen (OHT) in cell lines that express CreERt2 (#10 and #11), but not in cell line #5, which is CreERt2 negative (Figure 1b). Neither Rac2 nor Rac3 was detectable, regardless of Rac1 deletion (Figure 1b). All the $NRas^{Q61K}$-expressing melanocyte cell lines (#5, #10 and #11) were able to induce rapid AIG in soft agar and form large colonies (Figure 1c–e). Rac1 deletion reduced colony number by about 80% and the average colony size by about 60% (Figure 1c–e). Rac1-deleted $NRas^{Q61K}$-expressing melanocytes additionally showed reduced proliferation (Supplementary Figure S1A online). 12-*O*-Tetradecanoylphorbol 13-acetate acts as a growth factor for cultured melanocytes, but is not required for growth when melanocytes are transformed by constitutively active c-kit receptor (Larue *et al.*, 1992). Similarly, 12-*O*-tetradecanoylphorbol 13-acetate is not required for $NRas^{Q61K}$-expressing melanocytes growth on either 2D or in soft agar (Supplementary Figure S1 online). Thus, $NRas^{Q61K}$ confers Rac1-dependent AIG on primary melanocytes. This suggests an important, but previously undescribed, role for Rac1 in mediating oncogenic transformation induced by $NRas^{Q61K}$ in melanocytes.

$NRas^{Q61K}$ does not confer excess melanocyte accumulation in embryos, but loss of Rac1 leads to melanocyte proliferation defects

We next examined the number and position of melanoblasts in Tyr::$NRas^{Q61K}$-expressing embryos. Strikingly, Tyr::$NRas^{Q61K}$-expressing E15.5 embryos or P0.5 newborn pup skin did not show a statistically significant increase in the number of melanoblasts/melanocytes (Supplementary Figure S2 online). There was a significant reduction in melanoblast number in Rac1 f/f Tyr::Cre embryos regardless of the presence of Tyr::$NRas^{Q61K}$ (Supplementary Figure S2 online), indicating that $NRas^{Q61K}$ does not affect melanoblast numbers or position.

Expression of $NRas^{Q61K}$ causes Rac1-dependent hyperpigmentation in adult mice

Mice of the C57BL/6 strain normally have light skin and black fur, but when Tyr::$NRas^{Q61K}$ is expressed, they have black skin and darker fur (Ackermann *et al.*, 2005). Mice lacking Rac1 in melanocytes have white patches on the underside and along the dorsal midline and also a lightening of the paws and tails (Supplementary Figure S2C and D online; Li *et al.*, 2011). At P14, Tyr::$NRas^{Q61K}$ Rac1 f/f Tyr::Cre mice had similar pigmentation patterns to Rac1 f/f Tyr::Cre mice (Figure 2a–d and Supplementary Figure S3A and B online;Li *et al.*, 2011). Furthermore, the number and size of white patches on the dorsal skin between Tyr::$NRas^{Q61K}$ Rac1 f/f Tyr::Cre and Rac1 f/f Tyr::Cre mice was similar (Figure 2a and e and Supplementary Figure S3A online). Mice were born healthy at the expected Mendelian ratio. However, Tyr::$NRas^{Q61K}$ Rac1 f/f Tyr::Cre mice were smaller than control littermates and had a similar weight to Rac1 f/f Tyr::Cre as measured on P7 and P14 (Figure 2f and g and Methods). Histological analysis of P14 Tyr::$NRas^{Q61K}$ ventral skin revealed melanocytes in hair follicles, dermis and fatty tissue (Figure 2h). Thus, deletion of Rac1 in the melanocyte lineage from mice expressing $NRas^{Q61K}$ resulted in similar pigmentation defects as Rac1 deletion alone.

Expression of $NRas^{Q61K}$ promotes survival and accumulation of epidermal/dermal melanocytes via Rac1 over the hair cycle

Before birth, murine melanoblasts reside in the dermis and epidermis (Luciani *et al.*, 2011), but shortly after birth they reside only in hair follicles (Kelsh *et al.*, 2009). We investigated the origin of Tyr::$NRas^{Q61K}$ dermal melanocyte clusters (Figure 2h, arrow) and the effect of Rac1 loss during the first hair cycle, which is synchronous (Fuchs, 2007). Dorsal skin from control, Tyr::$NRas^{Q61K}$ control, Rac1 f/f Tyr::Cre and Tyr::$NRas^{Q61K}$ Rac1 f/f Tyr::Cre mice at P0.5, 3, 5, 14, and 21 was sectioned and stained with dopachrome tautomerase antibody to localize melanocytes. In control, Rac1 f/f Tyr::Cre and Tyr::$NRas^{Q61K}$ Rac1 f/f Tyr::Cre mice, melanocytes in epidermis and dermis gradually disappeared from P0.5 to P5 (Figure 3a, Supplementary Figure S4A and C online and data not shown), leaving only hair follicle melanocytes (Hirobe, 1984; Kelsh *et al.*, 2009). However, in Tyr::$NRas^{Q61K}$ control mice, melanocytes in epidermis survived from P0.5 and number of melanocytes in epidermis increased over the hair cycle (Figure 3a, Supplementary Figure S4B online), indicating that expression of $NRas^{Q61K}$ promotes Rac1-dependent postnatal melanocyte survival and/or growth in epidermis.

At P14, Tyr::$NRas^{Q61K}$ control (Figure 3c) and Tyr::$NRas^{Q61K}$ Rac1 f/f Tyr::Cre (Figure 3d) skin showed excess melanocytes in hair follicles (Figure 3b). However, in Tyr::$NRas^{Q61K}$ control mice, melanocytes also accumulated in clusters in the epidermis/dermis and fatty tissue (Figure 3c, arrows) but these were absent from Tyr::$NRas^{Q61K}$ Rac1 f/f Tyr::Cre mice (Figure 3d and e).

