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Rac1 drives melanoblast organization during mouse development by orchestrating pseudopod-driven motility and cell cycle progression

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Summary

During embryogenesis, melanoblasts proliferate and migrate ventrally through the developing dermis and epidermis as single cells. Targeted deletion of Rac1 in melanoblasts during embryogenesis causes defects in migration, cell cycle progression and cytokinesis. Rac1 null cells migrate markedly less efficiently, but surprisingly, global steering, crossing the dermal/epidermal junction and homing to hair follicles occur normally. Melanoblasts navigate in the epidermis using two classes of protrusion: short stubs and long pseudopods. Short stubs are distinct from blebs and are driven by actin assembly, but are independent of Rac1, Arp2/3 complex, myosin or microtubules. Rac1 positively regulates the frequency of initiation of long pseudopods, which promote migration speed and directional plasticity. Scar/WAVE and Arp2/3 complex drive actin assembly for long pseudopod extension, which also depends on microtubule dynamics. Myosin contractility balances the extension of long pseudopods by effecting retraction and allowing force generation for movement through the complex 3D epidermal environment.

Introduction

Mammalian melanoblasts are the precursor cells of melanocytes; they emerge from the neural tube during embryogenesis, and migrate and proliferate to populate the skin and hair follicles. In mice, melanoblasts reside in an area just near the neural tube, called the migration staging area, where they receive proliferation and survival signals from kit-ligand (kit-l), the ligand of their major tyrosine kinase receptor c-kit. Between embryonic day 8.5

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(E8.5) and E10.5, their migration is dorsolateral and after E10.5 they progress ventrally toward the face, ventral abdomen and the developing limbs. They also emerge upward through the developing dermis and into the epidermis at around E13.5. Over the next few days, they enter developing hair follicles and after birth epidermal melanoblasts reside only in the hair follicles (Mayer 1973; Jordan and Jackson 2000; Thomas and Erickson, 2008). Signaling to c-kit via kit-l triggers proliferation, survival and possibly motility via activation of Ras and downstream MAPK/ERK signaling (Mackenzie et al., 1997; Nishimura et al., 2002; Smalley, 2009; Steel et al., 1992). Recently, powerful mouse genetic tools became available to study melanoblast-specific gene alterations (Delmas et al., 2003; Yajima et al., 2006) and combined with high power ex-vivo microscopic methods (Mort et al., 2010) allow study of the mechanisms of migration of these fascinating cells.

Rac1 is the major ubiquitous isoform of Rac expressed in mammalian tissues, with Rac2 hematopoietic and Rac3 in brain (Didsbury *et al.*, 1989; Haataja *et al.*, 1997). Rac1 controls assembly of the actin cytoskeleton primarily via activation of the Scar/WAVE complex leading to Arp2/3 complex actin nucleation (Insall and Machesky, 2009) and activation of p21-activated kinase (PAK) and LIM kinase driving cofilin-mediated actin turnover (Delorme *et al.*, 2007). Both of these pathways contribute to normal and cancer cell migration. Loss of Rac1 in tissue culture and primary cells leads to a loss of lamellipodia and a general reduction of migration speed (Guo et al., 2006; McCarty et al., 2005; Vidali et al., 2006). Rac1 depletion also reduces focal adhesions and stress fibers in mouse embryonic fibroblasts (Delorme et al., 2007; Guo et al., 2006; Vidali et al., 2006). *In vivo*, we have limited knowledge of the role of Rac1 in migration. *Drosophila* oocyte border cells are unable to migrate when they express dominant negative Rac (Bianco et al., 2007; Duchek et al., 2001; Geisbrecht and Montell, 2004; Murphy and Montell, 1996a; Wang et al., 2010) and their motility is induced by photoactivation of a Rac analog (Wang et al., 2010). Border cells use cadherin-based adhesion to navigate as a small cluster among nurse cells and Rac triggers the generation of long protrusions in leader cells (Bianco et al., 2007; Duchek et al., 2001; Geisbrecht and Montell, 2004; Murphy and Montell, 1996a; Wang et al., 2010). Likewise, Rac1 inhibition in zebrafish germ cells inhibits actin brush formation, cell polarity and migration (Kardash *et al.*, 2010). Zebrafish embryonic neural crest cell migration toward SDF-1 is regulated by Rac1 (Theveneau et al., 2010). Mouse anterior visceral endoderm (AVE) cells require Rac1 for protrusion formation and collective migration to the developing anterior side of the embryo during body axis specification (Migeotte et al., 2010). Surprisingly, mouse neural crest cells do not require Rac1 for homing, but Rac1 loss impairs proliferation (Fuchs et al., 2009). Thus Rac1 emerges as an important controller of in vivo migration, but our mechanistic insight is limited.

Rac is activated downstream of Ras and can activate JNK1, possibly via Pak kinases to stimulate cell cycle progression (Olson et al., 1995). Both cell-cell and cell-matrix adhesion signal to increase cyclin D1 levels for progression through the cell cycle and Rac1, together with ERK regulates this (Fournier *et al.*, 2008; Klein *et al.*, 2008). Rac1 is tightly regulated in cytokinesis, both temporally and spatially and is inhibited at the cleavage furrow by centralspindlin, a GTPase activator (GAP) (Canman et al., 2008).

We describe here an important role for Rac1 in migration, proliferation and cytokinesis in mouse embryonic melanoblasts. Deletion of Rac1 in the melanocyte lineage has severe effects on coat color of mice, resulting from inability of cells to mount a Rac – Scar/WAVE – Arp2/3 mediated protrusion response for migration as well as delayed progression through the cell cycle. Surprisingly, though, Rac1 null melanoblasts can still use short stubby protrusions to migrate independently of this pathway by an actin-driven mechanism that is distinct from amoeboid or mesenchymal motility. We provide a detailed molecular analysis

of migration of melanoblasts in skin ex vivo, revealing an important role for Rac1 as a coordinator of the rate of protrusion generation during migration.

Results

Rac1 loss in melanoblasts causes coat color defects, indicating impaired migration and proliferation

To assess the functions of Rac1 in the melanocyte lineage *in vivo*, we crossed mice carrying a floxed allele of Rac1 (Corbetta et al., 2009; Walmsley et al., 2003) with mice expressing Cre recombinase under control of the tyrosinase promoter (Tyr::Cre) (Delmas et al., 2003) (Figure 1A) in a C57BL6/J background. Rac1 f/f Tyr::Cre$^{+/o}$ mice were born healthy at the expected Mendelian ratio, however, they were smaller than littermates (Figure S1A, B). Recombination in melanocytes was confirmed by genomic PCR (Corbetta et al., 2009) and the Rac1 null allele was only observed together with Tyr::Cre (Figure 1B).

Rac1 f/f Tyr::Cre$^{+/o}$ mice (n=15) exhibited a distinctive white patch of hair along their ventral region, ranging from half to whole the width of the underside (Figure 1C). More than 80% of the mice had at least one white patch along the dorsal midline (Figure 1C, white arrows) as well as white hairs among the black dorsal fur. The limbs and tail in all Rac1 f/f Tyr::Cre$^{+/o}$ mice were also hypopigmented (Figure 1D, E). Histological analysis of P14 control skin or black areas of the Rac1 f/f Tyr::Cre$^{+/o}$ mice showed melanocytes in hair follicles (Figures 1F and S1C), indicating that cells lacking Rac1 can still home correctly. Whereas, no melanoblasts were present in dorsal white patches or ventral skin of Rac1 f/f Tyr::Cre$^{+/o}$ mice (Figure 1G). Thus, Rac1 deficiency in melanocytes causes a pigmentation defect resulting from a lack of melanocytes in areas distal to their migration origin near the neural tube.

Rac1 null melanoblasts fail to complete population of the embryo skin

Mouse melanoblasts migrate out of the neural tube around E10 and continue to proliferate and migrate to fill the skin by birth. We asked whether loss of Rac1 affected melanoblast number and position between E11.5 and E15.5 using a DCT::LacZ transgene (Mackenzie et al., 1997). At E11.5, when the Tyr::Cre is expressed (Delmas et al., 2007; Delmas et al., 2003), control and Rac1 f/f Tyr::Cre$^{+/o}$ embryos showed no differences (Figure 2A, F). In E13.5 control embryos, the melanoblast population had expanded ventrally (Figure 2B, F). By E15.5, melanoblasts colonized almost the entire embryo (Figure 2C, F). However, by E13.5, there were fewer melanoblasts in Rac1 f/f Tyr::Cre$^{+/o}$ embryos than controls and this difference increased by E15.5 (Figure 2B, C, F). The E15.5 Rac1 f/f Tyr::Cre$^{+/o}$ embryos also displayed regions devoid of melanoblasts ventrally on their abdomens and on their limbs (Figure 2C, E). Additionally, Rac1 depleted melanoblasts appeared less elongated than controls (Figure 2D). Melanoblasts in Rac1 f/f Tyr::Cre$^{+/o}$ failed to cover the entire dorsal midline region on E15.5 (Figure S1I), likely due to a lack of dorsal migration of cells emerged on both sides of the neural tube in the migration staging area (Figure S1D). The central midline area is largely devoid of melanoblasts up until E13.5 (Figure S1E–G) and where normal cells fill in this region by E15.5 (Figure S1H–I) white patches devoid of melanoblasts are still present in Rac1 null embryos. Furthermore, Rac1 f/f Tyr::Cre$^{+/o}$ showed half the normal number of melanoblasts at P0.5 after birth (Figure 2G and H). Thus, Rac1 is important for melanoblast position and number during embryogenesis. However, melanoblasts lacking Rac1 can still proliferate, migrate, home to hair follicles and produce and secrete melanin to pigment the hair, albeit less efficiently than those expressing Rac1.

