

Rac1 mediates morphogenetic responses to intercellular signals in the gastrulating mouse embryo

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SUMMARY

The establishment of the mammalian body plan depends on signal-regulated cell migration and adhesion, processes that are controlled by the Rho family of GTPases. Here we use a conditional allele of *Rac1*, the only Rac gene expressed early in development, to define its roles in the gastrulating mouse embryo. Embryos that lack *Rac1* in the epiblast (*Rac1 Δ epi*) initiate development normally: the signaling pathways required for gastrulation are active, definitive endoderm and all classes of mesoderm are specified, and the neural plate is formed. After the initiation of gastrulation, *Rac1 Δ epi* embryos have an enlarged primitive streak, make only a small amount of paraxial mesoderm, and the lateral anlage of the heart do not fuse at the midline. Because these phenotypes are also seen in *Nap1* mutants, we conclude that Rac1 acts upstream of the Nap1/WAVE complex to promote migration of the nascent mesoderm. In addition to migration phenotypes, *Rac1 Δ epi* cells fail to adhere to matrix, which leads to extensive cell death. Cell death is largely rescued in *Rac1 Δ epi* mutants that are heterozygous for a null mutation in *Pten*, providing evidence that Rac1 is required to link signals from the basement membrane to activation of the PI3K-Akt pathway in vivo. Surprisingly, the frequency of apoptosis is greater in the anterior half of the embryo, suggesting that cell survival can be promoted either by matrix adhesion or by signals from the posterior primitive streak. Rac1 also has essential roles in morphogenesis of the posterior notochordal plate (the node) and the midline.

KEY WORDS: Rac, Morphogenesis, Embryogenesis, Apoptosis, Pten, WAVE complex, Mouse

INTRODUCTION

Cell fate determination is coupled to morphogenetic movements during mammalian embryogenesis (Arnold and Robertson, 2009; Nowotschin and Hadjantonakis, 2010; Srinivas, 2006). In order to understand the ontogeny of the body plan, it is essential to learn how signals are coupled to the machinery of morphogenesis. Morphogenesis is orchestrated by a small number of modular mechanical properties: cell-cell adhesion, cell-matrix adhesion, protrusion and contractility, all of which require appropriate regulation and dynamics of the cytoskeleton (Montell, 2008). Rho family GTPases are key regulators of all of these cellular processes.

Because of the many cellular functions of Rho family GTPases, cell type-specific expression of dominant-negative and constitutively active forms of the proteins was initially used to define their functions (Jaffe and Hall, 2005). Experiments in zebrafish and *Xenopus* have used dominant-negative forms of Rac1 or Rac1-interacting proteins to define the roles of Rac in gastrulating cells (Bakkers et al., 2004), neural crest cells (Kashef et al., 2009) and primordial germ cells (Kardash et al., 2010), and biosensors have confirmed that activated Rac1 is present at the leading edge of migrating germ cells and neural crest cells (Kardash et al., 2010; Matthews et al., 2008). Despite these elegant experiments, interpretation of the results has been clouded by the recognition that dominant-negative forms often inhibit more than

one GTPase (Wang and Zheng, 2007), and loss-of-function alleles have not been used to validate the specific functions of Rac proteins in early vertebrate morphogenesis.

In the mouse, tissue-specific gene-targeting experiments have indicated that Rho GTPases have more specific roles than anticipated and that these are highly dependent on cell type and developmental context (Heasman and Ridley, 2008). For example, in the developing vascular endothelium, Rac1 is essential for proper cell-cell and cell-matrix interaction, such that *Rac1* deletion disrupts adhesion, motility, permeability and capillary morphogenesis (Fiedler, 2009). By contrast, we showed recently that Rac1 is not required for the integrity or polarity of the visceral endoderm (Migeotte et al., 2010). Thus, to understand the mechanisms of action of Rac in embryonic morphogenesis, it is necessary to examine its functions as the body plan is specified.

Of the three mammalian Rac genes, *Rac1* is expressed ubiquitously and is the only Rac expressed early in embryogenesis (Wang and Zheng, 2007), so the phenotypes of *Rac1* mutants define the roles of Rac proteins in early mammalian development. *Rac1* null mutants die at the time of gastrulation, with extensive apoptosis between the epiblast and the visceral endoderm (Sugihara et al., 1998), but the initial studies did not define the developmental defects caused by loss of Rac1. We showed recently that Rac1 is required in the visceral endoderm for specification of the anterior-posterior body axis of the mouse embryo because it promotes directed collective migration of an extra-embryonic organizer: the anterior visceral endoderm (AVE) (Migeotte et al., 2010). This activity of Rac1 is mediated largely or completely by actin reorganization controlled by the WAVE complex, which promotes the formation of a branched actin network at the leading edge of migrating cells through the actin nucleator Arp2/3 (Takenawa and Suetsugu, 2007).

Nap1 (*Nckap1* – Mouse Genome Informatics) mutant embryos, which lack normal activity of the WAVE complex, have a set of characteristic defects in the migration of nascent mesoderm

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(Rakeman and Anderson, 2006). The WAVE complex can be activated by Rac in combination with acidic phospholipids (Eden et al., 2002; Lebensohn and Kirschner, 2009). Because the disruption of axis specification in *Rac1* null mutants causes early lethality, it has not been possible to analyze the function of Rac1 during gastrulation in null embryos. Here, we use a conditional allele to circumvent the early lethality of *Rac1* null embryos and test whether Rac1 acts upstream of the WAVE complex during mesoderm migration. We find that many aspects of early development proceed in the absence of Rac1 in the epiblast: the embryos form an anterior-posterior body axis, initiate all of the signaling pathways required for gastrulation, and form cardiac, lateral plate, extra-embryonic and paraxial mesoderm. However, in the absence of Rac1, as in *Nap1* mutants, the migration of mesodermal cells is greatly impaired, which leads to a deficit of paraxial mesoderm, a failure of somite formation and cardia bifida. The results suggest that Rac1 implements the signals that promote mesoderm migration through activation of the WAVE complex. In addition, we find that embryos that lack Rac1 in the epiblast show several phenotypes not seen in *Nap1* mutants. In particular, we find that Rac1 is important for cell survival, and that survival can be rescued by lowering the gene dosage of *Pten*, suggesting that Rac1 promotes survival through adhesion-dependent activation of the PI3 kinase pathway.

MATERIALS AND METHODS

Mouse strains

The *Rac1* conditional allele *Rac1^{tm1Tyb}*, a gift from Victor Tybulewicz (Walmsley et al., 2003), was crossed to mice expressing *Sox2-Cre* (Hayashi et al., 2002) or *Ttr-Cre* (Kwon and Hadjantonakis, 2009) to generate epiblast-deleted or visceral endoderm-deleted embryos, respectively. The genotype of the *Rac1 Δepi* (epiblast-deleted) embryos is *Sox2-Cre/+; Rac1^{cond}/Rac1^{null}*; the genotype of the *Rac1 ΔVE* (visceral endoderm-deleted) embryos is *Ttr-Cre/+; Rac1^{cond}/Rac1^{null}*. The *RRQ* gene-trap allele of *Nap1* has been described (Migeotte et al., 2010). The Afp-GFP line (Kwon et al., 2006) and the ubiquitous myristoylated-GFP (Rhee et al., 2006) were gifts from A.-K. Hadjantonakis. *Nodal-lacZ* (Collignon et al., 1996) was a gift from E. Robertson. Mice carrying a targeted null allele of *Pten* (Di Cristofano et al., 1998) were obtained from P. P. Pandolfi. All mice were maintained on a C3H background.