The transition from anagen to telogen leads to apoptosis of most follicular melanocytes (Sharov *et al.*, 2005). In the telogen phase of the first hair follicle cycle (P21), only the permanent part of the hair follicles remained in dorsal skin taken from control mice (Supplementary Figure S4A online). Most of follicular melanocytes remained in the dermis even

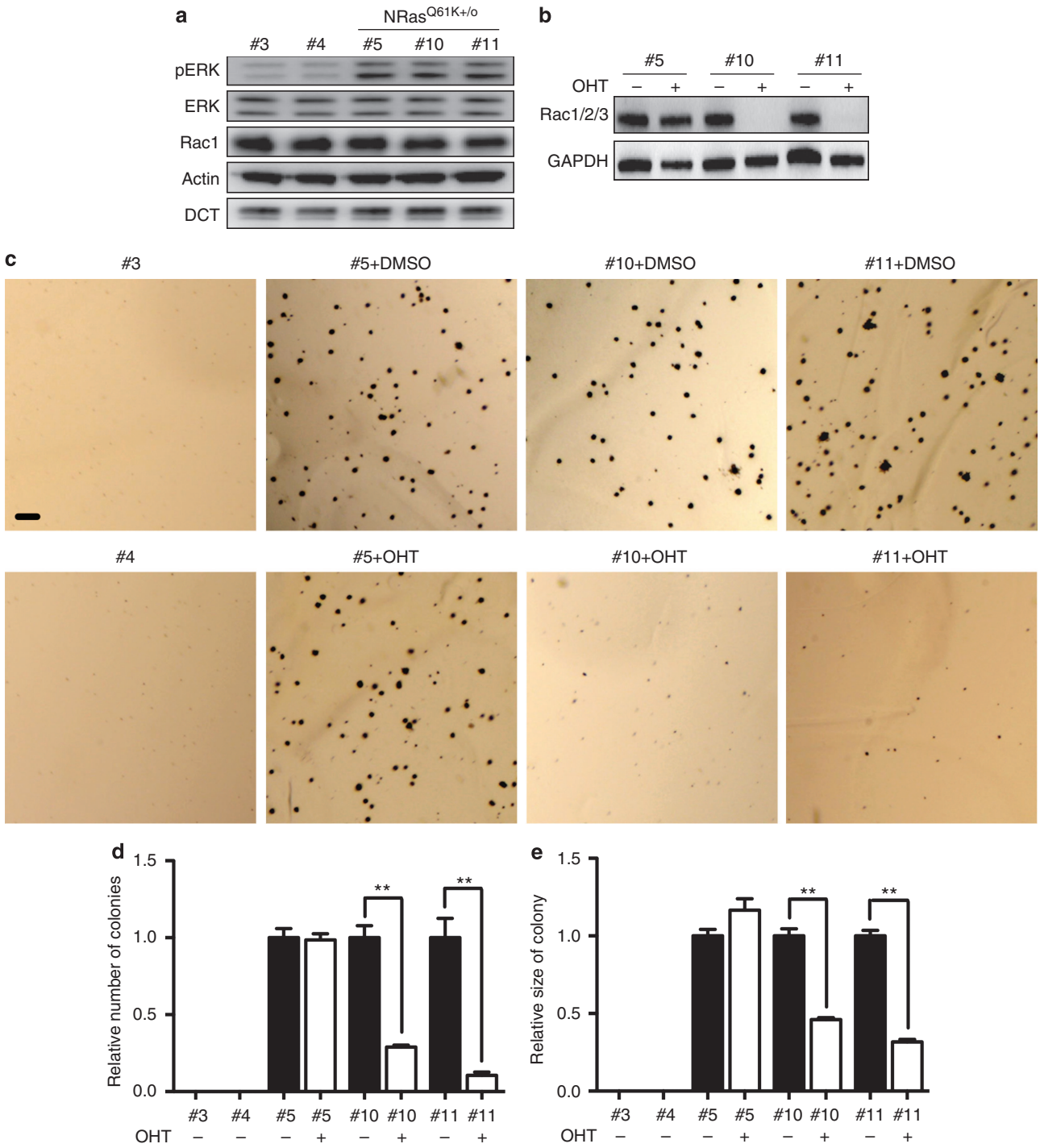


Figure 1. Rac1 is required for NRas^{Q61K}-induced anchorage-independent growth. (**a**) Western blots of primary melanocyte cell lines with antibodies as indicated. (**b**) Western blot of #5, #10, and #11 primary melanocyte cell lines treated with DMSO or 4-hydroxytamoxifen (OHT) for 5 days were probed with antibodies as indicated. (**c**) Representative images of primary melanocyte cell lines treated with DMSO or OHT were plated in soft agar in the presence of 200 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) for 2 weeks. (**d**) Relative number of colonies and (**e**) relative size of each colony in the presence of 200 nM TPA. All error bars show mean ± SEM from three independent experiments. Bar = 500 μm; $^{**}P<0.01$ by *t*-test. DCT, dopachrome tautomerase; ERK, extracellular signal–regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

after the hair follicles in Tyr::NRas^{Q61K} mouse degenerated (Figure 3f and Supplementary Figure S4B online, arrows). Which, however, failed to be maintained when Rac1 was deleted (Figure 4f, Supplementary Figure S4C online). Thus, NRas^{Q61K} protects dermal melanocytes from being cleared from the skin in a Rac1-dependent manner.