Melanoblasts migrate in embryo epidermis with Rac1 driven long pseudopods and Rac1 independent short stubs

To investigate the functions of Rac1 in melanoblast migration in vivo, mice carrying a Z/EG double reporter transgene (Novak et al., 2000) were crossed onto the Rac1 f/f Tyr::Cre$^{+/o}$ background to drive GFP-expression in the melanoblast lineage. E15.5 embryonic trunk skin was imaged as previously described (Mort et al., 2010). Skin explants consisted of epidermis, developing hair follicles and dermis with most melanoblasts in the epidermis (Movie 1). GFP-labelled melanoblasts in whole mounted skin explants appeared elongated (Figure 3A, B, Movie 1). They showed long protrusions (LP), (>cell body width) traversing the regions between adjacent keratinocytes, which had microtubule cores with several actin rich tips (Figure 3A). 3D reconstructions revealed a rounded cell body with frequently 1–2 LPs in the direction of migration (Figure 3B and Movie 1). Melanoblasts and keratinocytes make E-cadherin contacts in the developing mouse embryo epidermis (Figure 3C and (Jouneau et al., 2000). Loss of Rac1 did not detectably alter E-cadherin localization in Rac1 f/f Tyr::Cre$^{+/o}$ melanoblasts or position relative to keratinocytes (Figure 3C). Keratinocytes do not require Rac1 for adherence junctions in vivo (Benitah et al., 2005; Chrostek et al., 2006), so it is likely that loss of Rac1 in melanoblasts didn't completely disrupt their cell-cell contacts. In summary, melanoblasts exhibit elongated shape and extend actin-rich LPs between neighboring keratinocytes.

Live time-lapse imaging of embryo skin explants revealed severe motility defects in Rac1 null melanoblasts and the presence of short stubby protrusions (SSP). Control melanoblasts displayed at least one LP (average 0.9 per cell) compared with average 0.2 per cell for Rac1 nulls (Figure 3D white arrows LPs, yellow arrows SSPs, Figure 3E, Movie 1). Around 35% of Rac1 depleted and only 4% of normal melanoblasts showed no protrusions (Figure 3F) with more Rac1 null cells making SSPs (Figure 3F). LPs were long-lived, with an average lifetime of 1h (Figure 4A, B) but <30min in Rac1 nulls (Figure 4A, B) and the rate of LP initiation was nearly 3-fold decreased by Rac1 loss (4C, Movie 2). In contrast to *Drosophila* embryos or mouse AVE cells (Migeotte et al., 2010; Murphy and Montell, 1996b) around 90% of Rac1 depleted melanoblasts migrated despite having only SSPs (Figure 4D–F), but with average speed ^50% slower than controls (Figures 4D–F and Movie 2). The formation of SSPs was followed by cell body translocation in Rac1 nulls, unlike in controls where SSPs elongated into long pseudopods and cells frequently changed direction (Movie 2). SSP formation occurred completely independently of Rac1 (Figure 4 B, C). To distinguish SSPs from blebs, we created a conditional Lifeact expressing mouse to monitor F-actin dynamics specifically in melanoblasts ex-vivo (Supplementary Methods). Blebs arise by a dissociation between the cell cortex and plasma membrane, followed by a gradual recruitment of actin and myosin and bleb retraction (Charras, 2008). Live time-lapse video of melanoblasts expressing lifeact driven by Tyr::Cre expression revealed bright flashes of F-actin near the tips and in the bodies of both LPs and SSPs (Figure 4G yellow arrows and Movies 3). In contrast, 50μM LY294002, a PI-3-kinase inhibitor induced blebbing (Figure 4G and Movie 3). Blebs were clearly distinct in shape, as SSPs in Rac1 null cells were spiky (Figure 4G, yellow arrows, Movie 3). They also had distinct actin distribution; Actin accumulated at the neck region (Figure 4G, 15") and then later as they began to retract, actin accumulated near the periphery of blebs (Figure 4G, 30"), while it was distributed throughout SSPs (Figure 4G and Movie 3). Furthermore blebs occurred with high frequency (Figure 4H) and were much shorter-lived than SSPs (Figure 4I). Thus Rac1 null cells were impaired in long but not short pseudopod generation leading to a reduction in speed and protrusion lifetime. Rac1 loss also decreased the frequency of long but not short protrusion initiation, indicating a role for Rac1 as an initiator and potentiator of LPs that drive migration. SSPs are distinct from classical blebs and thus emerge as a Rac1-independent form of actin-based pseudopod.

Melanoblasts migrate individually in developing skin using long protrusions based on Arp2/3 complex and Scar/WAVE driven actin assembly

Inhibition of actin (latrunculin A) or tubulin polymerization (nocodazole) severely impaired migration (Figure 5A, B and Movie 4). Microtubule stabilization with taxol slowed migration (Figure 5B). In addition, latrunculin A severely inhibited both LPs and SSPs, whereas nocodazole or taxol only inhibited formation of LPs (Figures 5A, C, D and Movie 4). LPs formed by melanoblasts in skin are thus fundamentally different from lamellipodia, in that microtubule dynamics are required for normal protrusion extension and subsequent cell translocation (Ballestrem et al., 2000; Verkhovsky et al., 1999). However, the mechanism by which microtubules act is likely to be complex, as microtubules sequester RhoGEFs, such as GEFH1 and their depolymerization alters Rho activity and contractility (Redd et al., 2006; Takesono et al., 2010; Zhou et al., 2010). Indeed, the Rho-kinase inhibitor Y27632 restored LP formation in nocodazole treated explants and cells cultured in vitro but did not restore the migration speed (Figures 5A, C, D; Figure S3 B–D and Movie 4). Furthermore, LPs formed in the presence of Y27632 and nocodazole were devoid of visible microtubules (Figure S3B) indicating lack of an essential structural role. Thus, actin and tubulin dynamics are crucial for melanoblast migration in epidermis, and microtubule dynamics may regulate Rho in vivo.

Myosin-II mediated contraction drives cell body translocation, but not Rac1 mediated lamellipodia assembly in cultured cells (Ponti et al., 2004; Vidali et al., 2006). Y27632 or blebbistatin treated skin explants exhibited a reduction in myosin light chain phosphorylation (Figure S2C) consistent with previous reports (Betapudi et al., 2006; Fazal et al., 2005) but left keratinocyte organization intact (Figure S3A). Both Y27632 and blebbistatin halted melanoblast migration (Figure 5B), but increased the number of both SSPs and LPs (Figure 5A, C, D and Movie 4). The rate of LP formation increased to control levels (Figure 5F) and increased LP lifetime 2-fold (Figure 5E). Thus, myosin-II activity is important for the retraction dynamics of protrusions and for melanoblast translocation among keratinocytes in epidermis.

To test whether high contractility in Rac1 depleted melanoblasts might be inhibiting protrusion formation, we inhibited Rho-kinase with Y27632. Cells showed even slower migration than untreated Rac1 depleted or Y27632 treated control explants (Figure 5B, Movie 4), but they displayed a similar number of LPs to control cells (Figure 5A, C, D). These LPs were around 2-fold longer-lived than controls (Figure 5E) but cells barely moved (Figure 5B, Movie 4). Importantly, LPs in Rac1 depleted cells formed with a 3-fold lower frequency than controls, regardless of myosin inhibition (Figure 5F, Movie 4) and inhibiting myosin didn't increase the frequency of protrusion formation in normal melanoblasts (Figure 5F). Myosin light chain phosphorylation was similar between normal and Rac1 null melanocytes *in vitro* (Figure S2A). Thus, Rac and myosin are not apparently opposing each other, but rather when Rac is active, myosin-based contractility mediates protrusion retraction and enables LPs to generate force for movement. When Rac is absent, LPs rarely form and they are less resistant to contractile forces. Thus Rac controls the rate of protrusion formation and myosin generates contractile forces for motility.

Pak kinases drive motility downstream of Rac1 (Edwards et al., 1999; Smith et al., 2008; Yang et al., 1998), so we explored the role of Paks 1–3 and their downstream targets in migration. Treatment of skin explants with Pak inhibitor (IPA-3) (Deacon et al., 2008) and its downstream targets LIM kinase (LIMKi) (Scott et al., 2010) and ERK (U0126, a MEK1/2 inhibitor that inhibits ERK1/2 activation) showed no effect on melanobast motility despite reducing phosphorylation of their targets in skin explants (Figure 5, S2C, Movie 4 and data not shown). In addition, siRNA knockdown of Pak2, the Pak isoform expressed in melanocytes (Figure 6A), did not change melanocyte morphology on 3D collagen I matrix

(Figure 6B, F, G, H and Movie 5). Rac1 depletion in melanocytes did not alter the cofilin phosphorylation *in vitro* (Figure S3A). Thus, Rac1 regulates melanoblast migration in epidermis and formation of LPs through pathways other than Pak or its downstream targets.