Analysis of mutant embryos

Embryos were dissected in PBS containing 0.4% BSA. In situ hybridization and X-Gal staining were carried out as described (Eggenchwiler and Anderson, 2000). Whole-mount embryos were imaged using a Zeiss Axiocam HRC digital camera on a Leica MZFLIII microscope. For scanning electron microscopy, embryos were fixed overnight in 2.5% glutaraldehyde in PBS, washed in PBS and dehydrated in an ethanol series, and processed and imaged using standard procedures. For immunofluorescence, embryos were fixed in PBS containing 4% paraformaldehyde (PFA) for 1 to 3 hours on ice (and post-fixed in methanol at -20°C for 15 minutes for Rac1 staining). Fixed embryos were embedded in OCT (Tissue-Tek) and cryosectioned at 8 μm . Staining was performed in PBS containing 0.1% Triton X-100 (0.5% for Rac1 staining) and 1% heat-inactivated goat serum (10% for Rac1 staining). Whole-mount embryos were mounted in Vectashield (Vector Laboratories) or 1% agarose. Sections and whole-mount embryos were imaged on a Leica DM1RE2 inverted confocal microscope. Confocal datasets were analyzed using the Volocity software package (Improvision). For western blot analysis, embryos were dissected at E8.5 and snap-frozen after careful removal of extra-embryonic tissues; embryos were genotyped before protein extraction and western blot.

Explant analysis

Primary explant cultures of nascent mesoderm, epiblast and primitive streak were generated as described (Burdal et al., 1993). The explants were cultured for 24–48 hours on Matrigel (BD Biosciences) or an STO feeder layer. Explants were fixed for 10 minutes in PBS containing 4% PFA prior to immunostaining.

Antibodies

Antibodies were: rat anti-E-cadherin (cadherin 1 – Mouse Genome Informatics), 1:500 (Sigma); rabbit anti-laminin, 1:50 (Sigma); rabbit anti-GFP, 1:500 (Invitrogen); rabbit anti-ZO1 (Tjp1 – Mouse Genome Informatics), 1:200 (Zymed); mouse anti-vinculin, 1:200 (Sigma); rabbit anti-activated caspase 3, 1:500 (Cell Signaling) and 1:300 (Promega); rabbit anti-brachyury, 1/2000 (gift of Frank Conlon, UNV Chapel Hill, USA); rabbit anti-fibronectin, 1/800 (Sigma); rat anti- $\alpha 5$ -integrin, 1/200 (BD Biosciences); rabbit anti-phospho-histone H3, 1/200 (MCCF); mouse anti-Rac1, 1/1000 for western blot and 1/10 for immunofluorescence (Upstate); mouse anti-WAVE1 (Wasf1 – Mouse Genome Informatics), 1/500 (BD); rabbit anti- γ -tubulin, 1/5000 (Sigma). F-actin was visualized using FITC- and TRITC-phalloidin, 10 U/ml (Molecular Probes), and nuclei using DAPI (Sigma). Secondary antibodies were from Invitrogen.

Quantification of mitotic index and apoptosis

For quantification of mitotic index, we used ImageJ to count phospho-histone H3-positive cells, as well as the total number of cells (using DAPI), excluding the endoderm outer layer, in transverse sections of wild-type and *Rac1 Δepi* embryos. Statistics were obtained using a non-parametric unpaired *t*-test (GraphPad Prism). For quantification of apoptosis, we counted activated caspase 3-positive cells and the total number of cells in the anterior and posterior epiblast (defined as anterior or posterior to the distal tip of the embryo) in every tenth 1- μm confocal slice from *z*-stacks of whole-mount E6.5 *Rac1 Δepi* embryos, and in the extra-embryonic mesoderm and the embryonic mesoderm (defined as E-cadherin-negative cells), as well as the anterior and posterior epiblast, excluding the endoderm layer, in sagittal sections of E7.5 *Rac1 Δepi* embryos. For the *Pten* interaction study, we counted all cells except the outer endoderm layer, without regional specification.

RESULTS

Rac1 is required in the epiblast for embryonic morphogenesis

Rac1 null embryos die before E7.5 (Sugihara et al., 1998). We showed recently that Rac1 has an early function in the visceral endoderm layer of the early mouse embryo, where it is required for the collective migration of the AVE, the extra-embryonic organizer that defines the position of the primitive streak (Migeotte et al., 2010). Because this important early function of Rac1 leads to early lethality and prevents analysis of its role in later development, we generated embryos that have wild-type visceral endoderm but lack *Rac1* in the epiblast (Fig. 1A). The epiblast-specific mutants of *Rac1* were generated by combining a *Rac1* conditional allele with *Sox2-Cre*, which is active in the late blastocyst (Hayashi et al., 2002). These embryos, which we designate *Rac1 Δepi*, survived to E8.5 (early somite stage). *Sox2-Cre* is expressed 3 days before the onset of gastrulation; no Rac1 protein could be detected in E8.5 *Rac1 Δepi* embryos by western blotting (Fig. 1G), nor in the epiblast of E6.5 *Rac1 Δepi* embryos by immunofluorescence (Fig. 1I).

Although Rac1 was completely absent in epiblast-derived tissues, many aspects of early embryonic patterning and morphogenesis were initiated. The epiblast epithelium exhibited a normal organization and apical-basal polarity, and gastrulation initiated at a single, posterior position in the epiblast, as in wild type. Cardiac, paraxial, lateral plate, extra-embryonic and axial mesoderm and definitive endoderm were specified, as shown by expression of *Nkx2.5* (Fig. 1C), *Meox1* (Fig. 1B), *Twist* (not shown), *Flkl1* (*Kdr* – Mouse Genome Informatics; see Fig. S1F in the supplementary material) and *Shh* (see Fig. 6A).

Despite the normal initiation of development, *Rac1 Δepi* embryos displayed a characteristic set of morphological defects by E8.5. Some paraxial mesoderm was specified, but properly