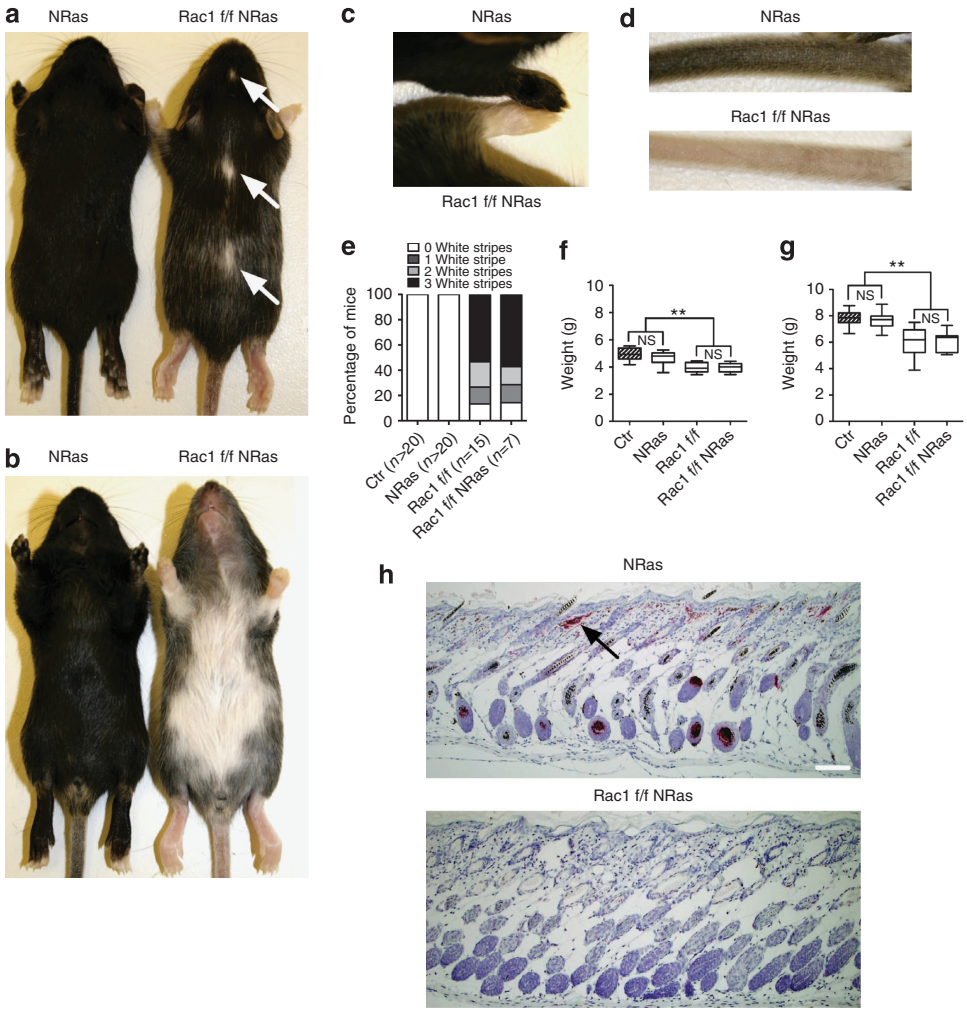


Figure 2. Mice expressing melanocyte lineage NRas^{Q61K} retain pigmentation defects caused by Rac1 deletion. Coat color of dorsal (**a**) and ventral side (**b**) of P14 Tyr::NRas$^{Q61K+/o}$ Rac1 f/f Tyr::Cre$^{+/o}$ (Rac1 f/f NRas) mouse with Tyr::NRas$^{Q61K+/o}$ (NRas) control (Ctr) littermate. Typical dorsal white patches Rac1 f/f NRas mouse (arrows). Coat color of forelimb (**c**) and tail (**d**) of Rac1 f/f NRas mouse with NRas Ctr littermate. (**e**) Quantification of white patches on dorsal side of mice. Body weight of mice at P7 (**f**) and P14 (**g**); $n=6$ mice from three different litters. Lower, median, and upper quartile are shown. (**h**) Ventral skin from P14 NRas Ctr and Rac1 f/f NRas mice with anti-dopachrome tautomerase (melanocytes). Typical cluster of dermal melanocytes in NRas Ctr mouse is shown with an arrow. $^{**}P<0.01$ compared with Ctr by *t*-test. Bar = 100 μm. NS, not significant.

NRas^{Q61K} does not affect the motility of melanoblasts in embryonic skin or their ability to cross the embryonic dermal/epidermal basement membrane

Melanoma metastasis has analogies with embryonic development (Hendrix *et al.*, 2007; Yang and Weinberg, 2008). NRas^{Q61K} is frequently found in human metastatic melanoma (Demunter *et al.*, 2001), thus may contribute to abnormal invasiveness and migration of melanoma cells (Mishra *et al.*, 2010). We studied the motility of melanoblasts in the epidermis of embryo skin explants by live imaging (Mort *et al.*, 2010). We asked whether NRas^{Q61K} could affect melanoblast motility and whether this was Rac1 dependent, as Rac1 is important for speed and protrusion formation of melanoblasts (Li *et al.*, 2011). Melanoblasts expressing NRas^{Q61K} had a similar morphology to control melanoblasts, showing both long and short protrusions (Figure 4a) in the same proportions (Figure 4g and h). NRas^{Q61K}-expressing melanoblasts extended dynamic short protrusions that elongated and often dictated the direction of migration (Figure 4c yellow arrows and Supplementary Movie S1 online). Loss of Rac1 in NRas^{Q61K}-expressing melanoblasts produced a phenotype indistinguishable from Rac1 null melanoblasts, with short stubby protrusions leading migration but rarely elongating (Figure 4c, yellow arrows, Supplementary Movie S1 online). Furthermore, by labeling melanoblasts with lifeact-GFP (Li *et al.*, 2011), we also confirmed that the short stubby protrusions in Tyr::NRas^{Q61K} Rac1 f/f Tyr::Cre melanoblasts were actin-rich spiky protrusions and not bleb-based (Figure 4d, yellow arrows, Supplementary Movie S2 online). Expression of activated NRas^{Q61K} did not measurably affect the migration speed (Figure 4b and e), persistence (Figure 4f), lifetime (Figure 4i), or frequency of protrusions (Figure 4j).

