RasGRF suppresses Cdc42-mediated tumour cell movement, cytoskeletal dynamics and transformation

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Individual tumour cells move in three-dimensional environments with either a rounded or an elongated 'mesenchymal' morphology. These two modes of movement are tightly regulated by Rho family GTPases: elongated movement requires activation of Rac1, whereas rounded/amoeboid movement engages specific Cdc42 and Rho signalling pathways. In siRNA screens targeting the genes encoding guanine nucleotide exchange factors (GEFs), we found that the Ras GEF RasGRF2 regulates conversion between elongated- and rounded-type movement. RasGRF2 suppresses rounded movement by inhibiting the activation of Cdc42 independently of its capacity to activate Ras. RasGRF2 and RasGRF1 directly bind to Cdc42, outcompeting Cdc42 GEFs, thereby preventing Cdc42 activation. By this mechanism, RasGRFs regulate other Cdc42-mediated cellular processes such as the formation of actin spikes, transformation and invasion in vitro and in vivo. These results demonstrate a role for RasGRF GEFs as negative regulators of Cdc42 activation.

Abnormal migration and invasion of tumour cells are key components of the metastatic phenotype. Individual tumour cells have different forms of movement: a 'mesenchymal' mode, undertaken by cells with an elongated morphology, which requires extracellular proteolysis at the leading edge, and so-called 'amoeboid' movement, in which cells have a rounded appearance with no obvious polarity. These modes of movement are interconvertible and Rho GTPases, key regulators of cell migration, have been identified as central elements in this transition. Elongated movement requires activation of Rac1 (RAS-related C3 botulinum toxin substrate 1), whereas rounded/amoeboid movement engages specific Cdc42 signalling pathways. Ameboid movement also requires RhoA–ROCK (Rho-associated kinase) signalling to induce the necessary levels of actomyosin contractility to deform the extracellular matrix and propel cell movement. Rho GTPases are activated by Rho GEFs that fall into two distinct families: those containing Dbl homology–pleckstrin homology (DH–PH) domains and DOCK (dedicator of cytokinesis) family GEFs. DOCK family GEFs lack DH–PH domains and their GEF activity is mediated by DHR (DOCK homology region) domains. In short interfering RNA (siRNA) screens to unveil regulators of Rho family GTPases controlling melanoma cell movement, we identified Dock10 as a Cdc42 activator specifically required for the rounded form of movement and Dock3 as a Rac1 GEF mediating elongated-type movement.

RasGRF2 controls the conversion between elongated and rounded movement

The panel of siRNAs used in these screens also targeted the genes encoding the GEFs of the RasGRF (RAS protein-specific guanine nucleotide-releasing factor) family. These archetypical Ras GEFs, in addition to a Cdc25 domain that catalyses guanine nucleotide exchange on Ras, also harbour DH–PH domains. In melanoma cells A375P and A375M2, which differ in the proportion of cells with a rounded morphology and amoeboid movement, downregulation of RasGRF2 increased the proportion of cells moving with a rounded morphology, and increased the levels of phosphorylated myosin light chain 2 (MLC2), a marker of rounded/amoeboid motility. Fig. 1a,b and Supplementary Movies S1 and S2). Similar results were obtained in melanoma cell lines WM266.4, WM1361 and SKMEL28 (Supplementary Fig. S1a). Conversely, overexpression of RasGRF2 in A375M2 cells led to an increase in the number of elongated cells (Fig. 1c and Supplementary Movies S3 and S4 and Fig. S2b), comparable to that induced by the ROCK inhibitor Y27632 (Fig. 1c and Supplementary Fig. S2b; ref. 3). To determine whether the GEF domains of RasGRFs were involved in these effects, we used deletion mutants for the Cdc25 and DH domains (Supplementary Fig. S2a). Notably, the ΔCdc25 mutant increased the number of elongated cells as efficiently as wild-type RasGRF2.

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RasGRF2, whereas the ΔDH mutant had no significant effect (Fig. 1c and Supplementary Movies S5 and S6). These results indicated that RasGRF2 regulates elongated/mesenchymal to rounded/amoeboid transition by a mechanism mediated by the DH–PH domain and independently of the Ras pathway.

**RasGRF GEFs inhibit Cdc42 activation**

As elongated movement involves Rac1 activation, we examined whether RasGRF2 regulates the activity of Rac1. We found that either reducing the level of RasGRF2 expression by siRNAs or overexpression of RasGRF2 had little effect on Rac1 activation levels in A375M2 (Fig. 1d and Supplementary Fig. 3a). Similar results were found in HeLa cells, where suppression of RasGRF2 also resulted in enhanced rounded morphology (data not shown). Furthermore, we saw no effect of RasGRF2 depletion or overexpression on the activation status of RhoA. These data imply that RasGRF2 does not act through regulation of Rac1 or Rho.