The main effector of actin nucleation downstream of Rac is the Scar/WAVE complex, which drives Arp2/3 mediated actin nucleation. RNA interference was used to deplete Arp2/3 complex (p34 siRNA), Scar/WAVE complex (Nap1 siRNA) in primary melanocytes *in vitro*. Knockdown of p34 or Nap1 (Figure 6D, E) caused a phenotype similar to inhibition of Rac1 (Figures 6F, G, H and Movie 5). Arp2/3 complex inhibitor (CK-869) (Nolen et al., 2009) but not CK312 (control) reduced melanoblast migration in explants without destroying keratinocyte actin organization (Figure 5B, Movie 4 and Figure S3). Melanoblasts in CK-869 treated explants resembled Rac1 deleted cells (Figure 5A–D). The frequency and lifetime of protrusion was also decreased (Figure 5E, F). GFP-p21-Arc (Arp2/3 complex) did not concentrate at the tips of SSPs in Rac1 null cells in vitro (Figure S3) but both GFP-mDia1 and mDia2 did. Surprisingly, this indicates that SSPs form independently of Arp2/3 complex, but that Scar/WAVE and Arp2/3 are the major effectors driving actin dynamics in LPs. Other effectors, such as mDia proteins, may predominate in the initiation of short stubs, but this requires further study.

Arp2/3 complex can also be activated by N-WASP to drive efficient invasive cell migration (Insall and Machesky, 2009; Machesky, 2008) and N-WASP is robustly expressed in primary melanocytes (Figure S3A). Surprisingly, melanoblasts lacking N-WASP in Z/EG$^{+/o}$ N-WASP f/f Tyr::Cre$^{+/o}$ explants (Snapper et al., 2001) showed no difference in migration and formation of LPs to melanoblasts in control explants (Figure 5 and Movie 4). Additionally, the Tyr::Cre deletion of N-WASP revealed no coat color changes in a C57Bl6/J background (Figure S4A) or changes in the number and position of melanoblasts in E15.5 DCT::LacZ N-WASP f/f Tyr::Cre$^{+/o}$ embryos (Figure S4B–E). Depletion of N-WASP in melanocytes *in vitro* showed normal morphology (Figures 6C, F, G, H and Movie 5) and GFP-N-WASP did not concentrate at the tips of LPs or SSPs in vitro (Figure S3). Thus, we propose that Rac1 regulates melanoblast migration in epidermis and formation of LPs independently of N-WASP.

Rac1 contributes to focal adhesion formation in vitro, but Rac1 depleted melanoblasts still contact the basement membrane in vivo

Fibroblasts require Rac1 for wide fan-shaped lamellipodia in culture (Delorme et al., 2007; Guo et al., 2006; Vidali et al., 2006). We thus examined cultured immortalized primary melanocytes from 1-day pups using conditional inducible Rac1 deletion. Rac1 deletion was induced with tamoxifen analog 4-hydroxytamoxifen (OHT), using Tyr::CreERT2$^{+/o}$ Ink4a-Arf$^{-/-}$ Rac1 f/f melanocytes plated on fibronectin (Ackermann et al., 2005; Serrano et al., 1996). Anti-DCT staining confirmed melanocyte identity (Figure S5A) and Rac1 was lost during 4 days of OHT treatment (Figure S5B). Control melanocytes continuously formed lamellipodia during migration (Movie 6) that were rich in Arp2/3 complex and Scar/WAVE complex (Figure S5 C–E). In contrast, Rac1-deleted melanocytes lacked stress fibers or lamellipodia (Figures S5C–E). Expression levels of WAVE complex (WAVE1, WAVE2 and Nap1) and Arp2/3 complex (p34) remained unchanged in OHT treated melanocytes (Figure S2A). No upregulation of other Rac family members (Rac2 and Rac3) (Figure S2A) and no alteration of activity of Cdc42 and RhoA were observed (Figure S2B). Rac1 depleted cells failed to form lamellipodia during migration with absence Arp2/3 or WAVE complex localization at the periphery (Figures S5D, E and Movie 6) and had fewer and smaller focal adhesions (Figure S5D) and impaired migration (Figure S5F). However, in vivo similar numbers of control and Rac1 null melanoblasts contacted the basement membrane (BM) at E15.5 (Figures S6A). Levels of E-cadherin and ZO-1 were normal in cultured Rac1 depleted melanocytes (Figure S2A). Thus, loss of Rac1 in cultured melanocytes parallels

observations in fibroblasts that lamellipodia and focal adhesions are affected (Guo et al., 2006), but Rac1 depleted melanoblasts can still position themselves relative to the basement membrane in vivo.

Melanoblast motility across the basement membrane and in skin is not invasive

We were surprised that loss of N-WASP did not produce any apparent defects in melanoblast migration in the skin and it raised the question of whether melanoblasts use invasive matrix-degrading migration during embryogenesis. N-WASP and Rac are essential for the formation of invadopodia by melanoma cells and for invasion of cultured cells into collagen gels (Li et al., 2010; Nakahara et al., 2003; Yamaguchi et al., 2005). Treatment of Z/EG$^{+/o}$ Tyr::Cre$^{+/o}$ control skin explants with MMP inhibitor (GM6001), did not affect formation of LPs or cell migration speed (Figure 5 and Movie 4), indicating that MMPs are not required at least for E15.5 melanoblast migration in epidermis.

Mouse melanoblasts migrate across the developing epidermal BM from dermis to epidermis between E11.5 and E13.5. However, normal pigmentation (Figure S4A) in the Tyr::Cre$^{+/o}$ N-WASP f/f mouse suggests that melanoblasts do not need invadopod-like structures mediated by N-WASP. To confirm this, the trunk region of X-gal stained Dct::LacZ control, Rac1 f/f Tyr::Cre$^{+/o}$ and N-WASP f/f Tyr::Cre$^{+/o}$ embryos were sectioned and the proportion of melanoblasts in the three distinct skin layers: dermis, epidermis and epidermal/dermal junction was determined. On E13.5 and E15.5, control, Rac1 depleted or N-WASP depleted embryos had similar melanoblast distribution (Figure S6B, C), indicating that this movement doesn't require matrix degradation by invadopod-like structures.

Rac1 is required for normal cell cycle progression and cytokinesis of melanoblasts during embryogenesis

We further investigated the reason for depleted numbers of melanoblasts in Rac1 null embryos. The number of melanoblasts undergoing the cell-cycle or apoptosis were determined by co-expression of DCT (melanoblast) and Ki67 (proliferation) or cleaved caspase3 (CC3, apoptosis) in E15.5 control or Rac1 f/f Tyr::Cre$^{+/o}$ embryos. A similar percentage of control and Rac1 null melanoblasts expressed either Ki67 or CC3 (Figure 7A and data not shown), indicating that Rac1 depleted melanoblasts aren't dropping out of the cell cycle or apoptosing. However, we observed a significant decrease in BrdU incorporation into Rac1 depleted melanoblasts when animals were given either a 2hr or 24hr pulse before sacrifice (Figures 7B and C). Furthermore, there was a significant reduction in the percentage of phospho-histone 3 (PH3, mitosis) positive melanoblasts in Rac1 f/f Tyr::Cre$^{+/o}$ embryos (Figure 7D). Cultured primary melanocytes also proliferated more slowly than controls (Figure 7E,F). Flow cytometry analysis indicated shifting to the G1 phase with reduced S phase as in Rac1 nulls (Figure 7G,H). Thus, melanoblast Rac1 is required for normal G1-S phase cell cycle progression but not cell cycle entry or prevention of apoptosis.

Time-lapse videos revealed alterations in cytokinesis of Rac1 deficient melanoblasts that likely also contribute to growth rate slowing. Normal melanoblasts in Z/EG$^{+/o}$ Tyr::Cre$^{+/o}$ explants undergo cytokinesis with a cleavage furrow appearing ~10 minutes after rounding and separation of daughter cells following ~30min later (Figures S7A and B). Rac1 depleted melanoblasts furrowed with normal timing, but exhibited a 3-fold delay in time to separation (Figures S7A, B and Movie 7) and the two daughter cells migrated apart more slowly (Figure S7C). Despite the strong delay in cytokinesis, Rac1 f/f Tyr::Cre$^{+/o}$ melanoblasts did not become multinucleate (data not shown) indicating that Rac1 is not essential for cytokinesis but required for efficient daughter cell separation in vivo. No obvious delay of cytokinesis was found in Rac1 depleted melanocytes in 2D (Figure S7D, E and Movie 7).

Thus Rac1 exerts control on cytokinesis in the tissue environment, which isn't seen on rigid 2D surfaces.

Discussion

The loss of Rac1 in the melanocyte lineage results in severe coat color defects in mice and our studies reveal important roles for Rac1 in melanoblast migration, cytokinesis and progression through the cell cycle in vivo. Rac1 deleted melanoblasts migrated more slowly and thus failed to reach areas distal to the sites of emergence from the neural tube, resulting in depigmented patches in the adult. The unique 3D architecture of the skin dictates that melanoblasts use Rac1 to regulate how frequently the cells form long protrusive pseudopods, driven by Scar/WAVE and Arp2/3 complex. These protrusions contain microtubule bundles and actin and aid translocation among keratinocytes. Our analysis uncovered a role for short stubby protrusions in vivo that form independently of Rac1, Scar/WAVE or Arp2/3 complex and that in normal cells can act as precursors to Rac1-driven long protrusions. In the absence of Rac1, SSPs drive slower migration in vivo, which is distinct from either amoeboid or mesenchymal motility and the Scar/WAVE and Arp2/3 actin nucleation machinery can be at least partially dispensed with. We also implicate Rac in progression through the cell cycle and control of the rate of completion of cytokinesis in vivo. Our studies in the melanocyte lineage offer unique insight into the roles of Rac1 in vivo and the control of migration within the complex environment of the developing epidermis.