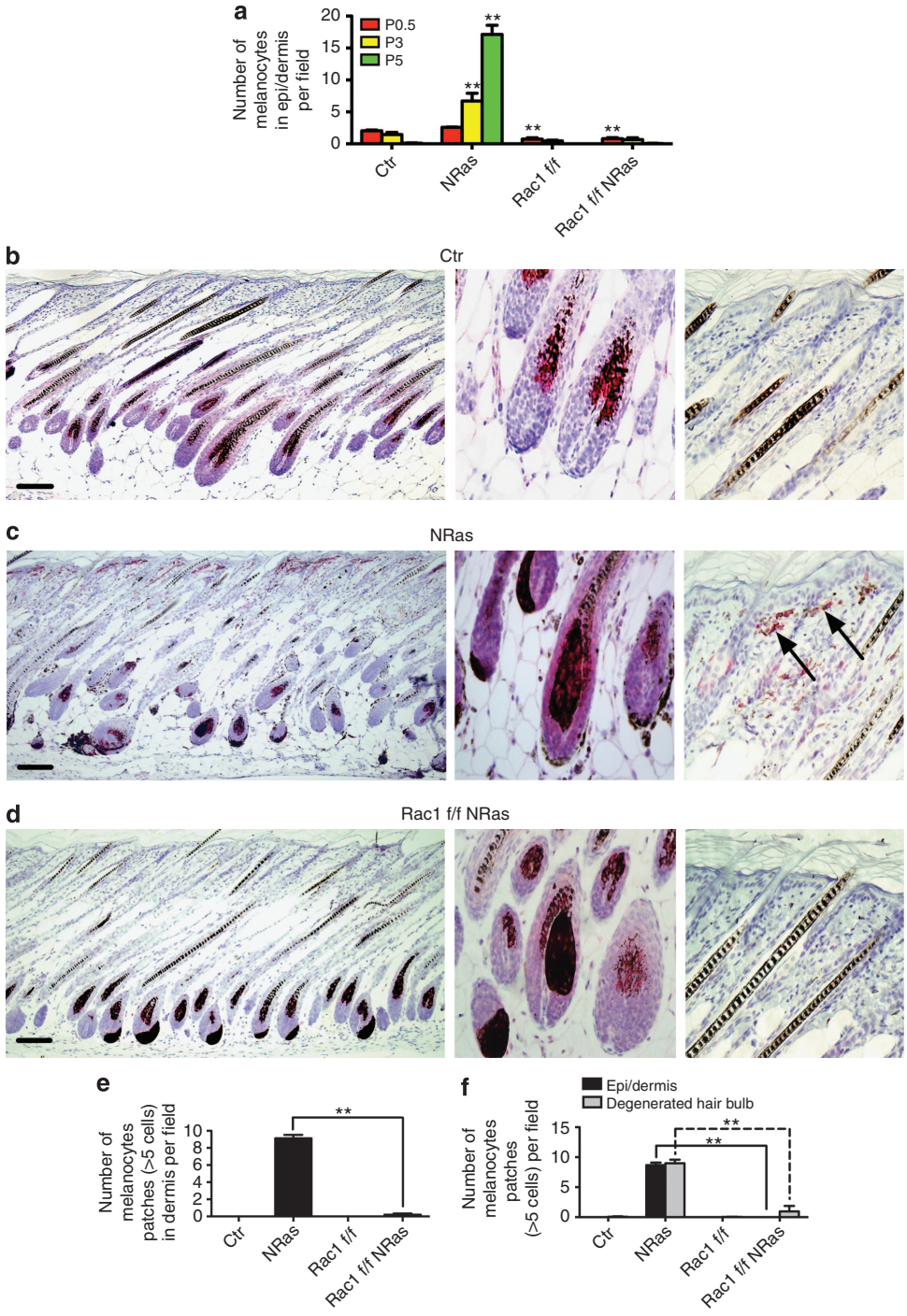


Figure 3. Rac1 is required for NRas^{Q61K}-induced survival of murine dermal melanocytes. (**a**) Number of melanocytes in epi/dermis at P0.5, P3, and P5 per field (× 10 objective) from (⩾3 pups, 3 litters). Dorsal skin from P14 control (Ctr) (**b**), NRas Ctr (**c**), and Rac1 f/f NRas (**d**) mice with anti-dopachrome tautomerase (melanocytes). High-magnification images of hair follicle and dermis shows typical cluster of excess dermal melanocytes in NRas mouse (arrow). (**e**) Number of patches (⩾5 cells) of melanocytes in dermis per field at P14 (from ⩾3 pups, 3 litters). (**f**) Number of patches (⩾5 cells) of melanocytes in dermis or former hair bulb per field at P21 (from ⩾3 pups, 3 litters). $^{**}P<0.01$ by *t*-test. Bars = 100 μm.

Therefore, we conclude that NRas^{Q61K} does not affect melanoblast motility in any detectable way. Furthermore, there was no effect of expression of NRas^{Q61K} on the ratio of melanoblasts in the dermis versus epidermis (Figure 4k and l).

In summary, NRas^{Q61K} expression has no detectable effect on melanoblast motility or basement membrane crossing during embryogenesis, whereas signaling through Rac1 is limiting for melanoblast proliferation and motility.

Rac1 is required for NRas^{Q61K}-induced invadopodia formation and invasion

We asked whether expression of NRas^{Q61K} could enhance the ability of melanocytes to degrade extracellular matrix and whether matrix-remodeling capacity was Rac1 dependent. Invadopodia are actin-based membrane protrusions formed in invasive cancer cells that have a matrix degradation activity (Buccione *et al.*, 2004). They are rich in filamentous actin, cortactin, and Arp2/3 complex (Linder, 2007). To examine whether NRas^{Q61K} could induce invadopodia formation, wild-type melanocyte cell lines (#3 and #4) and NRas^{Q61K} melanocyte cell lines (#10 and #11) were cultured on cross-linked gelatin matrix. Wild-type melanocytes (#3 and #4) showed similar morphology compared with NRas^{Q61K}-expressing melanocyte cell lines, but did not display invadopodia (Figure 5a). In contrast, NRas^{Q61K}-expressing melanocyte cell lines (#5, #10 and #11) formed copious invadopodia (Figure 5b). We found ~45% of NRas^{Q61K}-expressing melanocytes and only 4% of normal melanocytes contained invadopodia (Figure 5c). Furthermore, the matrix degradation for individual cells was negligible in controls (#3 and #4) as compared with NRas^{Q61K}-expressing melanocytes (Figure 5d).

#10 and #11 NRas^{Q61K}-expressing melanocytes treated with OHT to induce Rac1 deletion showed about 60% reduction in the percentage of cells degrading matrix (Figure 5c) and 80% reduced degradation (Figure 5a and e). OHT treatment did not affect invadopodia formation by #5 melanocytes, which do not delete Rac1 (Figure 5d). In addition, OHT-treated #10 and #11 melanocytes failed to invade in organotypic assays (Figure 5f and g). We conclude that Rac1 is important for melanocyte invadopodia formation and invasion downstream of NRas^{Q61K}.