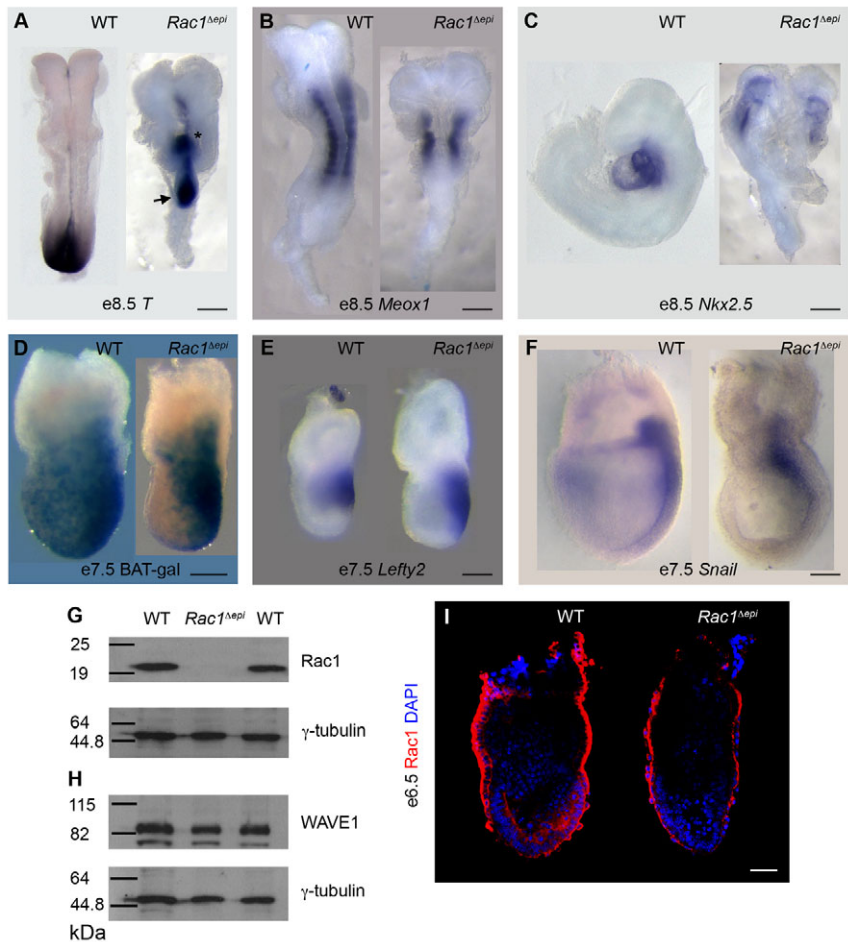


Fig. 1. Rac1 is required in the epiblast for the normal development of mesodermal tissues.

(A) Compared with wild type (WT, left), *Rac1* Δ epi mouse embryos (right) had a short trunk and an open neural tube. Brachyury (*T*)-expressing cells formed a bulge in the region of the primitive streak (arrow). The asterisk marks the abnormal posterior notochordal plate (PNC). Posterior view. (B,C) *Rac1* Δ epi embryos expressed the paraxial mesoderm marker *Meox1* (B), but failed to form well-defined somites. The mutants expressed the cardiac mesoderm marker *Nkx2.5* (C) but showed cardia bifida. (B) Posterior view; (C) wild type, lateral view; *Rac1* Δ epi, anterior view. (D-F) Signaling pathways that regulate gastrulation appeared normal in *Rac1* Δ epi mutants: BAT-gal staining (D) reflects activation of the Wnt canonical pathway, *Lefty2* (E) is a target of the Nodal pathway, and *Snail* (F) is a target of the Fgf pathway. Lateral view, anterior to the left. (G) Western blot for Rac1. No Rac1 protein could be detected in extracts from *Rac1* Δ epi embryos. (H) Western blot for WAVE1. The stability of the WAVE complex was not altered in *Rac1* Δ epi embryos. (G,H) Molecular weights for size markers are shown in kDa. (I) Extended view from five sections of whole-mount confocal z-stacks of E6.5 embryos stained for Rac1. Although there is signal in the outer (visceral endoderm) layer, no Rac1 was detected in the epiblast of *Rac1* Δ epi embryos. Scale bars: 200 μ m in A-C; 100 μ m in D-F; 50 μ m in I.

organized somites did not form, although mutant embryos reached the size of somite stage wild-type embryos (Fig. 1B). Cardiac mesoderm was specified, but the two lateral anlage of the heart did not fuse at the midline (cardia bifida, Fig. 1C). The neural plate was present but did not initiate neural tube closure. This syndrome of defects was strikingly similar to the phenotype of *Nap1^{khlo}* mutant embryos, which lack normal activity of the WAVE complex (Rakeman and Anderson, 2006). To determine whether the morphological phenotypes of *Rac1* Δ epi embryos arise because Rac1 acts upstream of the WAVE complex in epiblast-derived tissues, we characterized the cellular basis of the *Rac1* Δ epi phenotype.

Gastrulation signaling pathways are activated normally in *Rac1* Δ epi embryos

Gastrulation of the mouse embryo, which is the process that generates the mesoderm and endoderm germ layers from the epiblast epithelium, depends on the integrated activity of the Wnt, TGF β and Fgf signaling pathways (Arnold and Robertson, 2009). Because Rac1 has been implicated in signaling downstream of a number of membrane receptors, including receptor tyrosine kinases and Wnt receptors (Wu et al., 2008), we tested the activity of the major gastrulation signaling pathways in *Rac1* Δ epi embryos. As shown by the expression of the Wnt reporter BAT-gal in *Rac1* Δ epi embryos, Rac1 is not required for canonical Wnt signaling at gastrulation (Fig. 1D). The Nodal pathway also appeared normal in the primitive streak of mutant embryos, as assessed by the

expression of the Nodal target *Lefty2* (Fig. 1E) and the *Nodal-lacZ* transgene, which is an autoregulatory target of Nodal signaling (Fig. 6E).

Fgf signaling is crucial for the epithelial-to-mesenchymal transition (EMT) at gastrulation: Fgf signaling activates transcription of *Snail* (*Snail* – Mouse Genome Informatics) (Ciruna and Rossant, 2001), and the Snail transcription factor downregulates the expression of E-cadherin (Ciruna and Rossant, 2001). *Snail* was expressed normally in *Rac1* Δ epi embryos (Fig. 1F) and E-cadherin was not expressed in the mesoderm layer of the mutant embryos (Fig. 2B), indicating that these targets of Fgf signaling were unaffected in the mutants. Other aspects of the EMT also appeared to be normal in *Rac1* Δ epi embryos: cells changed in morphology from an epithelial to a mesenchymal cell type and ZO1 expression was lost from the mesoderm layer (Fig. 2A).

One aspect of the EMT, the degradation of the basement membrane at the position of the streak, was somewhat disrupted in *Rac1* Δ epi embryos. Basement membrane breakdown at the streak, marked by the absence of laminin beneath the epiblast, took place in both the mutant and wild-type embryos (Fig. 2C). However, large laminin aggregates attached to cells that had ingressed through the primitive streak were found on the surface of nascent mesoderm of the enlarged streak (Fig. 2C). Such aggregates were not found in *Nap1^{khlo}* or in *Nap1^{RRQ}* (a stronger *Nap1* allele) embryos (Migeotte et al., 2010) (see Fig. S2C in the supplementary material), suggesting that this phenotype represents a WAVE-independent activity of Rac1. This defect in the degradation of