Melanoblasts move using short protrusions that mature into long pseudopods

Short stubby protrusions might initiate by stochastic actin assembly reactions if we consider the cytoplasm as an excitable medium (Betz et al., 2009; Weiner et al., 2007) or by some form of signaling. Rho might signal short protrusion initiation, as it can precede Rac in motile cultured cells (Pertz et al., 2006) and downstream might lie diaphanous related formins (DRFs), which coordinate actin and microtubule assembly and dynamics (Palazzo et al., 2001). Indeed, GFP-mDia1 or GFP-mDia2 localized to the tips of LP and SSP (Figure S3B). However, further studies are required to elucidate the role of formins in melanoblast motility. Treatment of skin explants with broad formin inhibitor (SMIFH2) (Rizvi et al., 2009) resulted in massive cell death within half hour incubation even at 100nM inhibitor (Data not shown), indicating toxicity or that formin function may be crucial for melanoblast survival. Perhaps SSPs are driven by proteins such as Mena/VASP, which can support extension of actin-based unbranched networks (Urban et al.; Urban et al., 2010). SSPs are distinct from blebs (Fackler and Grosse, 2008), as they generally are thin and spiky or jagged in shape (rather than lobule-shaped like blebs) and they are rich in dynamic filamentous actin. SSPs are thus Rac1 independent actin structures that can act as precursors to LPs.

Melanoblast LPs traverse between keratinocytes, often spanning 2 or 3 cell widths and existing in multiple planes. Like neurite processes, they are microtubule-rich and have actin-rich tips. However, while Rac1 and the Scar/WAVE complex are important for lamellipodia of neuronal growth cones, the growth of long microtubule-rich processes from neuronal cells in culture wasn't ablated by loss of Rac1 or inhibition of Arp2/3 complex (Strasser et al., 2004; Tahirovic et al., 2010). Chick embryo muscle precursors have parallels with melanoblasts. Horwitz and colleagues showed that these cells extend long stable protrusions similar in lifetime to melanoblasts (50 min vs 60 min in our study) and migrate with similar average speed from the somite to the limb buds (37μm/hr vs 30μm/hr in our study). They also found that dominant negative Rac stopped migration and limited protrusion (Knight et al., 2000).

Rac1 controls the frequency of long pseudopod extension

Rac1 wasn't required for pseudopodia, but rather controlled the frequency at which SSPs progressed into LPs. Unexpectedly, when myosin was inhibited, Rac1 was no longer required for long pseudopod generation; but it still strongly controlled the frequency of generation of long pseudopods. This only became apparent when we inhibited myosin activity in skin explants by adding Y27632 or Blebbistatin. Cells generated LPs at a very reduced rate (over Rac1 wt cells) but failed to translocate. Partially activated Scar/WAVE and Arp2/3 complex might be able to generate long pseudopodia in a Rac-independent manner when cortical tension is low, but this is unlikely to lead to migration. This may depend on other signaling, such as via phosphorylation (Lebensohn and Kirschner, 2009) or phospholipid or adapter protein interactions (Chen et al., 2010). While Rac1 is a major controller, it is likely that multiple signal inputs modulate the Scar/WAVE complex.

Rac1 is not important for melanoblast positioning within the skin layers

Adhesion to extracellular matrix was likely affected by loss of Rac1, since in culture, melanocytes depleted for Rac1 showed reduced focal adhesions. This could alter the lifetime of LPs and their ability to generate pulling force for translocation. However, Rac1 null melanoblasts contacted the basement membrane in embryo skin, and E-cadherin and actin still appeared as normal. We can't be sure that melanoblasts are making E-cadherin junctions with keratinocytes, but the extension of LPs into the spaces between keratinocytes, and parallels with other studies (Geisbrecht and Montell, 2002; Kardash et al., 2010) suggests that E-cadherin is a good candidate for future studies.

Melanoblasts first emerge in the dermis, a complex matrix of collagen and proteoglycans and they migrate there until around E13.5, when they cross what appears as a collagen-rich BM into the epidermis. At E13.5, the epidermis is only ~1 cell-layer thick (Figure S6B) but by E15.5, multiple keratinocyte layers are present and around half of melanoblasts are touching the BM, but ~70% have crossed over and are above the BM (Figure S6C). This motility appears independent of N-WASP, matrix metalloproteases and Rac1, which are important for the assembly of invadopodia (Li et al., 2010; Nakahara et al., 2003; Yamaguchi et al., 2005) and for the invasion of cancer cells into 3D matrix (Kurisu et al., 2004; Li et al., 2010). Thus early dermal ECM and epidermal BM are likely permissive to cell transmigration similar to lymphatic endothelial BM and dendritic cells (Pflicke and Sixt, 2009). During melanoma formation, the acquisition of a BM degrading ability is thus unlikely to be a simple reversion to the normal melanoblast genetic programme. Melanoma cells require N-WASP for efficient invasion in vitro and use MT1-MMP to make invadopodia (Li et al., 2010), but melanoblasts do not appear to depend on similar invasive behavior during embryogenesis.

Rac1 is required for normal cell cycle progression and cytokinesis of melanoblasts during embryogenesis

Rac1 is important for G1 progression of melanoblasts during normal development. A major controller of melanoblast proliferation is c-kit, a tyrosine kinase receptor, which signals to Ras and activates entry into the cell cycle via PI3-kinase, Akt and MEK pathways (Smalley, 2009). Rac1 acts downstream of Ras in concert with PI3-kinase and regulates G1 progression in vitro (Olson et al., 1995). However, unlike melanoblasts, anterior visceral endoderm cells can dispense with Rac1 for proliferation (Migeotte et al., 2010) and murine neural crest cells have increased apoptosis with loss of Rac1 (Thomas et al., 2010). We wondered if Rac1 loss affects stem cell survival of melanocytes in hair follicles, as was the case for keratinocytes (Benitah et al., 2005; Chrostek et al., 2006), but expression of Tyr::Cre in the nervous system (Tonks et al., 2003) caused a shakiness of Rac1 deleted mice that required culling at around 3 weeks, before they could be aged over several hair cycles.

There is little precedent for the positive involvement of Rac in control of cytokinesis, so it was surprising to find a 3-fold increase in time to divide in Rac1 null cells in vivo. Cytokinesis is driven by a combination of pinching off at the cleavage furrow and relaxation of cortical tension at the spindle poles (Werner and Glotzer, 2008). However, Rac drives the assembly of the cortexillin-actin networks in dividing *Dictyostelium* cells (Faix, 2002) and recently Insall and colleagues found that Scar/WAVE proteins enabled cells to repolarize and crawl apart following cleavage, suggesting a late requirement for Rac at opposite poles of the dividing cells (King et al., 2010). In *C. elegans* early cleavage furrow formation, Rac1 is inhibited by a complex containing the RacGEF CYK4 and centralspindlin, so that Rho can effect contractility (Canman et al., 2008). Rac1 depleted melanoblasts dividing in skin rounded up with the normal timing, but took far longer to divide, indicating a likely requirement for Rac1 in adhesion of the dividing cell to the surrounding milleu and repolarization of the dividing cells to then crawl apart. This defect wasn't seen in cultured cells, indicating that it was specific to the epidermal environment. So while in early cytokinesis, Rac1 may be inhibited at the furrow, later cytokinesis may in some tissue environments utilize activation of Rac for adhesion, polarization and migration of the two daughter cells.

Materials and Methods

Transgenic mice and genotyping

All experiments were performed according to UK Home Office regulations. The Rac1 floxed mice in C57BL6/J background were previously described (Walmsley et al., 2003). Tyrosinase Cre A (Tyr::Cre) mice in C57BL6/J background were previously described (Delmas et al., 2003). The Tyr::Cre transgene is integrated on the X chromosome (Delmas et al., 2003). Therefore, only Rac1 f/f Tyr::$Cre^{+/o}$ males were analyzed as Rac1 null, which will carry one copy of Tyr::Cre and Rac1 f/f Tyr::$Cre^{+/o}$ females were excluded. There are no phenotypes associated with Rac1 f/+ Tyr::$Cre^{+/o}$ animals. Therefore, heterozygous genotype with genotypes: Rac1 +/+ Tyr::$Cre^{+/o}$, Rac1 +/+ Tyr::$Cre^{o/o}$, Rac1 f/+ Tyr::$Cre^{o/o}$ served as control for this study. All other mouse strains used in this study were previously described and are listed in Supplementary Methods.

Antibodies and inhibitors

Primary antibodies and inhibitors were used according to the manufacturer's suggestions, except where indicated in Supplementary Methods. The sources are also indicated in Supplementary Methods.

Ex-vivo imaging of melanoblast migration

Experimental set up was as previously reported with modifications (Mort et al., 2010). Briefly, a freshly dissected E15.5 embryonic skin sample was sandwiched between a nuclepore membrane (Whatman) and a gas permeable Lumox membrane in Greiner Lumox culture dish (Greiner Bio-One GmbH) so that the epidermal side of skin was in contact with lumox membrane. To immobilize the sample, Growth Factor Reduced Matrigel (BD Bioscience) was used to cover the whole assembly and incubated at 37°C for 10 min. Culture medium (Phenol red free DMEM supplied with 10% FBS and 100 μg/ml primocin (InvivoGen) was added. Drugs/inhibitors were added to Matrigel and medium 1 hr before imaging. Timelapse images were captured using an Olympus FV1000 or Nikon A1 confocal microscope in a 37°C chamber with 5% CO_2 at 20× or 60× magnification for 5 hr.