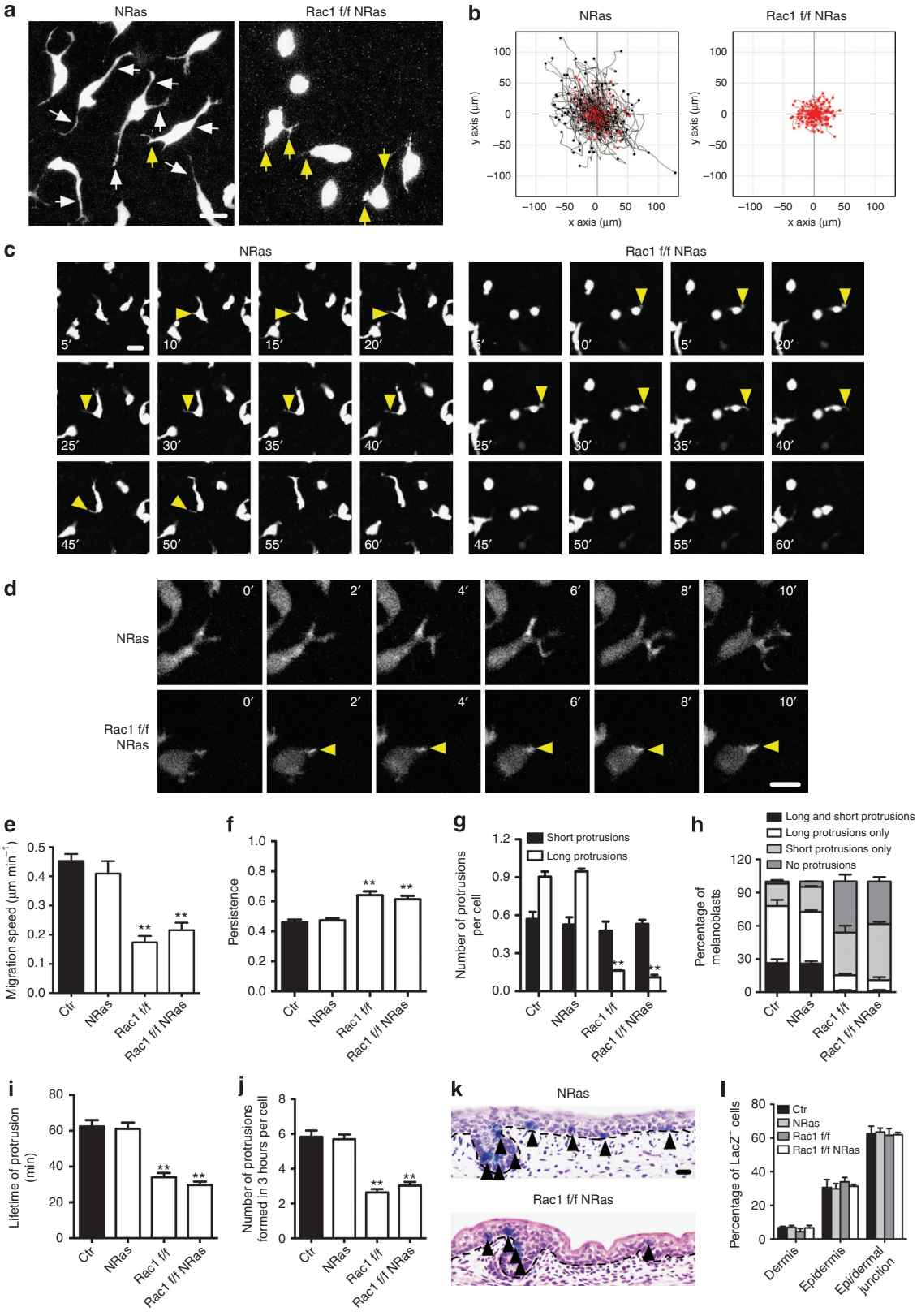
Genetic deletion or pharmacological inhibition of Rac1 impairs NRas^{Q61K}-induced melanoma tumor growth and formation of lymph node metastasis

Although Rac function is required for KRas^{G12D}-induced primary squamous cell skin and lung tumor initiation (Kissil *et al.*, 2007; Samuel *et al.*, 2011), the role of Rac1 in NRas^{Q61K}-induced tumor growth and invasiveness *in vivo* has not been addressed. We transplanted #11 NRas^{Q61K} Rac1 f/f melanocytes subcutaneously into the left flank of nude mice (CD-1). The growth of Rac1-deleted allografts on day 1–3 of tamoxifen treatment was similar to vehicle, likely because it requires 3 days to deplete endogenous Rac1 (Li *et al.*, 2011). However, allograft growth halted at day 5 after tamoxifen treatment (Figure 6a). BrdU incorporation was reduced by 60% in Rac1-deleted allografts (Figure 6b and c). Pak2 (Mouse melanocytes do not express Pak1/3 (Li *et al.*, 2011).), Erk1/2, and AKT activation were reduced in Rac1-deleted allografts. Although the other Rac1-dependent signaling pathways (JNK, p38, and NF-κB; Jaffe and Hall, 2005) remained unchanged (Figure 6d). Rac-specific inhibitors (NSC23766 or EHT1864) showed strong inhibition of Rac1 activity *in vivo* (Figure 6e) and significant inhibition of allografts growth (Figure 6a) without inducing body-weight loss (Supplementary Figure S5A online). Proliferation was decreased by about 50% (Figure 6b). Consistent with Rac1 deletion results, both NSC23766- and EHT1864-treated allografts showed a strong decrease in Pak2, Erk1/2, and AKT activation (Figure 6e). Interestingly, analysis of vehicle-treated mice systematically revealed pigmentation of left axillary and inguinal skin-draining lymph nodes (Figure 6f and Supplementary Figure S5B online), a characteristic of the metastatic process. Tumor-draining lymph nodes were enlarged (Figure 6f and g and Supplementary Figure S5B online), and contained large numbers of dopachrome tautomerase-positive pigmented proliferating melanocytic cells (Figure 6h) and disrupted architecture (Supplementary Figure S5C online). However, in tamoxifen, NSC23766- or EHT1864-treated mice, lymph nodes retained their normal architecture and weight (Figure 6f and g and Supplementary Figure S5B, C online). All vehicle-treated mice (6/6) had melanocytes in lymph nodes (Figure 6h and i, Supplementary Figure S5C online). Whereas, none of mice (0/6) treated with tamoxifen and only 1 in 4 mice treated with either NSC23766 or EHT1864 showed melanocytes in lymph nodes (Figure 6h and i, Supplementary Figure S5C online). Together, these data indicate that Rac1 activity is required for NRas^{Q61K}-induced tumor growth and lymph node spread *in vivo*, which suggests that Rac1 may be a valid therapeutic target against NRas^{Q61K}-induced metastatic melanoma.

DISCUSSION

NRas^{Q61K} does not affect either migration or proliferation during embryogenesis nor compensate for Rac1 loss

As NRas^{Q61K} is a major driver of human melanoma (Hendrix *et al.*, 2007), we investigated whether expression of NRas^{Q61K} would alter the growth, survival, or motility of melanoblasts during embryogenesis. However, NRas^{Q61K}-expressing embryos showed a normal distribution and numbers of melanoblasts until birth. Although we cannot formally discount the possibility that NRas^{Q61K} may enhance melanoblast proliferation and simultaneously promote apopotosis, we did not observe any apoptotic melanoblasts using live *ex vivo* imaging (Supplementary Movie S1 online) or by histology (not shown). This may indicate a high basal level of NRas activity in melanoblasts during embryogenesis, as cells are proliferating and populating the skin and that KitL (kit ligand) is expressed in the embryo epidermis and decreases after birth (Balch *et al.*, 2011). Other factors besides Ras may be limiting for the growth and accumulation of melanoblasts such that expressing NRas^{Q61K} does not further enhance proliferation or survival. Melanoblasts require the membrane-bound form of KitL for survival and migration in the epidermis, suggesting cell–cell interactions might be involved (Abraham *et al.*, 2012). Rac1 has been shown to mediate the formation of dendritic structures in melanoma cells in response to melanocyte-stimulating hormone and UV light *in vitro* (Scott and Cassidy, 1998; Balch *et al.*, 2011). However, whether dendritic structures mediated by Rac1 in melanocytes or melanoma cells *in vitro* are analogous to long protrusions mediated by Rac1 during melanoblast migration *in vivo* is still not clear. We emphasize here that, in addition to our previous study (Li *et al.*, 2011), Rac1 is an important


a
NRas
Rac1 f/f NRas
b
NRas
Rac1 f/f NRas
y axis (μm)
x axis (μm)
c
NRas
Rac1 f/f NRas
d
NRas
Rac1 f/f NRas
e
Migration speed (μm min−1)
Ctr
NRas
Rac1 f/f
Rac1 f/f NRas
f
Persistence
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Long protrusions only
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Percentage of melanoblasts
i
Lifetime of protrusion (min)
j
Number of protrusions formed in 3 hours per cell
k
NRas
Rac1 f/f NRas
l
Ctr
NRas
Rac1 f/f
Rac1 f/f NRas
Percentage of LacZ+ cells
Dermis
Epidermis
Epi/dermal junction

mediator of dynamic long protrusions, which contribute to melanoblast migration in epidermis.