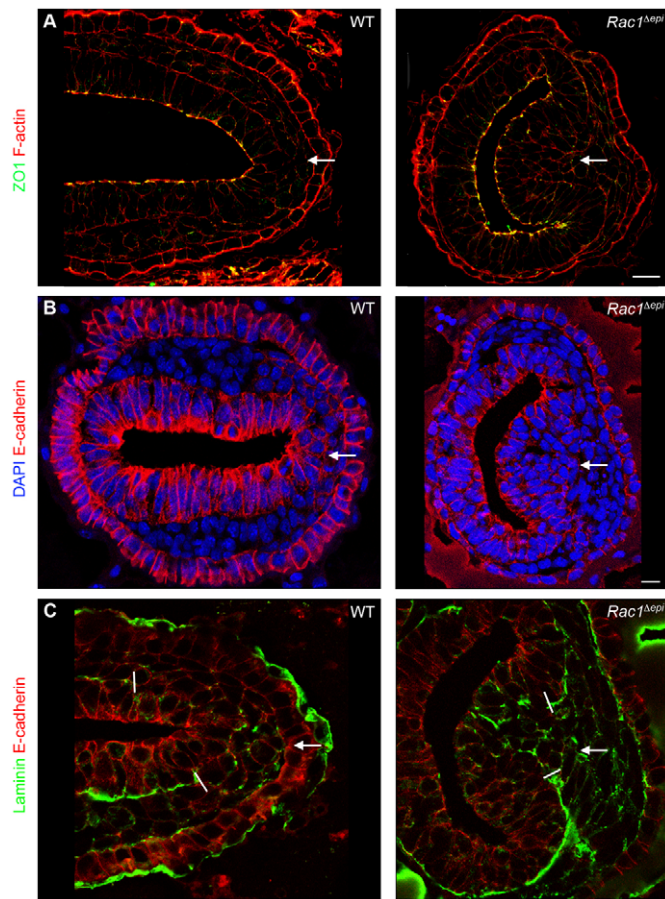


Fig. 2. Migration of the nascent mesoderm is impaired in *Rac1* Δ *epi* embryos. (A,B) The epithelial-to-mesenchymal transition initiated in the primitive streak (marked by arrows in all panels) of *Rac1* Δ *epi* mouse mutants, as shown by the downregulation of ZO1 (A, green) and E-cadherin (B, red) expression in the mesodermal layer, and the change in cell shape from epithelial to mesenchymal (A, F-actin, red). However, mesenchymal cells accumulated at the streak, resulting in an enlarged primitive streak that bulged into the amniotic cavity of the mutants. (C) Breakdown of the basement membrane (laminin, green) occurred in the enlarged primitive streak of *Rac1* Δ *epi* mutants; white bars mark the extent of the region lacking basement membrane. Numerous aggregates of laminin were associated with the mesenchymal cells that accumulated at the primitive streak. Anterior to the left; posterior (primitive streak) to the right. Scale bars: 20 μ m.

basement membrane components is consistent with observations that Rac1 can promote the endocytosis of extracellular proteins, which leads to their degradation (Ahram et al., 2000).

Rac1 is important for mesoderm migration

Completion of the gastrulation EMT depends on coordinated rearrangements of the actin cytoskeleton that allow the nascent mesoderm to migrate away from the primitive streak. The *Nap1*^{khlo} primitive streak is enlarged owing to an accumulation of cells that have downregulated E-cadherin properly but fail to move away from the streak (Rakeman and Anderson, 2006). Similarly, in E7.5 *Rac1* Δ *epi* embryos, cells with the morphology and molecular markers of nascent mesoderm accumulated at the posterior of the embryo, creating a very enlarged streak, often forming a ball of mesenchymal cells in the amniotic cavity, instead of migrating into

the mesodermal wings between epiblast and visceral endoderm (Fig. 2). Brachyury (*T*)-expressing cells of *Rac1* Δ *epi* embryos accumulated at the primitive streak (Fig. 1A), similar to the phenotype of *Nap1*^{khlo} embryos. As in *Nap1*^{khlo} embryos, primitive streak markers [*T*, *Fgf8*, *Tbx6*, *Sprouty1* (*Spry1*), *Lhx1*; see Fig. S1 in the supplementary material] were expressed normally at E7.5, suggesting that cells were correctly specified but failed to migrate away from the primitive streak. Migration of the extra-embryonic mesoderm appeared to be less impaired in the *Rac1* Δ *epi* embryos (as judged by *Flk1*; see Fig. S1F in the supplementary material). The strong similarity of these *Rac1* Δ *epi* phenotypes to those seen in *Nap1*^{khlo} embryos argues that these aspects of the *Rac1* phenotype are due to inappropriate regulation of the WAVE complex in epiblast-derived cells. In contrast to *Nap1*^{khlo} mutant embryos, in which the WAVE complex is not detectable owing to the absence of the Nap1 subunit, the WAVE complex was stable in *Rac1* Δ *epi* embryos (Fig. 1H), as expected if Rac regulates the activity rather than stability of the WAVE complex (Takenawa and Suetsugu, 2007).

Although fewer cells arrived in the mesodermal wings of *Rac1* Δ *epi* embryos, there was a thin layer of mesoderm around the entire embryonic circumference of most embryos. Nascent mesoderm cells express α 5-integrin and secrete the extracellular matrix protein fibronectin, to which they adhere and which acts as a substrate for migration (Burdal et al., 1993). Both proteins were expressed correctly in the mesoderm of *Rac1* Δ *epi* embryos in the enlarged primitive streak and the thin mesodermal wings (see Fig. S2A,B in the supplementary material). As visualized by scanning electron microscopy (SEM), the mutant mesoderm cells were highly abnormal in morphology. The wild-type mesoderm cells had many thin protrusions, consistent with their highly migratory character, whereas mutant cells were round, with just a few short protrusions (Fig. 3B).

Defects in matrix adhesion in *Rac1* null cells

The behavior of cells from *Nap1* mutant embryos has been analyzed in explant cultures, where mutant cells lack normal lamellipodia and are, instead, surrounded by short protrusions (Rakeman and Anderson, 2006) (Fig. 3D). When we attempted to culture cells from E7.5 *Rac1* Δ *epi* embryos, the cells failed to adhere to either fibronectin or Matrigel matrices, consistent with previous reports on *Rac1* null mutants (Sugihara et al., 1998). Because mutant epiblast and mesoderm cells survive better in the embryo, we cultured explants from E7.5 GFP-expressing *Rac1* Δ *epi* embryos on an STO feeder layer. These explants showed better adhesion and survival, but, unlike wild-type explants, mutant cells failed to undergo EMT and exit the explants (Fig. 3A). The mutant cells were less elongated and protrusions were scarce and small, but details of cell behavior could not be analyzed because of the feeder layer.

Younger, E6.5 epiblast explants survived on Matrigel for short periods (less than 24 hours), which permitted analysis of mutant cell behavior. In wild-type explants, cells exited the explants after a few hours; cells at the explant borders lost E-cadherin expression and extended prominent membrane ruffles and filopodia (Fig. 3C,D). However, *Rac1* Δ *epi* embryo explants were small, as most explanted cells did not adhere, and only a few cells exited the explant; the cells at the border of the explant were round and lacked lamellipodia and filopodia (Fig. 3C). Staining for vinculin, a component of focal adhesion complexes, showed that, in wild-type explants, the complexes formed stripes of puncta parallel to the direction of migration and at the tip of filopodia, the dynamic