Melanoblast migration speed in skin explants

Individual cells were tracked manually using ImageJ, Manual Tracking plugin, with distance/time measurements taken every 5 min for 3 hr. The migration speed (cell path/time) was calculated using Chemotaxis Tool plugin. More than 60 cells were examined from each of at least 3 different skin explants from different embryos. Mean values ± s.e.m and statistical analysis were calculated and plotted using Graphpad Prism (Graphpad Software) and significance was determined using two-tailed unpaired t-tests.

Cell culture, blotting, imaging and siRNA transfection

Primary mouse melanocytes were cultured as previously reported (Larue et al., 1992) and standard siRNA methods were used as described in the Supplementary Methods. Immunoblotting, immunohistochemistry, live cell imagning and immunofluorescnece of cells and tissues were carried out using standard methods and are described in Supplementary Methods.

Whole-mount staining of embryos

Embryos or the dorsal skin from new born pups were fixed and stained as described in Supplementary Methods.

Melanocyte growth and flow cytometry

Analysis of melanocyte growth and flow cytometry analysis of cell cycle were carried out using standard methods and are described in Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Rac1 and the Scar/WAVE Complex drive pseudopod-based motility of melanoblasts
- Rac1 depleted melanoblasts move using unique actin-based stubs and not blebs
- Rac1 controls pseudopod frequency but is dispensible for pseudopod formation
- Loss of Rac1 delays melanoblast cell cycle progression and cytokinesis

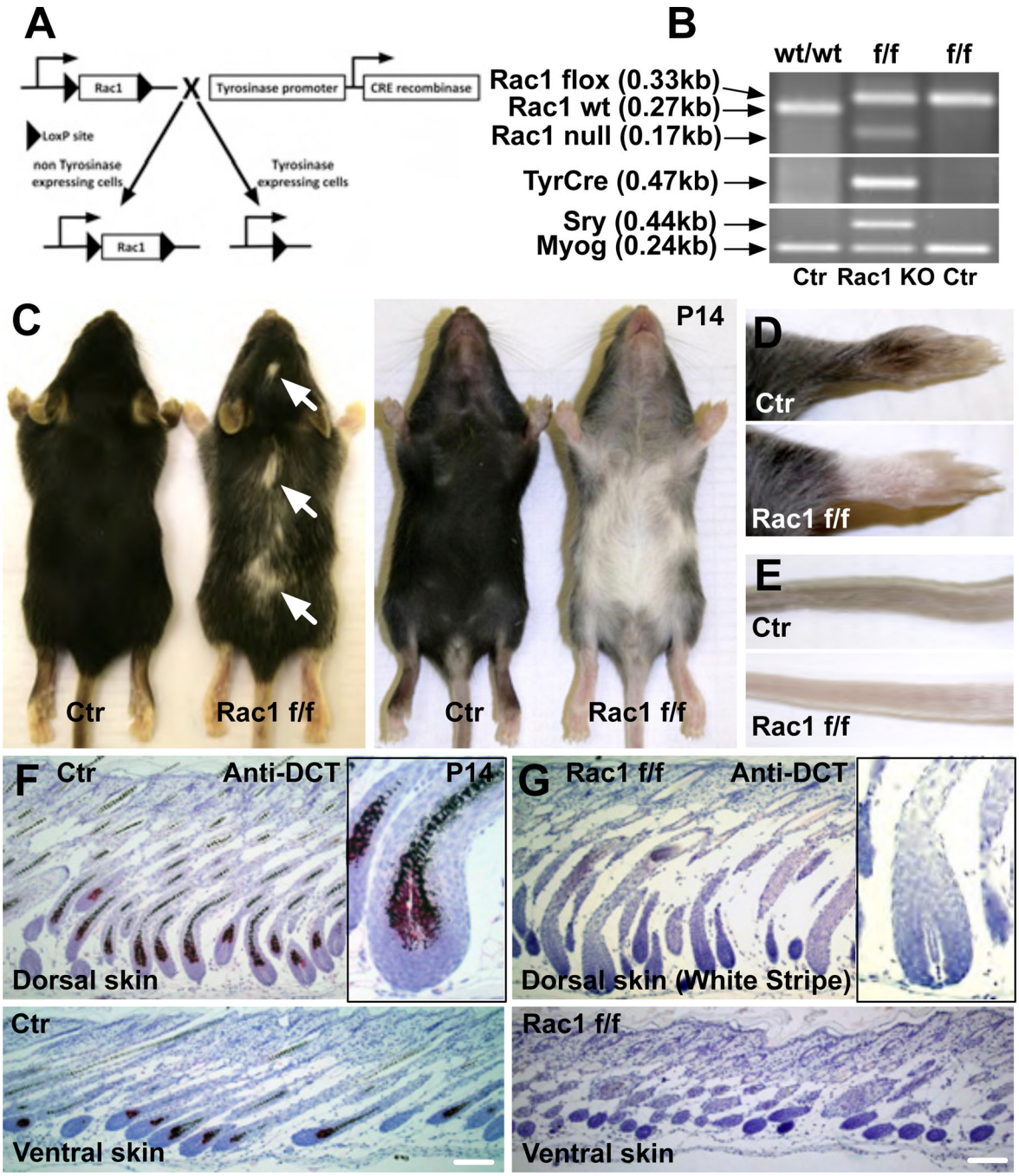

Figure 1. Rac1 f/f Tyr::Cre$^{+/o}$ show absence of melanoblasts in dorsal and ventral patches

(A) Gene targeting strategy for generating Rac1 f/f Tyr::Cre$^{+/o}$ mice. Tyr::Cre mediates excision of Rac1 in the melanocyte lineage. (B) A representative genotyping assay: wild-type (left lane), Rac1 f/f Tyr::Cre$^{+/o}$ (middle lane) and Rac1 f/f Tyr::Cre$^{o/o}$ (right lane). Male-specific maker Sry and universal maker Myog were used to sex mice. (C) Coat-color of P14 Rac1 f/f Tyr::Cre$^{+/o}$ (Rac1 f/f) mouse with control littermate (Ctr). White arrows point to dorsal white patches. (D) Forelimb of P14 Rac1 f/f Tyr::Cre$^{+/o}$ mouse with control. (E) Tails of control and Rac1 f/f Tyr::Cre$^{+/o}$. (F) Dorsal and ventral skin of P14 control and (G) Rac1 f/f Tyr::Cre$^{+/o}$; dorsal white skin patch and ventral skin with anti-DCT (melanocyte). Insets show hair follicles. Scale bars 100μm. See also Figure S1.