Rac1 is required for NRas^{Q61K}-induced survival of dermal melanocytes

Adult mice expressing NRas^{Q61K} in the melanocyte lineage accumulated more dermal and hair follicle melanocytes than normal mice. Rac1 deletion in melanocytes of NRas^{Q61K}-expressing mice resulted in loss of dermal melanocytes similar to normal mice, suggesting that Rac1 has a very important role downstream of NRas in regulating melanocyte survival in the dermis. However, we failed to detect any apoptotic melanocytes in dermis with cleaved caspase-3 antibody in both P0.5 and P3 skin sections (data not shown). Cells may be rapidly cleared or die via a non-apoptotic mechanism.

c-Kit signaling triggers proliferation and survival via activation of Ras via mitogen-activated protein kinase/extracellular signal–regulated kinase signaling (Mackenzie *et al.*, 1997) and PI3 kinase. KitL accumulates in the hair bulbs of murine anagen hair follicles with very little or no expression in the adult epidermis (Peters *et al.*, 2003). Lack of KitL survival signals in the epidermis after birth may explain why in normal mice, melanocytes cannot survive in epidermis. However, expression of activated NRas^{Q61K} in melanocytes may overcome the requirement for KitL and thus promote melanocyte survival in epidermis in a Rac1-dependent manner. It will be of interest to see whether the Rac1 function can be compensated by ectopic expression of KitL in epidermis (Kunisada *et al.*, 1998) or by deleting Rac1 in mice lacking the Ras-GAP Nf1 (McCalmont and Bastian, 2012).

Rac1 is required for NRas^{Q61K}-induced AIG and invasive matrix-degrading capacity of melanocytes

Knockdown of Rac1 in melanoma cells resulted in defective proliferation *in vitro* (Bauer *et al.*, 2007) and Rac1 is important downstream of KRas^{G12D} in a lung cancer model (Kissil *et al.*, 2007). We explored the possibility that Rac1 was required for AIG conferred by NRas^{Q61K} on primary mouse melanocytes in culture. NRas^{Q61K}-expressing melanocytes showed Rac1-dependent AIG (Figure 1). Survival of AIG may be important for melanoblasts during their migration through the dermis and epidermis. This could point to an importance of Rac1 in melanoma development, progression, and metastatic spread, as AIG may be important for these processes.

In addition to its contribution to AIG, Rac1 was surprisingly important for invasive and matrix degradative capacity of cultured melanocytes (Figure 5). Normal immortalized melanocytes did not form invadopodia or degrade gelatin matrix, but NRas^{Q61K}-expressing melanocytes did so in a Rac1-dependent manner (Yamaguchi *et al.*, 2005; Linder, 2007). Rac1 has been previously implicated in invadopodia using dominant negatives (Nakahara *et al.*, 2003; Furmaniak-Kazmierczak *et al.*, 2007) but to our knowledge it has not been previously reported that Rac1 is essential for invadopodia and can work downstream of NRas^{Q61K} in melanoma cells. This has important implications for understanding how Ras confers invasion potential on cells and raises the question of which Rac1 targets are key in invadopodia formation.

Rac1 inhibition as potential therapy for melanoma

Finally, loss of Rac1 or its pharmacological inhibition halted tumor growth *in vivo* and prevented metastatic spread, suggesting that Rac1 is a key downstream effector of NRas^{Q61K} in melanoma tumor growth and progression. It should be noted that although sentinel node biopsy is now widely recommended for the staging of melanomas and has proven prognostic value (Balch *et al.*, 2011), some interesting controversies remain. For example, two recent studies highlight lymph node spread of an apparently benign melanocytic nevus, raising the possibility that even in nevi that are not genetically unstable, lymph node spread can occur (Abraham *et al.*, 2012; McCalmont and Bastian, 2012). However, larger studies such as Balch *et al.* (2001) do identify lymph node spread as a significant predictor of survival. In our study, Rac1 loss or downregulation by treatment of mice with Rac inhibitors NSC23766 or EHT1864 decreased key growth signaling pathways via PAK2, AKT, and extracellular signal–regulated kinase, and slowed proliferation in tumors, as well as halting lymph node spread. Thus, we propose that Rac1 is an important candidate target for melanoma tumor growth and metastasis downstream of NRas and targeting Rac1 or its effectors could be effective therapies to pursue.

MATERIALS AND METHODS

Transgenic mice and genotyping

All experiments were performed according to UK Home Office regulations. See also Supplemental Materials online.

Ex vivo imaging of melanoblast migration

Experimental setup was previously reported (Li *et al.*, 2011).

Whole-mount staining and immunohistochemistry

Whole-mount staining of dopachrome tautomerase-LacZ-positive mouse embryos and dorsal skin was previous reported

Figure 4. Expression of NRas^{Q61K} does not affect melanoblast migration in epidermis. All experiments show embryo skin explants or embryos. (**a**) Combined Z-stack confocal images of melanoblasts from Z/EG NRas and Z/EG Rac1 f/f NRas embryo skin. Long (≥cell body width) and short (≤cell body width) protrusions, are shown with white and yellow arrows, respectively. (**b**) Three hour tracks of melanoblasts. Black tracks migrated faster and red slower than average of control (Ctr). (**c**) Live imaging of cell protrusion (yellow arrow) dynamics. (**d**) Live imaging of melanoblast in GFP-Lifeact NRas Ctr or GFP-Lifeact Rac1 f/f NRas skin. Yellow arrows indicate protrusions. (**e**) Migration speed. (**f**) Persistence. (**g**) Number of long/short protrusions per melanoblast. (**h**) Proportion of melanoblasts with long/short protrusions. (**i**) Lifetime of growing protrusions. (**j**) Frequency of protrusion formation. (**k**) Photos and (**l**) quantification of melanoblasts in E15.5 DCT::LacZ embryos. Melanoblasts are indicated with black arrows. Black dotted lines represent epi/dermal junction. Error bars indicate mean ± SEM. $^{**}P<0.01$, by *t*-test. Bars (**a**, **c**) = 10 μm and (**k**) 20 μm.