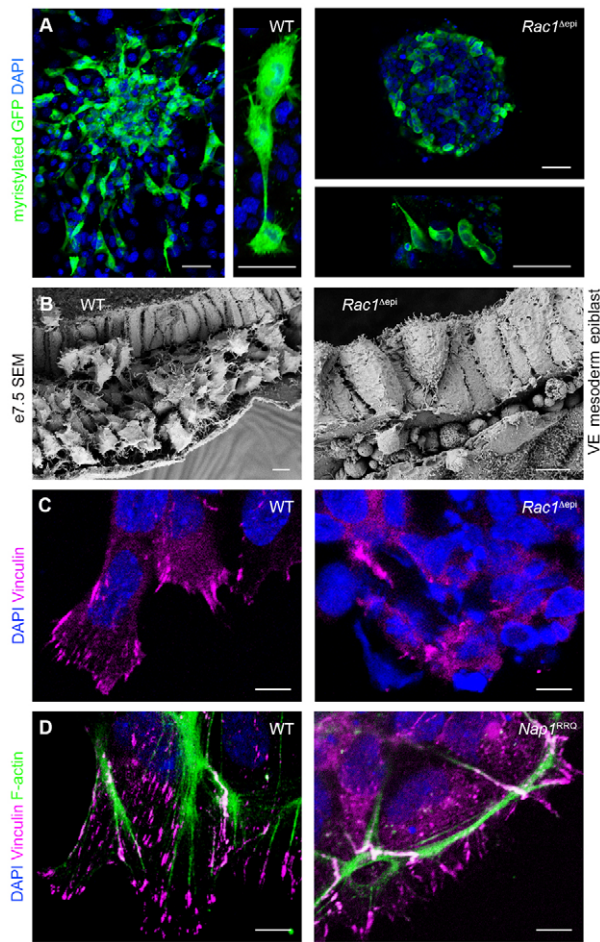


Fig. 3. *Rac1* mutant mesoderm cells fail to migrate. (A) Explants of E7.5 epiblast, primitive streak and mesoderm from wild-type and *Rac1* Δ *epi* mouse embryos expressing myristoylated-GFP were plated on an STO feeder layer and cultured for 24 hours. Wild-type cells migrated away from the explants and displayed long projections and large lamellipodia. By contrast, mutant explants failed to expand. Mutant cells were round and displayed rare filopodia but no lamellipodia. A high-magnification image of a few cells is displayed to the right of (for WT) and beneath (for the mutant) the low-magnification views. (B) E7.5 embryos were processed for scanning electron microscopy (SEM) of the three germ layers. In contrast to the many projections on cells in the mesoderm layer of the wild-type embryo, *Rac1* Δ *epi* mesoderm cells were round and lacked nearly all filopodia. (C) Explants derived from the epiblast of E6.5 embryos were cultured on Matrigel for 24 hours. The cells at the border of wild-type explants were mesenchymal in morphology, had lost E-cadherin expression (not shown) and developed lamellipodia and filopodia that had numerous focal adhesions, as marked by vinculin (magenta). By contrast, in *Rac1* Δ *epi* explants, cells did not migrate away from the explants, displayed no projections and had only diffuse, large focal adhesions. (D) *Nap1*^{RRQ} mutant cells from E6.5 embryos cultured on Matrigel for 24 hours migrated out of the explants less efficiently than did wild type. Similar to *Nap1*^{khlo} mutant cells (Rakeman and Anderson, 2006), actin stress fibers (green, phalloidin) surrounded the cells. *Nap1*^{RRQ} cells lacked lamellipodia but displayed filopodia with focal adhesions. Scale bars: 50 μ m in A; 5 μ m in B; 10 μ m in C,D.

pattern expected for migrating cells that attach and detach constantly (Fig. 3C,D). Some vinculin staining was observed in *Rac1* Δ *epi* explants, but it was present in a diffuse pattern (Fig. 3C). Focal adhesions in explanted *Nap1*^{RRQ} mutant cells (homozygous

for the strong allele of *Nap1*) were present near the tips of filopodia, but were less numerous than in wild-type cells (Fig. 3D), consistent with their WAVE-dependent defect in the formation of lamellipodia.

Cell death in *Rac1* Δ *epi* embryos is region specific and is prevented by lowering the level of *Pten*

The embryonic region of *Rac1* Δ *epi* mutants was smaller than that of wild type from E7.5 onwards. We observed a decrease in proliferation in the *Rac1* Δ *epi* mutants (see Fig. S3 in the supplementary material): the mitotic index obtained by staining E7.5 sections for phospho-histone H3 was $7.8 \pm 1.5\%$ in mutants (from 1171 cells) versus $11.9 \pm 2.9\%$ in wild type (from 889 cells) ($P=0.014$). Large numbers of caspase 3-positive cells were detected in the mesoderm and at the basal side of the epiblast of *Rac1* Δ *epi* embryos (Fig. 4A,B). At E7.5, $\sim 11\%$ (from 7881 cells) of mesodermal cells were positive for activated caspase 3.

Remarkably, cell death in the epiblast was not uniform: there was a threefold higher frequency of apoptotic cells in the anterior half (27%, 3383 cells) than in the posterior half of the *Rac1* Δ *epi* embryo (9%, 2525 cells). There was a somewhat lower rate of apoptosis in E6.5, as compared with E7.5, *Rac1* Δ *epi* embryos (8.0% of cells positive for caspase 3; total $n=10,658$), when mutants were morphologically indistinguishable from wild type (Fig. 4C). However, a similar enrichment of apoptotic cells in the anterior epiblast was already apparent at this stage: there were 2.7 ± 0.3 -fold more apoptotic cells in the anterior than posterior half of the embryo ($n=4$ embryos). Loss of the WAVE complex does not cause the same phenotype: *Nap1*^{RRQ} mutant embryos exhibited only slightly higher levels of apoptosis than the wild type at E7.5 and apoptotic cells were scattered throughout the three germ layers, being most numerous in the visceral endoderm (Fig. 4A). Thus, Rac1 is required to prevent cell death in a WAVE-independent, region-specific manner in the early embryo.

It was shown recently that Rac1 ablation in embryonic stem (ES) cell-derived embryoid bodies leads to massive apoptosis of epiblast cells in contact with the basement membrane (He et al., 2010). Those experiments indicated that Rac1 acts downstream of integrins and upstream of Akt. We reasoned that if a similar pathway were acting in the embryo, increasing phospho-Akt might rescue the cell death phenotype. We generated *Rac1* Δ *epi* embryos that were also heterozygous for *Pten*, a negative regulator of the PI3K-Akt pathway. Remarkably, lowering the gene dosage of *Pten* rescued much of the cell death phenotype (Fig. 5B,C): whereas $11.3 \pm 1.8\%$ of cells (from 23,848 cells) in E7.5 *Rac1* Δ *epi* were positive for caspase 3 in this experiment, only $4.0 \pm 1.7\%$ of cells (from 20,719 cells) in E7.5 *Rac1* Δ *epi*; *Pten*^{-/+} were caspase 3 positive. This provides evidence that Rac1 acts upstream of the PI3K-Akt pathway to promote cell survival in the embryo. Despite the strong rescue of cell death, the morphological phenotype of the *Rac1* Δ *epi*; *Pten*^{-/+} embryos was indistinguishable from that of the *Rac1* Δ *epi* single mutants (Fig. 5A), indicating that the abnormal morphology of the mutant embryos is not secondary to cell death.

Rac1 is required for organization of the posterior notochordal plate and midline

The mouse embryo posterior notochordal plate (PNC; also referred to as the node) and axial midline arise from the anterior primitive streak and are important organizers of the left-right and mediolateral body axes (Kinder et al., 2001; Lee and Anderson, 2008). Marker expression indicated that the organization of midline structures was abnormal in *Rac1* Δ *epi* embryos (Fig. 1A; Fig. 6).