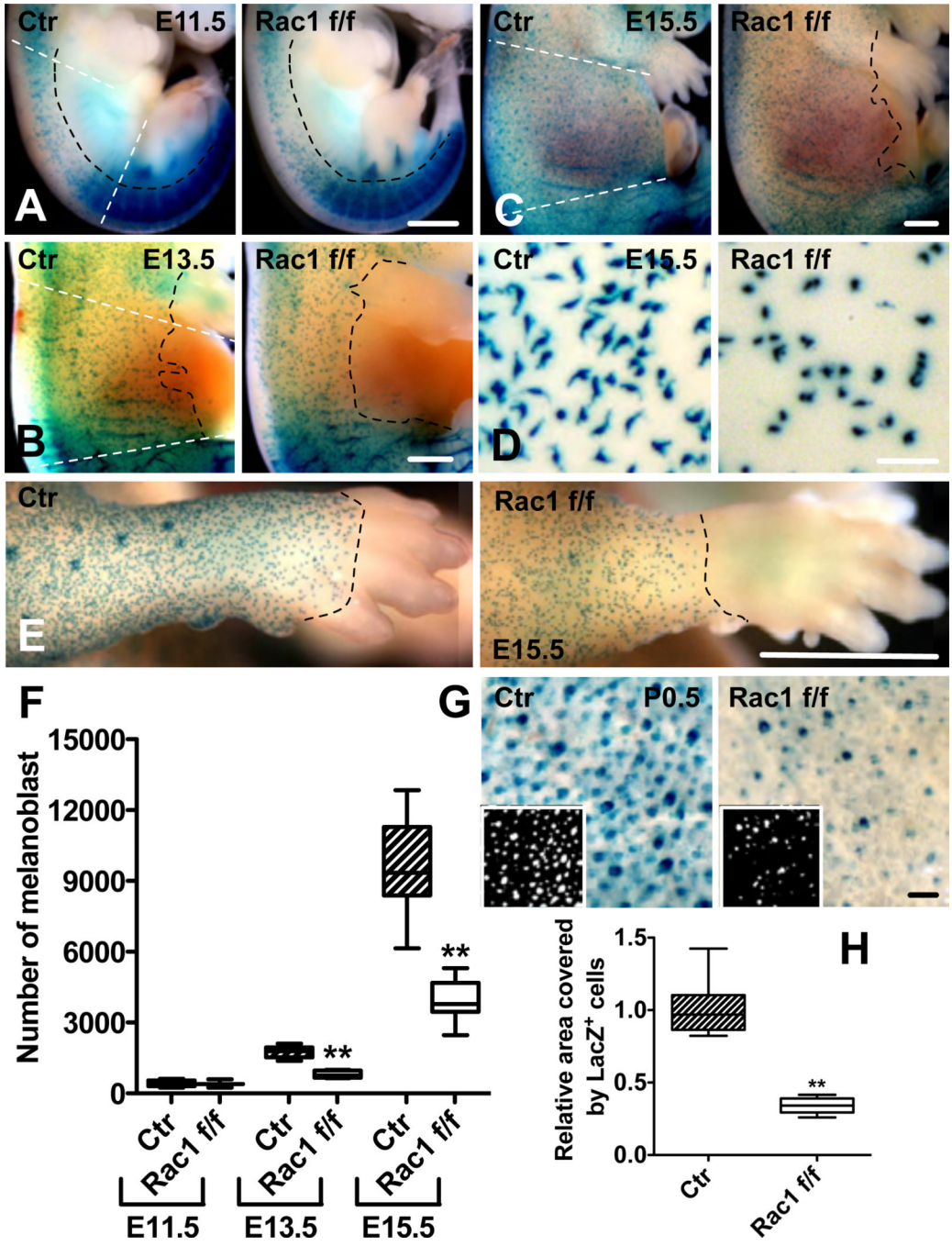

Figure 2. Loss of Rac1 alters melanoblast number and position from E13.5

(A–C) β-galactosidase stained whole mount DCT::LacZ control (left) and Rac1 f/f Tyr::Cre$^{+/o}$ (right) embryos at E11.5, E13.5 and E15.5. White dotted lines in A, B and C indicate the trunk regions for quantification as shown in (F). Images represent (>= 5 embryos, 4 litters) (D) Enlarged images of E15.5 trunk regions (E) Enlarged images of forelimb from E15.5 DCT::LacZ control (left) and Rac1 f/f Tyr::Cre$^{+/o}$ (right) embryos (F) Number of melanoblasts in DCT::LacZ control and Rac1 f/f Tyr::Cre$^{+/o}$ littermate embryos at E11.5, 13.5 and 15.5 from (>=5 embryos, 4 litters). Lower quartile, median, and upper quartile are shown. **, P< 0.01 by t-test (G) Representative β-galactosidase stained dorsal skin samples DCT::LacZ control (left) and Rac1 f/f Tyr::Cre$^{+/o}$ (right) pups at P0.5. Each

image is from (>= 3 pups, 3 litters). Insets show representative thresholded images used in quantification. (H) Relative area covered by melanocytes per image for control and Rac1 f/f Tyr::Cre$^{+/o}$. Data derived from (5 images each from >=3 pups, 3 litters). Lower quartile, median, and upper quartile are shown. **, P< 0.01 by t-test. Scale bars are (A, B, C and E) 1mm, (D) 50μm (G) 100μm. See also Figure S1.

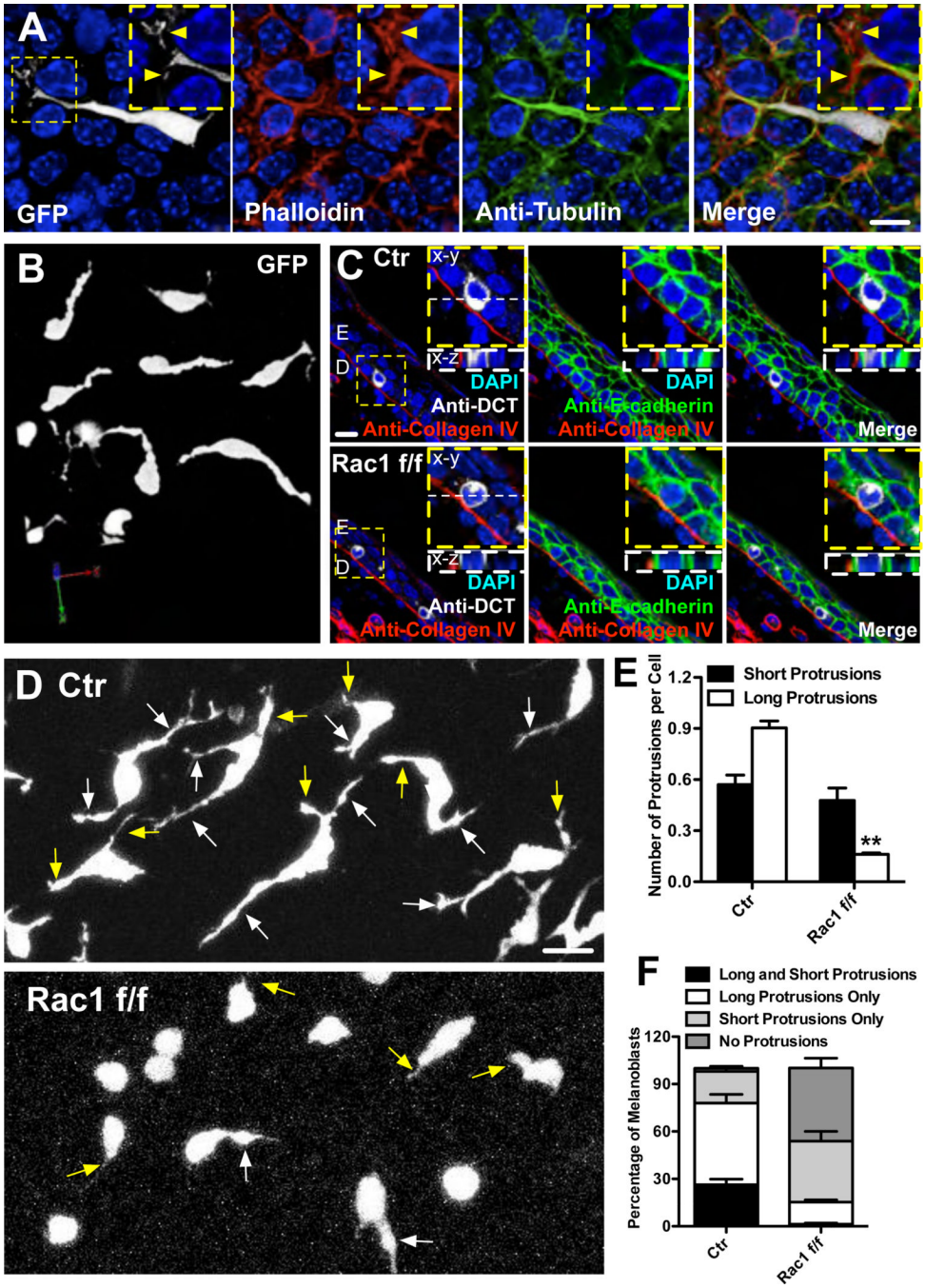

Figure 3. Epidermal melanoblasts display an elongated shape with long protrusions

(A) Combined Z-stack image (1μm depth) of E13.5 Z/EG Tyr::Cre$^{+/o}$ control embryo stained with phalloidin, anti-tubulin and DAPI. Insets show distal tips (yellow arrowed). (B) 3D reconstruction of E15.5 GFP-melanoblasts (see also Movie 1) (C) Representative combined Z-stack images (1μm depth) of sections from E15.5 control and Rac1 f/f Tyr::Cre$^{+/o}$ embryos stained for Collagen IV, E-cadherin, DCT and DAPI. “E” denotes epidermis and “D” denotes dermis above and below the red line of collagen IV staining that delineates the basement membrane. Insets show X–Y and X–Z projections (D) Combined Z-stack images (1μm depth) of melanoblasts in skin explants from Z/EG$^{+/o}$ Tyr::Cre$^{+/o}$ and Z/EG$^{+/o}$ Rac1 f/f Tyr::Cre$^{+/o}$ embryos. Long/short protrusions are white and yellow arrowed

respectively. (E) Number of long/short protrusions per melanoblast n>60 cells per explant >= 3 explants per genotype. (F) Proportion of melanoblasts with long/short protrusions. Error bars indicate Mean ± SEM. **, P< 0.01, by t-test. Scale bars are 10μm.