Cdc42 activation can lead to elevated levels of phosphorylation of MLC2 and the rounded/amoeboid form of cell movement. To determine whether Cdc42 activation was affected by alterations in RasGRF2 expression, we used HeLa, Jurkat and A375M2 cells that endogenously express this GEF (refs 14,15; V.S-M. and C.J.M., unpublished results). RasGRF2 overexpression markedly inhibited Cdc42 activation. Conversely, reducing RasGRF2 levels with siRNAs increased the activation status of Cdc42, an effect particularly noticeable under basal conditions and less pronounced in growing cells. (Fig. 2a,c and Supplementary Fig. S3b). In agreement with these results, we found that levels of phosphorylated Pak1 (p21 protein (Cdc42/Rac)-activated kinase 1), a downstream effector of Cdc42 (ref. 16) inversely correlated with levels of RasGRF2 expression (Fig. 2b and Supplementary Fig. S3c).

As an additional control for off-target effects of siRNA, we showed that the effects of siRNA-mediated downregulation of endogenous RasGRF2 on Cdc42 activation and phospho-MLC2 levels could be overcome by the ectopic expression of murine wild-type RasGRF2 and a ΔCdc25 mutant, but not by a ΔDH mutant (Supplementary Fig. S3d). Cdc42 activation in response to stimulation with ionomycin or TNF-α was largely unaffected by abrogating RasGRF2 expression (Fig. 2c). In contrast, RasGRF2 downregulation resulted in a marked increase in Cdc42–GTP levels following stimulation by lysophosphatidic acid (LPA). We also analysed whether RasGRFs impact on Cdc42 activation resulting from the overexpression of Cdc42 GEFs. Overexpression of RasGRF1 or RasGRF2, but not another Ras GEF, Sos1 (son of sevenless homologue 1), markedly reduced Cdc42 activation induced by Dbl (also known as Mcf2, mcf.2 transforming sequence), Ost (also known as Ptprv, protein tyrosine phosphatase, receptor type, V) and Dock10. Phosphorylated Pak1 levels were affected similarly (Fig. 2d). Rho–GTP

**Figure 1** Depletion of RasGRF2 affects cell morphology and movement in a Rac/Rho-independent manner. (a,b) Bottom left, histograms showing the percentage of elongated A375M2 (a) and A375P (b) cells, after transfection of three siRNAs against RasGRF2 (no. 7–10). (800 cells per experiment; n = 4 in A375M2 and n = 5 in A375P; error bars indicate s.e.m.; *P < 0.05, **P = 0.01 and ***P < 0.001 by Student’s t-test.) Top left, immunoblots, representative of 4 or 5 independent experiments, showing the efficiency of RasGRF2 downregulation. Positive control cells (C+) were transfected with a cDNA for RasGRF2. Right, histograms (top) showing the increase in phospho-MLC2 after transfection of two siRNAs against RasGRF2 (no. 7,10) relative to control (C) cells as quantified from western blots (representative blot shown at the bottom). (Error bars indicate s.e.m.; *P = 0.05 and **P < 0.01 by Student’s t-test.) Middle, images of mock- or RasGRF2-siRNA-transfected A375M2 cells plated on a thick layer of collagen. Scale bars, 100 μm in low-magnification images and 20 μm in high-magnification images. (c) Overexpression of RasGRF2 potentiates elongated morphology. A375M2 cells were stably transfected with vector or RasGRF2 wild type, ΔCdc25 and ΔDH mutants. RI, vector-transfected cells treated with Y27632. (800 cells per experiment; n = 4; error bars indicate s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001 and ns P > 0.05 by Student’s t-test.) Western blot confirms expression of indicated proteins. (d) Rac1 and Rho activation levels in A375M2 and HeLa cells after depletion (siRNA, si) or overexpression of RasGRF2, in cells exponentially growing or serum-starved for 24 h. Uncropped images of blots are shown in Supplementary Fig. S8.
To investigate the mechanism whereby RasGRFs prevent Cdc42 activation under basal conditions and in response to some stimuli. HeLa cells, control or RasGRF2 depleted (siRNA, si), were serum-starved (St.) and stimulated with the indicated agonists. Iono., ionomycin. (d,e) RasGRF GEFs inhibit Cdc42 and Pak1 but not RhoA or Rac1 activation. COS-7 cells were transfected with HA-tagged GTPases in addition to Ras GEFs, plus vector (Vec.) or the indicated Ras family GEFs onco-Dbl, Ost, Dock10DHR2 domain and Vav1. Graph shows fold increase in phosphorylated PAK1 levels as quantified from western blot performed as shown above the graph (error bars indicate s.e.m; n = 3;  * P < 0.05,  **P < 0.001 and ns P > 0.05 by Student’s t-test, relative to the values in the respective controls (C)). Representative western blot used for quantification is shown. (c) Depletion of RasGRF2 increases Cdc42 activation under basal conditions and in response to some stimuli. HeLa cells, control or RasGRF2 depleted (siRNA, si), were serum-starved levels were unaffected under the same circumstances, whereas Rac activation stimulated by Vav1 was slightly enhanced by the Ras GEFs (Fig. 2e). Overall, these results indicate that RasGRFs specifically affect Cdc42 activation and not that of Rho and Rac1.