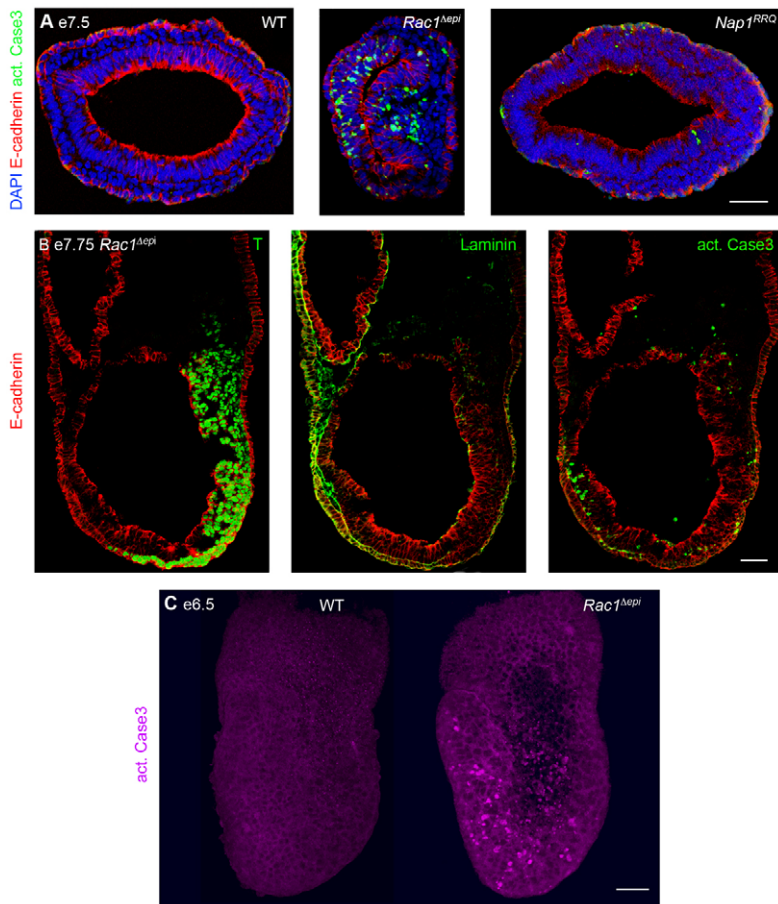


Fig. 4. Localized apoptosis in *Rac1* Δ *epi* embryos.

(A) Transverse sections of E7.5 mouse embryos stained for activated caspase 3 (green). At this stage, wild-type embryos showed virtually no detectable apoptosis, whereas extensive apoptosis was apparent in *Rac1* Δ *epi* embryos in the primitive streak, the thin mesodermal wings and the epiblast. Note the high level of cell death in the anterior epiblast (anterior is to the left). *Nap1*^{RRQ} embryos had only a few apoptotic cells, mostly in the visceral endoderm. (B) Sagittal sections of an E7.75 *Rac1* Δ *epi* embryo stained for brachyury protein (which marks the primitive streak, node and midline), laminin and activated caspase 3 (all in green). Extensive apoptosis is apparent in the anterior epiblast. (C) Three-dimensional reconstruction of whole-mount confocal z-stacks of E6.5 embryos stained for activated caspase 3 (magenta). In wild-type embryos, a few apoptotic cells could be seen shedding into the amniotic cavity. *Rac1* Δ *epi* embryos, which are morphologically indistinguishable from wild type at this stage, showed a high rate of apoptosis, most strongly in the anterior region of the epiblast. Scale bars: 50 μ m.

As the rate of cell death in the *Rac1* Δ *epi* was relatively low in the distal cells that give rise to the PNC and midline (Fig. 4B), it was possible to characterize the role of *Rac1* in the organization of these midline tissues.

The midline was broad in E8.5 *Rac1* Δ *epi* mutants, as shown by the expression pattern of *Shh* (Fig. 6A) and *FoxA2* (Fig. 6B), which are expressed in the PNC and notochord. At late E7.5, *Shh* (Fig. 6C) and *FoxA2* (Fig. 6D) were expressed in distal regions of *Rac1* Δ *epi* mutants, suggesting that cells with PNC identity had been specified. The *Nodal-lacZ* transgene is expressed in the wild-type E7.5 primitive streak and becomes restricted to a horseshoe-shaped region (the crown cells) surrounding the posterior side of the PNC by E7.75 (Collignon et al., 1996) (Fig. 6E). In *Rac1* Δ *epi* mutants, *Nodal-lacZ* was expressed in the primitive streak and the prospective PNC region (Fig. 6E), but did not resolve into the horseshoe shape. Those dispersed distal *lacZ*-positive cells are likely to be misplaced visceral endoderm-derived crown cells, but could also be epiblast-derived cells inappropriately expressing *Nodal*. By E8.5, the mutant embryos were severely dysmorphic, which precluded analysis of later markers of left-right asymmetry in the lateral plate or heart.

Cells of the prospective PNC in *Rac1* Δ *epi* mutants were arranged in clusters of apically constricted cells [as shown by staining for F-actin (Fig. 7A) and by SEM (Fig. 7B,C)] and grew the long cilia typical of the PNC (Fig. 7D), but the cells failed to coalesce to make the normal PNC. Cells of the notochordal plate express brachyury, and brachyury was expressed near the prospective PNC and midline of *Rac1* Δ *epi* embryos. However, the region that expressed brachyury was broad and short (Fig. 7A,E),

which could reflect a defect in convergent extension in the mutants. However, the *Rac1* Δ *epi* midline was much broader than has been described in planar cell polarity (PCP) mutants (Song et al., 2010; Ybot-Gonzalez et al., 2007), as individual brachyury-expressing cells were present on the lateral sides of the embryo, far from the midline (Fig. 7A).

At gastrulation, most of the visceral endoderm cells that cover the early embryo are dispersed by intercalating definitive endoderm cells, but a subset of visceral endoderm cells aligns around the developing midline (Kwon et al., 2008). We crossed the *Afp-GFP* transgene, which is expressed specifically in cells of the visceral endoderm (Kwon et al., 2006), into the mutant background to help define the organization of the midline in the mutants (Fig. 7E). In the *Rac1* Δ *epi* mutants, the visceral endoderm cells (which are wild-type for *Rac1*) were dispersed over most of the embryo, which demonstrated that *Rac1* mutant definitive endoderm was specified, exited from the primitive streak and successfully intercalated with the cells of the outer visceral endoderm layer. However, *Afp-GFP*-expressing cells were present on top of the PNC and midline, and were not aligned flanking the midline structures, indicating that the mutant PNC and midline were not able to direct the proper behavior of the visceral endoderm cells, either because of their structural anomalies or because of a defect in *Rac1*-dependent signaling. In the reciprocal experiment, we deleted *Rac1* only in the visceral endoderm using the *Ttr-Cre* transgene (Kwon and Hadjantonakis, 2009). A relatively normal PNC and midline developed in these embryos, but *Rac1* mutant visceral endoderm-derived *Afp-GFP*-expressing cells were present on top of the PNC and midline and mutant VE cells failed to form a border around the midline

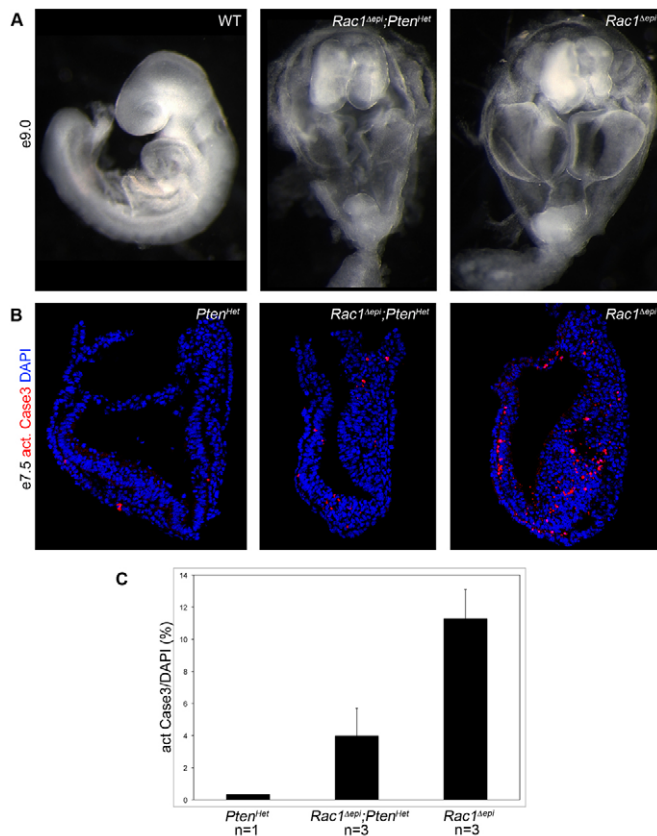


Fig. 5. Heterozygosity for *Pten* rescues apoptosis in *Rac1* Δ *epi* embryos. (A) At E9.0, the morphology of *Rac1* Δ *epi* and *Rac1* Δ *epi*; *Pten*^{+/+} mouse embryos was undistinguishable. (B) Sagittal sections of *Rac1* Δ *epi* and *Rac1* Δ *epi*; *Pten*^{+/+} embryos stained for activated caspase 3 (red). Apoptosis is reduced in double mutants, without regional differences. (C) Quantification of B. Error bars indicate s.d.