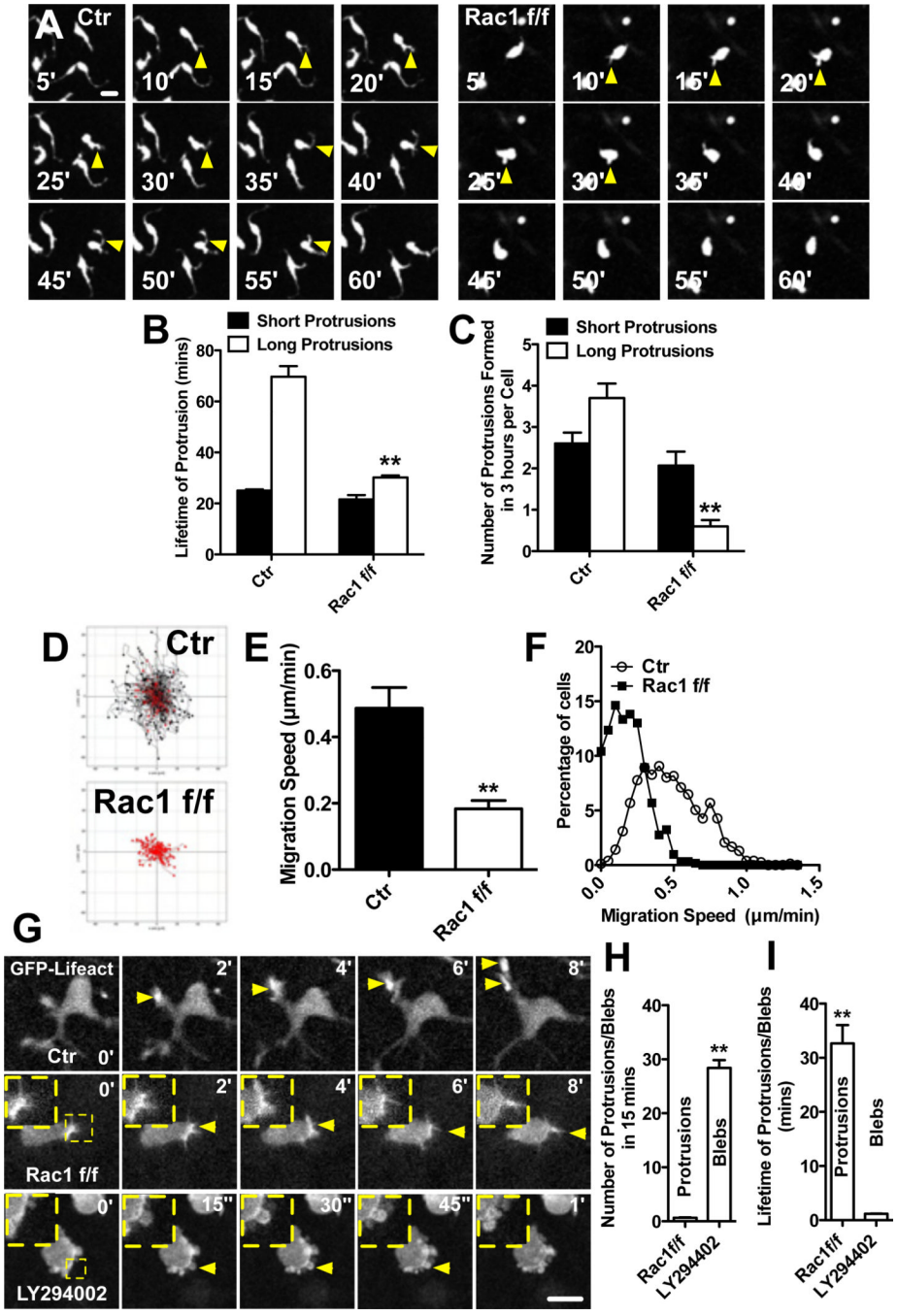

Figure 4. Loss of Rac1 in melanoblasts decreases the migration speed and reduces the number, lifetime and frequency of protrusions

(A) Live cell imaging of protrusion dynamics in explants. Images captured every 5 min, yellow arrows - protrusions. (B) Lifetime of actively growing protrusions formed in 20 cells per explant from >= 3 explants. (C) Frequency of protrusion formation. (D) 3 hr tracks of individual melanoblast migration in skin explants, black tracks migrated faster than average control, red indicates slower. (E) Migration speed. (F) Speed distribution. >300 melanoblasts from three explants were randomly selected and mean migration speed over 3hr was plotted according to frequency in the population. (G) Live cell imaging of F-actin dynamics in GFP-Lifeact f/f Tyr::Cre$^{+/o}$ control or GFP-Lifeact f/f Rac1 f/f Tyr::Cre$^{+/o}$ explants or control

explant treated with 50μM Ly294002. Yellow arrows - protrusions/blebs. (H) Frequency of blebs and short protrusions (I) Lifetime of blebs and protrusions. Error bars indicate Mean ± SEM. **, P< 0.01, by t-test. Scale bars are 10μm.

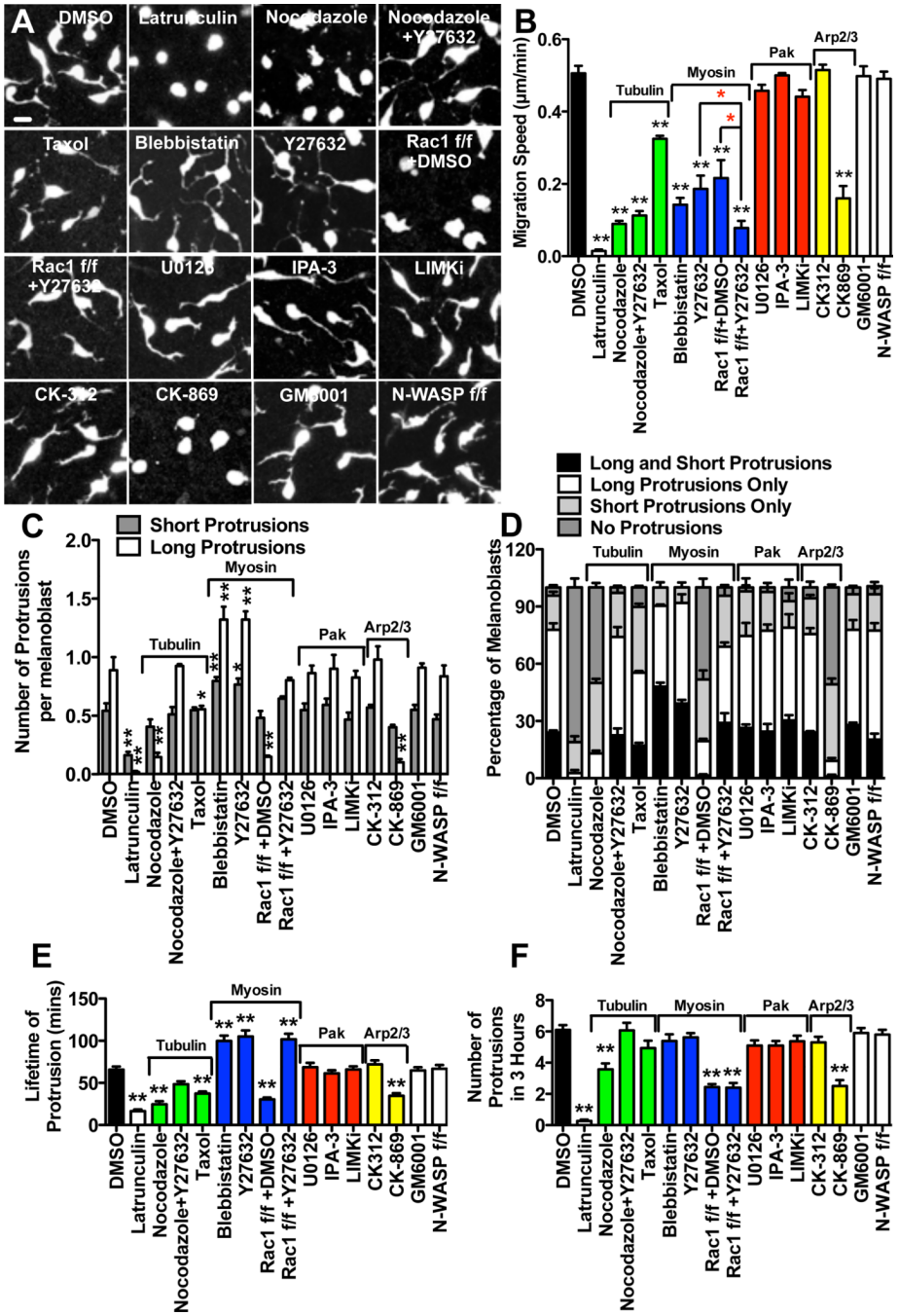

Figure 5. Melanoblast migration and protrusions are regulated by actin, Arp2/3 complex, myosin, microtubules, but not PAK signaling, metalloproteases or N-WASP

(A) Combined Z-stack images (1μm depth) of Z/EG$^{+/o}$ Tyr::Cre$^{+/o}$ control or Z/EG$^{+/o}$ Rac1 f/f Tyr::Cre$^{+/o}$ (Rac1 f/f) melanoblasts in skin explants treated with inhibitor and/or of genotype as indicated. (B) Migration speed. (C) Number of long and short protrusions per melanoblast for >60 cells per explant from >= 3 explants per genotype. (D) Proportion of melanoblasts with short/long protrusions. (F) Lifetime of actively growing protrusions. (F) Frequency of protrusions formed in 20 cells per explant from >= 3 explants per condition. Error bars indicate Mean ± SEM. **, P< 0.01. *, P<0.05 by t-test. Scale bar 10μm. See also Figures S2–4.