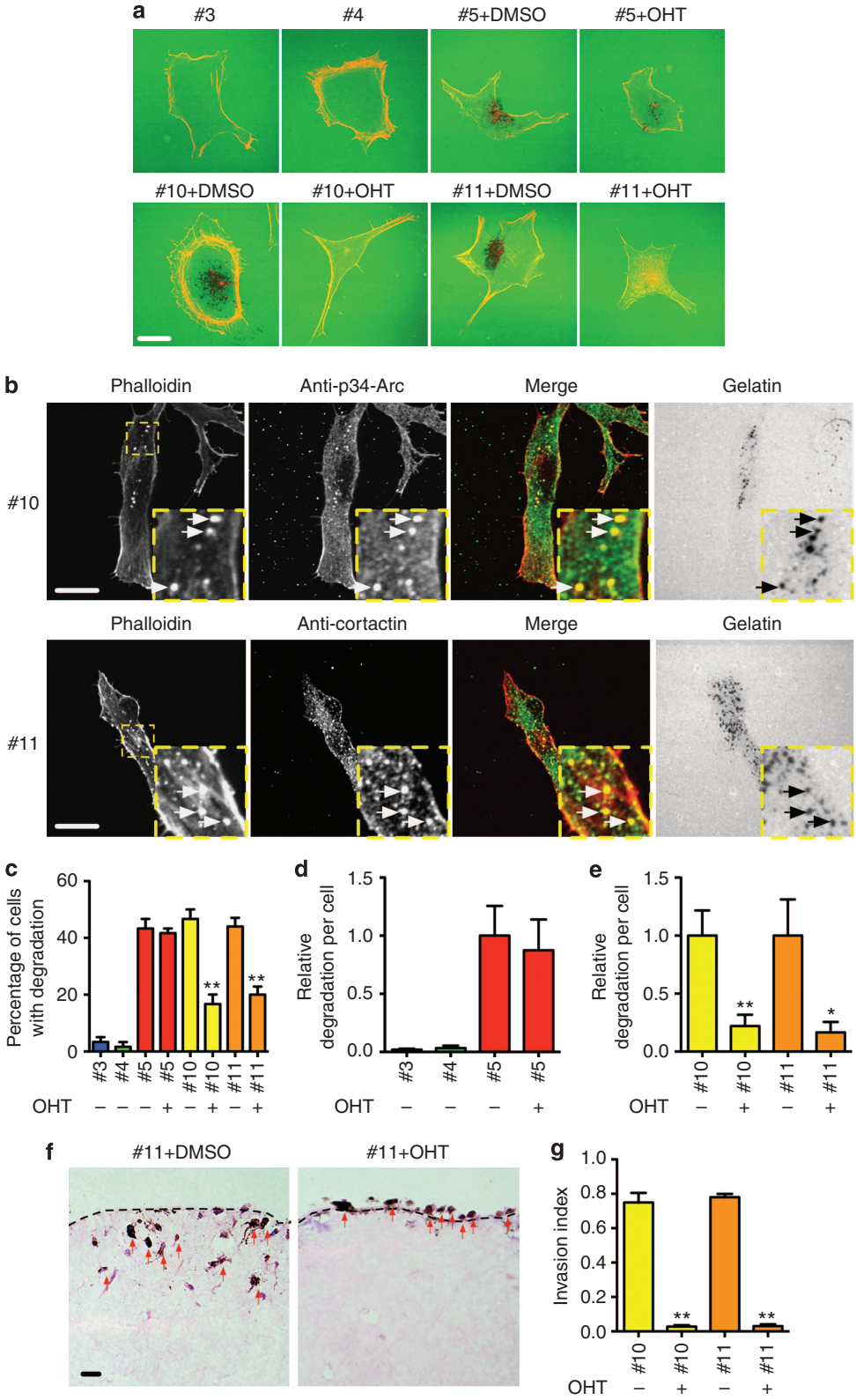

Figure 5. Rac1 is required for NRas^{Q61K}-induced invadopodia formation and invasion in melanocytes. (**a**) #3, #4, #5, #10, and #11 primary melanocyte cell lines treated with DMSO or 4-hydroxytamoxifen (OHT) on cross-linked gelatin and stained with rhodamine phalloidin. (**b**) #10 or #11 melanocyte cells on cross-linked gelatin stained with anti-p34 (Arp2/3 complex), anti-cortactin, and phalloidin. Arrows indicate invadopodia. (**c**) Percentage of cells degrading matrix. (**d**) Relative matrix degradation to DMSO control #5 (**e**) or #10 or #11. All error bars show mean ± SEM from 30 cells per experiment, $n = 3$ independent experiments. (**f**) Organotypic invasion assays and quantification (**g**) for invasion of #10 or #11 melanocytes treated with DMSO or OHT. Melanocytes are indicated by red arrows. $^{**}P < 0.01$ and $^{*}P < 0.05$ by *t*-test. Bars = 10 μm (**a**, **b**) and 20 μm (**f**). Insets show invadopodia.