RasGRF GEFs directly bind to Cdc42

To investigate the mechanism whereby RasGRFs prevent Cdc42 activation, we determined whether there was a direct interaction between RasGRFs and Cdc42. For RasGRF2, we used cell lines that endogenously express this GEF; for RasGRF1 we used homogenates of mouse brain, where this isoform is enriched. In all cases, immunoprecipitation of RasGRFs pulled down Cdc42, indicating that the proteins are in a complex. Reciprocal co-immunoprecipitations yielded similar results (Fig. 3a). The interaction seemed to be specific, as RasGRFs did not associate with other GTPases, such as Rap1 (also known as Rap1a, RAS-related protein-1a), nor did Cdc42 bind to the Ras GEF Sos1. The specificity of the interaction was further demonstrated in HeLa and Jurkat cells, where RasGRF2 could not be detected in RhoA or Rac1 immunoprecipitates or Cdc42 in Sos1 immunoprecipitates (Supplementary Fig. S4a). As previously observed, RasGRF1 and RasGRF2 interacted with wild-type Cdc42 and with the S17N mutant, which has reduced affinity for nucleotides and exhibits increased binding to GEFs, but not to a constitutively GTP-bound Q61L mutant (Supplementary Fig. S4b). Abrogating RasGRF2 expression enhanced Cdc42 activation by LPA but not by ionomycin (Fig. 2c). Significantly, both ligands activated H-Ras to similar levels (Supplementary Fig. S4c) through RasGRF-mediated reactions (Fig. 3b). These data therefore show that RasGRFs selectively interact with the inactive forms of Cdc42 and that this interaction can be regulated by agonist stimulation.

To further characterize the interaction between RasGRFs and Cdc42, we used deletion mutants of RasGRF2 (Supplementary Fig. S2a). RasGRF2 mutants lacking either the full DH domain or the PH2 domain, including the portion overlapping with the DH domain, were deficient for binding to Cdc42N17 (Fig. 3b). Furthermore, using constructs consisting of green fluorescent protein (GFP) fused to the DH domains of RasGRF1 and 2, we demonstrated that the DH domain...
Figure 3 RasGRF GEFs associate with Cdc42 in vivo. (a) RasGRF and Cdc42 form a complex. Lysates from cell lines and mouse brain homogenates (Mm brain hom.) were immunoprecipitated for RasGRF (top panels) or Cdc42 (bottom panels). Immunoprecipitates (IP) and corresponding total lysates (TL) were probed by immunoblotting. Control immunoprecipitation using pre-immune serum (PI) was included. (b) RasGRF2 requires a full DH domain for binding to Cdc42. COS-7 cells were transfected with HA–Cdc42N17 plus GFP, GFP-tagged RasGRF1 and 2, or their corresponding DH mutants. Proteins bound to immunoprecipitated Cdc42 were revealed by immunoblotting. Vec., vector-transfected cells. Uncropped images of blots are shown in Supplementary Fig. S8.

RasGRF on Cdc42 activation were mediated by effects on other GTPases. RasGRF2 is not related to its Ras GEF or Rac GEF activities. Cdc42–, Ras–, Rac1– and RhoA–GTP levels were determined in HeLa cells transfected with AU5–Cdc42N17 and onco-Dbl, plus RasGRF2 or its Cdc25 and DH mutants. (c) RasGRFs outcompete Cdc42 GEFs for binding with Cdc42. COS-7 cells were transfected with AU5–Cdc42N17 plus Dbl in addition to Ras GEFs. Cdc42 was anti-AU5 immunoprecipitated and associated proteins were revealed by immunoblotting. Vec., vector-transfected cells. Uncropped images of blots are shown in Supplementary Fig. S8.

RasGRF2 ΔDH mutants. Proteins bound to immunoprecipitated Cdc42 were revealed by immunoblotting. Vec., vector-transfected cells. Uncropped images of blots are shown in Supplementary Fig. S8.