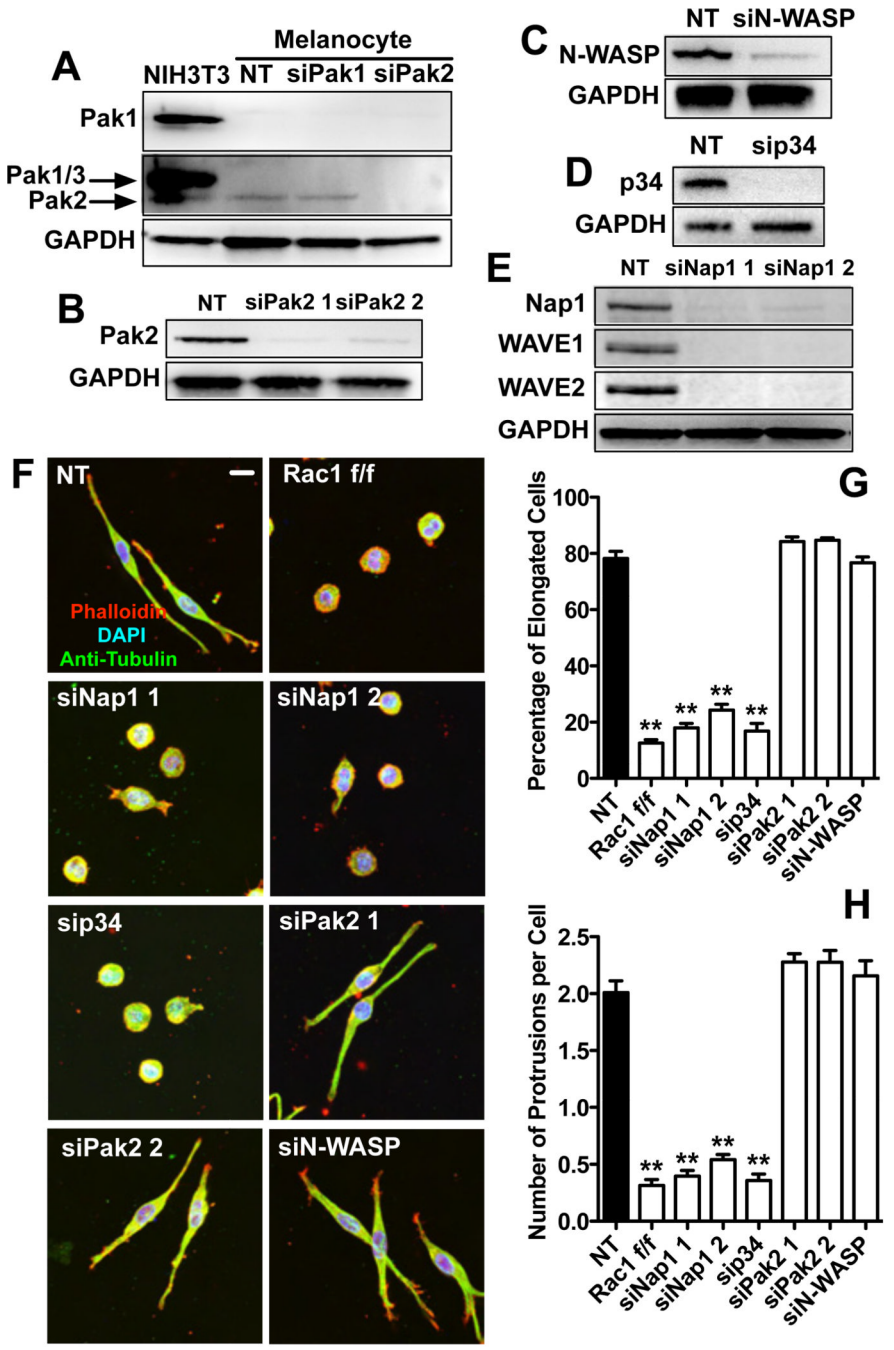

Figure 6. Primary murine melanocytes cultured on collagen require Rac1-WAVE-Arp2/3 pathway for long protrusions

(A) Immunoblots of primary mouse melanocytes transfected with NT, Pak1 and Pak2 siRNA. The same blot was probed with anti-Pak1, anti-Pak1/2/3 and anti-GAPDH as loading control. NIH3T3 mouse fibroblast lysate was used as positive control for Pak1. (B–D) Immunoblots of siRNA treated melanoblasts as indicated (B) Pak2, (C) N-WASP and (D) p34-Arc. (E) Immunoblots showing knockdown of Nap1 in melanocytes; anti-WAVE1 and anti-WAVE2 show the loss of WAVE complex. GAPDH - loading control. (F) Rac1 f/f Tyr::CreERT2$^{+/o}$ Ink4a-Arf$^{-/-}$ (#4) primary melanocytes treated with OHT or siRNA as indicated on 3D collagen I matrix, showing actin (phalloidin), microtubules (anti-tubulin) or

DNA (DAPI) (G) % cells with elongated morphology and (H) Number of protrusions per cell for >300 cells × 3 repeats. Error bars indicate Mean ± SEM. **, P< 0.01 by t-test. Scale bar 10μm. See also Figure S5, S6.

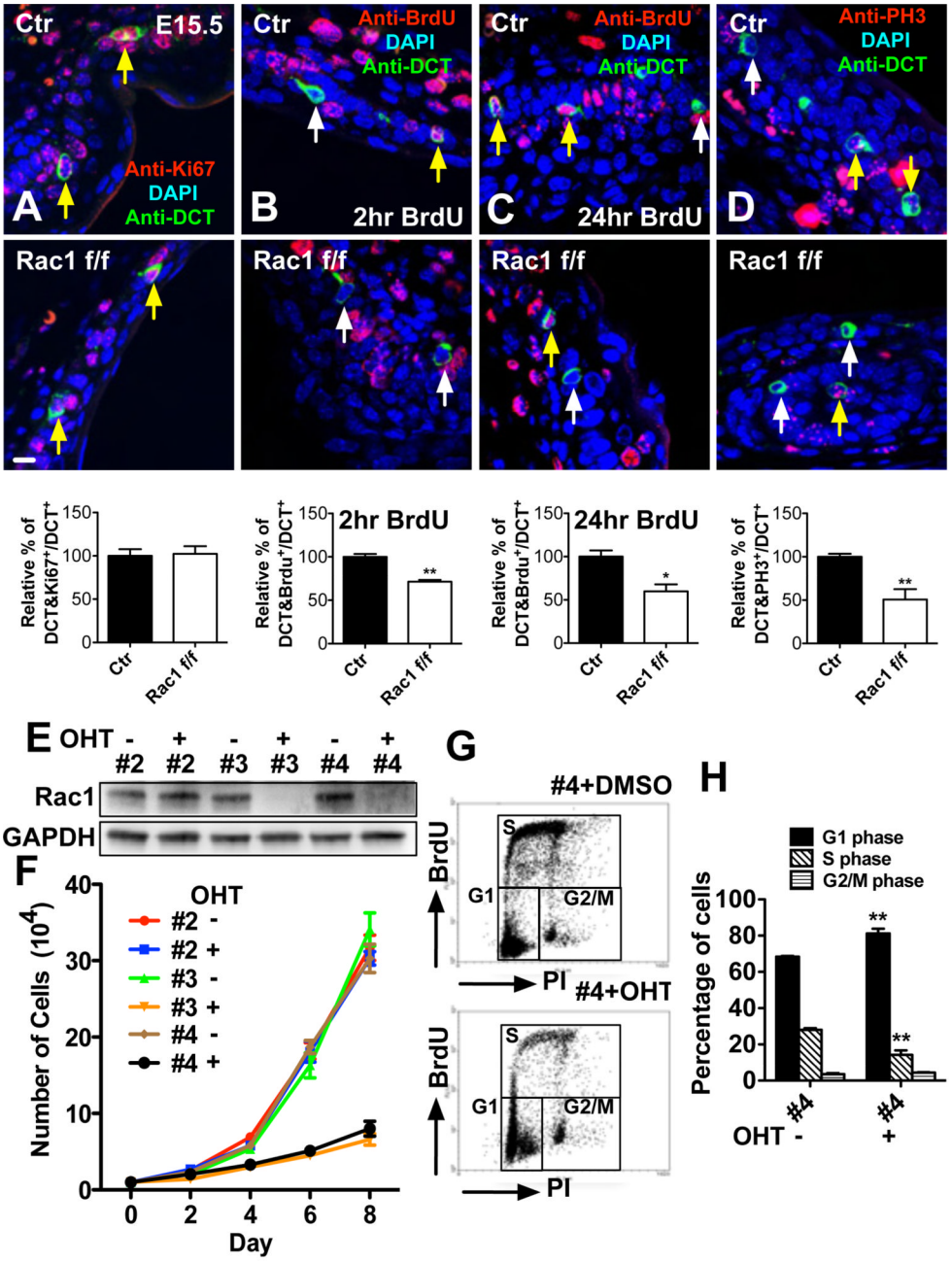

Figure 7. Rac1 is required for melanoblast cell cycle progression in vivo

(A–D) Representative images of E15.5 control (left) and Rac1 f/f Tyr::Cre$^{+/o}$ (right) with anti-Ki67 (proliferation) (A), anti-BrdU (proliferation) (B and C) or anti-PH3 (phospho-histone 3, mitosis) (D) with anti-DCT (melanoblast) and DAPI (DNA). The DCT-positive cells expressing Ki67, BrdU or PH3 are yellow arrowed and DCT-positive cells that do not co-localize with Ki67, BrdU or PH3 are white arrowed. At least 3 embryos per genotype and 200 cells per embryo analyzed. Error bars indicate mean ± SEM. **, P< 0.01 *, P<0.05 by t-test. (E) Western blots from Rac1 f/f Tyr::CreERT2$^{o/o}$ Ink4a-Arf$^{-/-}$ (#2) and two independent Rac1 f/f Tyr::CreERT2$^{+/o}$ Ink4a-Arf$^{-/-}$ primary melanocyte cell lines (#3 and #4) treated with DMSO or OHT probed with anti-Rac1 and anti-GAPDH (loading control).

(F) Growth curve of melanocyte primary lines (#2, #3 and #4) treated with DMSO or OHT. Each point (Mean ± SEM) is from 3 replicates of 3 independent experiments. (G) Representative experiment from Rac1 f/f Tyr::CreERT2$^{+/o}$ Ink4a-Arf$^{-/-}$ (#4) melanocytes treated with DMSO or OHT were pulse-labeled for 2 h with BrdU, and analyzed by flow cytometry for BrdU incorporation and propidium iodide (PI) labeling. (H) % cells in each phase as calculated from (G). N= 3 independent experiments. Error bars: Mean ± SEM. **, P< 0.01 by t-test. Scale bar 10μm. See also Figure S7.