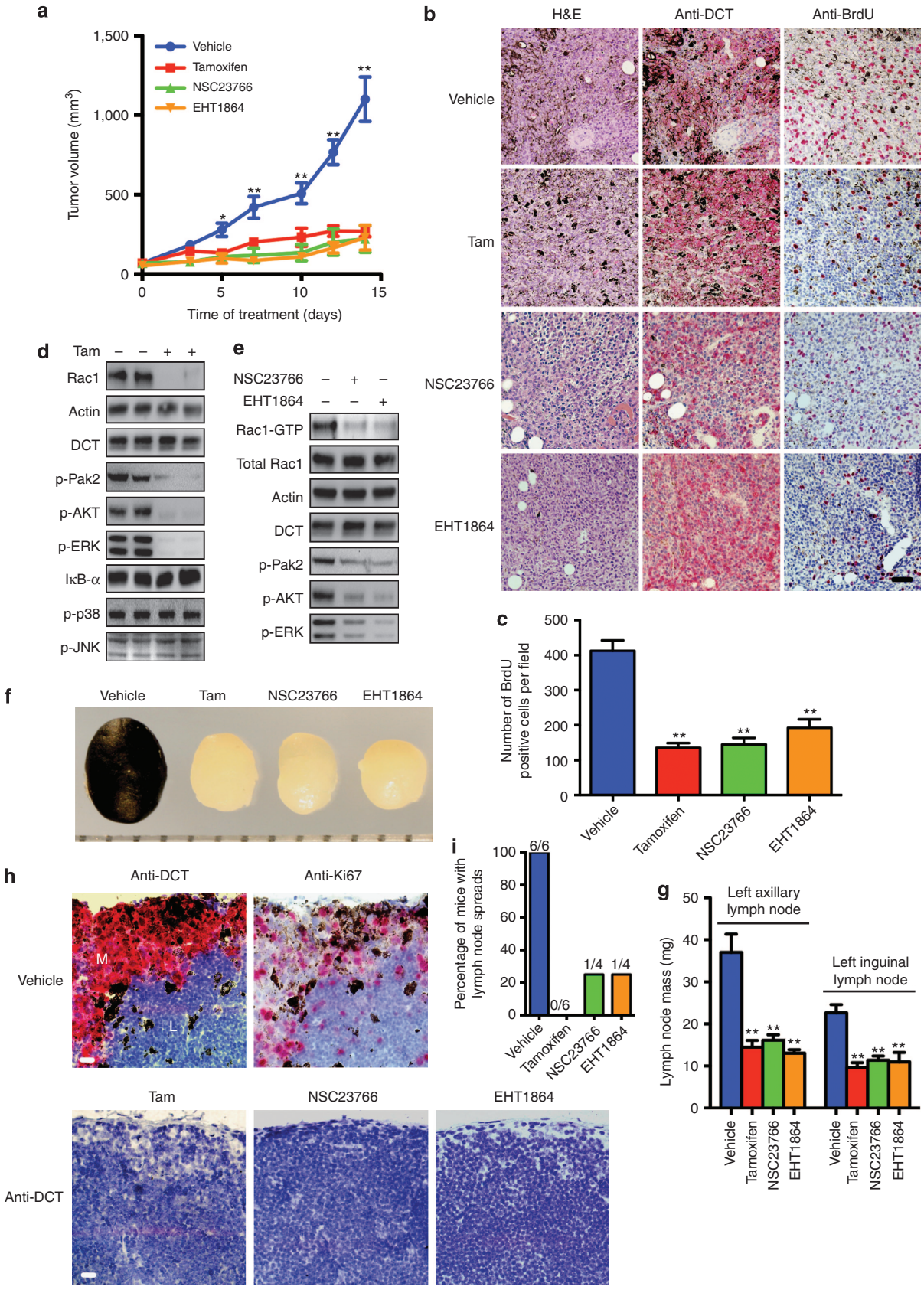

Figure 6. For caption refer page 2620.

Figure 6. Rac1 activity is required for NRas^{Q61K}-induced melanoma growth and lymph node spread. (**a**) Response of allografts to drug treatment ($n \geq 4$) on day 3 of NSC23766 or EHT1864 treatment ($P < 0.05$). (**b**) Drug-treated allografts stained with H&E, anti-dopachrome tautomerase (DCT), and anti-BrdU. (**c**) BrdU-positive cells per field (≥ 3 tumors). (**d**) Western blot of vehicle/tamoxifen (Tam)-treated allografts. (**e**) Rac1 activity and western blot of vehicle-, NSC23766-, or EHT1864-treated allografts. (**f**) Left inguinal lymph node and (**g**) average lymph node mass for drug-treated mice ($n \geq 4$). (**h**) Lymph nodes with anti-DCT or with anti-Ki67. L = lymph node tissue; M = melanocytes. (**i**) Percentage of mice with DCT-positive lymph nodes. Error bars show mean ± SEM, $^{**}P < 0.01$ and $^{*}P < 0.05$ by *t*-test. Bars = 50 μm. H&E, hematoxylin and eosin.

(Li *et al.*, 2011). Immunohistochemistry was done with standard methods (see Supplementary Material online).

Growth in soft agar

A total of 1×10^4 cells were plated in 1 ml of 0.35% SeaPlaque agarose/DMEM/10% fetal bovine serum (Lonza Rockland, Rockland, ME) on top of 1.5 ml of 0.7% SeaPlaque agarose/DMEM/10% fetal bovine serum in 6-well dishes. Once solidified, the agarose was covered with 2.5 ml F-12/10% fetal bovine serum with or without 200 nM 12-*O*-tetradecanoylphorbol 13-acetate, which was changed twice weekly. Images were captured 2 weeks later at room temperature using Zeiss Stemi 2000C (Carl Zeiss, Welwyn Garden City, UK) dissection microscope at ×0.65 magnification. The number of colonies (>0.1 mm in diameter) and the relative size of colonies were determined 2 weeks after plating.

Fluorescent gelatin degradation assay

Gelatin degradation assay was done as previously described (Li *et al.*, 2010).

Cell culture

Primary mouse melanocytes were isolated from 1-day-old Rac1 f/f Ink4a$^{-/-}$ Tyr::CreERT2$^{+/o}$ (#3 and #4), Tyr::NRas$^{Q61K+/o}$ Rac1 f/f Ink4a$^{-/-}$ Tyr::CreERT2$^{+/o}$ (#10 and #11), and Tyr::NRas$^{Q61K+/o}$ Rac1 f/f Ink4a$^{-/-}$ Tyr::CreERT2$^{o/o}$ littermate mice and cultured as previously reported (Li *et al.*, 2011).

Effector domain pulldown assay

The relative levels of GTP-bound Rac1 in tumors treated with vehicle or Rac inhibitors (NSC23766 or EHT1864) were determined by an effector pulldown assay as described previously (Vidali *et al.*, 2006).

Organotypic invasion assay

Organotypic cultures were set up as described (Edward *et al.*, 2005). See Supplementary Materials online for modifications.

Tumor transplantation and *in vivo* treatment studies

For tumor grafting experiments, #11 primary immortalized mouse melanocytes cell line were introduced into mice by subcutaneous injection of $\sim 1 \times 10^6$ cells in 0.1 ml. Phosphate-buffered saline into the lower flank of mouse (CD-1 nude females 6-week-old, Charles River Lab, Wilmington, MA). See also Supplementary Materials online.

Assaying proliferation *in vivo*

Proliferation levels were assessed by measuring bromodeoxyuridine (BrdU; Amersham Biosciences, Piscataway, NJ) incorporation. Mice were injected with 250 μl of BrdU 2 hours before being killed. Immunohistochemical staining for BrdU was then performed using an anti-BrdU antibody (BD Bioscience, Oxford, UK). At least three mice were used from each treatment for analysis.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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