structures. These observations indicate that Rac1 is required for the normal organization of both the streak-derived midline cells and the visceral endoderm cells around the midline.

DISCUSSION

Although Rac proteins have been implicated in a host of different developmental processes, the expression of only a single Rac gene in the early mouse embryo and the availability of a conditional allele allowed us to define specific aspects of early mammalian development that require Rac activity. We find that Rac is not necessary for the transcriptional response to developmental signaling pathways, but that it does mediate morphogenetic responses to those signaling pathways.

Rac1 is required for WAVE-dependent embryonic morphogenesis and the migration of nascent mesoderm

We previously showed that the WAVE complex is required for the migration of nascent mesoderm cells away from the primitive streak. Because the morphology of *Nck1*; *Nck2* double mutants is reminiscent of the *Nap1*^{khlo} phenotype (Bladt et al., 2003), we suggested that Nck proteins were likely to activate the WAVE complex in nascent mesoderm (Rakeman and Anderson, 2006). We now provide evidence that Rac1 acts upstream of the WAVE complex to control mesoderm migration. The defects in mesoderm

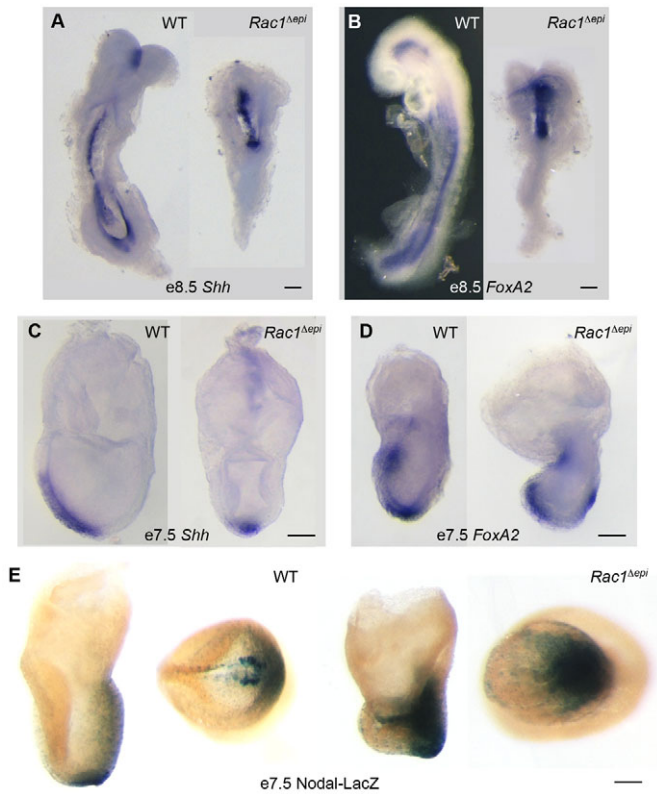


Fig. 6. Rac1 is required for normal expression of midline markers. (A,B) In situ hybridization for *Shh* (A) and *FoxA2* (B), which are expressed in the floor plate, posterior notochord, notochordal plate and gut endoderm, highlights the broad midline in E8.5 *Rac1* Δ *epi* mouse mutants. Ventral view, except for the wild-type embryo in B, which is shown in lateral view. (C,D) At E7.5, the PNC was specified in *Rac1* Δ *epi* mutants, as shown by expression of *Shh* (C; wild type, lateral view; mutant, anterior view) and *FoxA2* (D; anterior to the left). (E) *Nodal-lacZ* was expressed in the primitive streak and PNC regions of the mutant, as in wild type, but crown cells did not organize into a crescent around the PNC. Lateral (left, anterior to the left) and distal (right) views are shown. Scale bars: 100 μ m.

migration in *Rac1* Δ *epi* and *Nap1*^{khlo} embryos lead to identical developmental defects: nascent mesoderm cells accumulate in a bulge at the primitive streak, only a small amount of unsegmented paraxial mesoderm is specified, the lateral heart anlage fail to fuse at the midline, and the neural tube fails to close. Given that Rac1 acts upstream of the WAVE complex in cultured cells (Lebensohn and Kirschner, 2009), we propose that both Rac1 and Nck proteins are required to activate the WAVE complex to enable the actin remodeling required for migration of the nascent mesoderm.

Remarkably, mesoderm cells that completely lack Rac1 can migrate, albeit inefficiently, as some mesoderm cells navigate the entire circumference of the embryo so that mesodermal wings encircle the embryo, as was also seen in *Nap1* mutant embryos (Rakeman and Anderson, 2006). These mesoderm cells might use lamellipodia-independent pseudopods to migrate, as has been observed in *Rac1* null fibroblasts (Vidali et al., 2006). By contrast, we have shown that the collective migration of the AVE is absolutely dependent on Rac1-mediated protrusions (Migeotte et al., 2010), demonstrating that the mechanism of migration is cell type-dependent.

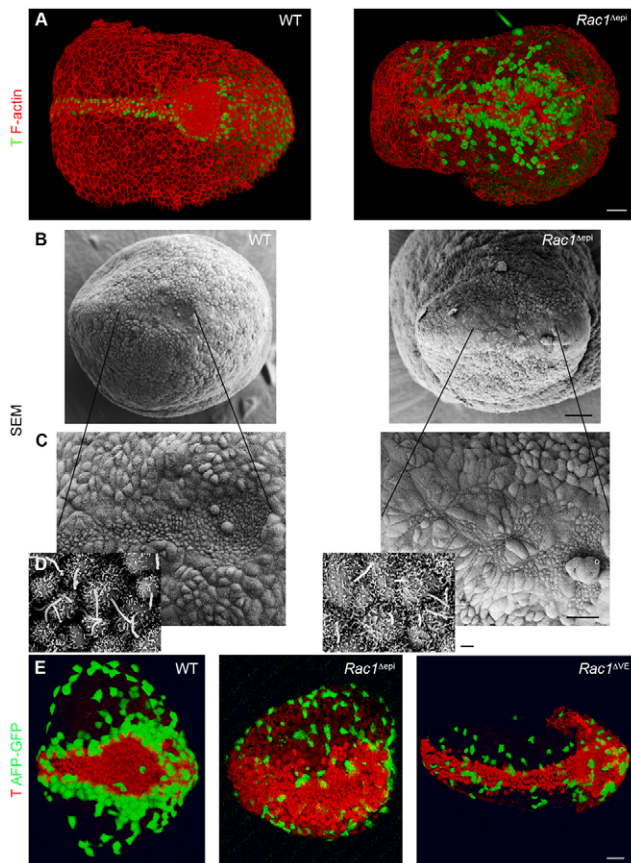


Fig. 7. Disrupted organization of the midline in *Rac1* Δ *epi* embryos. (A) Three-dimensional rendering of confocal stacks of mouse embryos stained for F-actin (red) and brachyury protein (green), which is expressed in the epiblast-derived cells of the PNC and midline. In wild-type embryos, apically constricted brachyury-expressing cells formed a 4-cell-wide continuous midline. Brachyury-expressing cells covered a broad domain in *Rac1* Δ *epi* mutants and did not form a coherent group. (B–D) SEM views of the PNC and midline. Ciliated cells with constricted apical surfaces were present in the PNC and midline of *Rac1* Δ *epi* mutants, but they failed to coalesce into a single PNC and a tightly packed midline. Instead, several clusters of PNC cells were present in the PNC region, no pit was formed, and the midline was broad, irregular and kinked. (E) Three-dimensional renderings of confocal stacks of E7.75 embryos carrying an *Afp-GFP* transgene (green), which is expressed exclusively in the visceral endoderm, stained for brachyury (red). In wild-type embryos, visceral endoderm-derived cells are excluded from the PNC and midline and form a continuous border around them (Kwon et al., 2008). In *Rac1* Δ *epi* embryos, visceral endoderm-derived cells (which are wild-type for *Rac1*) failed to align around mutant brachyury-expressing cells. In embryos in which *Rac1* was deleted specifically from the visceral endoderm using Ttr-Cre, the morphology of the PNC and midline was normal. However, mutant visceral endoderm cells were found ectopically on top of the wild-type PNC and midline, and failed to form a continuous border. All images are ventral views, anterior to the left. Scale bars: 50 μ m in A,B,E; 20 μ m in C; 1 μ m in D.

The signals that activate Rac1 to promote mesoderm migration in the mouse are not known. In *Drosophila*, Fgf signaling promotes mesoderm migration (Beiman et al., 1996; Gisselbrecht et al., 1996) in a process that requires the three *Drosophila* Rac proteins (van Impel et al., 2009). Our data show that transcriptional targets

of Fgf signaling are expressed normally at gastrulation in the absence of Rac1, but our findings are consistent with the possibility that Rac1 mediates a branch of Fgf signaling that controls mesoderm migration. In *Drosophila* mesoderm migration, it is believed that the Rho GEF Pebble provides a link between the Fgf receptor and Rac (van Impel et al., 2009), and it will be important to test whether the homologous GEF (Ect2) (Hansen et al., 2003) links Fgf receptor signaling and Rac-dependent mesoderm migration in the mouse.

WAVE-independent roles of Rac1 in matrix adhesion and cell survival

In contrast to *Nap1* mutant cells, which adhere to matrix in culture, *Rac1* mutant cells show a nearly complete failure to adhere to, and spread on, matrix. We were not able to culture mutant E7.5 epiblast or mesoderm explants on any matrix, although the mutant cells showed some ability to adhere to a feeder layer of cells. Cells from younger mutant embryos were able to adhere to matrix to some extent, but did not form the small focal adhesion complexes normally associated with lamellipodia in migrating cells (Lock et al., 2008). Because of the strong defect in WAVE-dependent migration, we cannot assess the contribution of the lack of matrix adhesion to the *Rac1* Δ *epi* mesoderm migration phenotype.

It is likely that the defect in adhesion contributes to the high level of apoptosis that we observed in the epiblast and nascent mesoderm in the *Rac1* Δ *epi* embryos. Our findings in the embryo are consistent with data obtained after conditional inactivation of *Rac1* in ES cell-derived embryoid bodies, where there were very high levels of apoptosis in epiblast cells adjacent to the basement membrane (He et al., 2010). Those authors provided evidence that Rac1 is activated through integrin-mediated recruitment of the Crk-Dock180 (Dock1) complex, and that activation of the PI3K-Akt signaling pathway promotes cell survival. We found that lowering the gene dosage of *Pten* (in order to increase levels of phospho-Akt) in *Rac1* Δ *epi* embryos largely prevents apoptosis in epiblast and mesoderm cells that lack all Rac1. Thus, our data provide strong *in vivo* support for the model that Rac1 in the epiblast is required to link signals from the basement membrane to activation of the PI3K-Akt pathway. Despite this important connection between Rac1 and the Akt pathway, the final morphology of E8.5 *Rac1* Δ *epi* embryos is not modified by *Pten* heterozygosity; thus, the morphological phenotype is due primarily to a *Pten*-independent role of Rac1 in cell migration.

A previous study noted that there was a high rate of cell death on the basal side of the epiblast of *Rac1* null embryos, where the mesoderm should be (Sugihara et al., 1998). The early death and the streak localization defect in null embryos prevented a more detailed analysis. In *Rac1* Δ *epi* embryos, we observed similar numbers of cells positive for activated caspase 3 in the ectoderm and mesoderm. In contrast to the case with embryoid bodies, we were able to observe the spatial pattern of cell death in whole embryos and found that the likelihood of apoptosis depends on the position of the cell in the embryo, with cells in the anterior of dying at about three times the frequency of cells in the posterior of the embryo. The source of this difference in cell survival is not clear, although the signals localized to the posterior of the embryo that promote formation of the primitive streak (Wnt, Nodal and Fgf) can also be pro-survival signals. Thus, cells in the anterior of the embryo, which see lower levels of streak-derived signals, might rely more heavily on Rac-dependent signals from the extracellular matrix for survival.

Rac1 in midline morphogenesis

Formation of the PNC and midline organizers of the embryo depends on a set of complex morphogenetic movements (Lee and Anderson, 2008). Despite the defects in gastrulation and the high rate of cell death, the PNC and midline fates are specified in headfold stage *Rac1* Δ epi embryos, which allowed us to examine the role of Rac1 in the morphogenesis of these tissues. One of the defects in the *Rac1* Δ epi embryos is that the clusters of PNC precursors fail to coalesce to form a single concave nodal pit, reminiscent of the node of embryos that lack the FERM domain protein Lulu (Epb4.115) (Lee et al., 2010).

A second defect in the *Rac1* Δ epi midline is that brachyury-expressing cells are spread across a wide domain flanking the midline of the embryo. Narrowing and elongation of the midline depend on PCP-mediated convergent extension movements in zebrafish and *Xenopus* (Roszko et al., 2009). In mouse *Vangl1*; *Vangl2* double mutants, which show strong PCP phenotypes, there is a defect in convergent extension of the axial mesoderm (Song et al., 2010) and Rac has been implicated as a downstream effector of PCP in vertebrates (Roszko et al., 2009). Thus, the broad midline we observe in *Rac1* Δ epi mutants could be consistent with a role for Rac downstream of planar polarity proteins during convergent extension in vivo. However, the midline of *Rac1* Δ epi embryos is much broader than that of mouse PCP mutants, suggesting that other processes required for midline morphogenesis also depend on Rac1. One possible scenario is that the ectopic brachyury-expressing mutant cells represent midline cells from the mid-gastrula organizer (Kinder et al., 2001) that migrate with nascent mesoderm and fail to reach the midline because of the general defect in mesoderm migration. These observations, in addition to the absence of the normal border between the epiblast-derived midline and the adjacent visceral endoderm, indicate that Rac1 has multiple roles in midline morphogenesis.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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