Rac1 and RhoA activation (Fig. 3e). Overexpression of RasGRF2 wild type and ΔDH potentially activated H-Ras, as expected, but had no effects on Rac1 and RhoA activation. Moreover, overexpression of RasGRF2 ΔCdc25, incapable of activating H-Ras, markedly reduced Cdc42–GTP levels, but had no impact on Rac1 and RhoA activities (Fig. 3e). Overall, these results indicate that the effects of RasGRFs on Cdc42 activation are direct, depending on the interaction between RasGRFs and Cdc42.
RasGRFs antagonize Cdc42 binding to its activating GEFs

As we have shown that the DH domain of RasGRF interacts with Cdc42 but does not stimulate Cdc42 activation, we monitored whether RasGRF1/2 interfered with the ability of Cdc42 GEFs to interact with Cdc42. We conducted competition assays using Cdc42N17, as it shows maximal interaction with GEFs such as Dbl and Dock10 (Supplementary Fig. S5a). In the presence of RasGRF1 or RasGRF2, association of Cdc42N17 with Cdc42 GEFs Dbl, Ost and Dock10 was markedly diminished (Fig. 3f and Supplementary Fig. S5b). This was not seen with the DH–PH-containing Ras GEF, Sos1 (Fig. 3f). We also observed that mutants of RasGRF1 and 2 lacking their DH domains did not interfere with Cdc42 binding to Dbl (Fig. 3h and Supplementary Fig. S5c). Furthermore, Dbl bound to Cdc42N17 in vitro was progressively displaced by increasing concentrations of a purified peptide spanning RasGRF1 DH–PH domains (Supplementary Fig. S5d). These results confirmed that RasGRF2 association to Cdc42 through its DH domain is sufficient for its inhibitory function by preventing Cdc42 binding to its activating GEFs.

RasGRFs regulate Cdc42-mediated transformation and cytoskeletal processes

Next, we evaluated the participation of RasGRF1/2 in cellular processes controlled by Cdc42. Cdc42 is required for fibroblast transformation by Dbl (ref. 26), whereas transformation by Vav1 depends on Rac1 and RhoA (ref. 27). Expression of RasGRF1 or RasGRF2 diminished the transforming capacity of Dbl but not Vav1, as analysed in NIH3T3 fibroblasts by foci formation and soft-agar colony assays (Fig. 4a and Supplementary Fig. S6a,b). These results were recapitulated by the ∆Cdc25 but not by the ∆DH mutant, in agreement with the effects of these mutants on Cdc42 activation. Cdc42 activation can lead to the formation of filopodia25; therefore, we analysed the impact of RasGRF1/2 on this process. The expression of the Dock10 DHR2 domain in NIH3T3 cells potently induced filopodia formation, in a Cdc42-dependent fashion; this effect was impaired when RasGRF1, RasGRF2 or their ∆Cdc25 mutants were co-expressed, but not by the ∆DH mutants (Fig. 4b and Supplementary Fig. S7a). In contrast, the lamellipodia and profuse actin stress fibres observed in Vav1-transfected cells were unaltered by expression of RasGRFs (Supplementary Fig. S6b). In a parallel approach, we examined the formation of filopodia when endogenous RasGRF2 expression was attenuated using siRNAs. In normal growth conditions, HeLa cells have some filopodia; however, knocking down RasGRF2 by RNA interference remarkably increased the number of these structures, an effect abolished by the inhibition of Cdc42 either by short hairpin RNA (shRNA) or an inhibitory mutant (Fig. 5a,b and Supplementary Fig. S7b). Conversely, overexpression of RasGRF1 or RasGRF2 almost completely abolished the formation of filopodia, to a similar extent to that following suppression of Cdc42 expression by shRNA. Overall, these results demonstrate that RasGRF family GEFs have a profound influence in cellular processes regulated by Cdc42.

RasGRF2 regulates invasion by melanoma cells

Finally, we investigated the impact of RasGRF2 on cell invasion, a process highly dependent on Rho GTPase signalling. Depletion of RasGRF2 in a set of melanoma cell lines resulted in increased invasive capacity into a three-dimensional (3D) collagen I matrix

Figure 4 RasGRF GEFs regulate Cdc42-mediated transformation and filopodia formation in NIH3T3 cells. (a) RasGRF expression blocks Cdc42-mediated transformation. NIH3T3 cells were transfected with onco-Dbl plus the indicated cDNAs. The bars represent the number of transformed foci, mean ± s.d. of five independent experiments, expressed as a percentage relative to those induced by onco-Dbl alone. (*** P < 0.001, ** P < 0.01, * P < 0.05 and ns P > 0.05 by Student’s t-test.) (b) RasGRFs impair Dock10-induced filopodia formation. Representative confocal micrographs of NIH3T3 cells transfected with Dock10DHR2–GFP plus the indicated constructs. Cells were immunostained to monitor the expression of RasGRFs (blue) and stained with phalloidin (red) to mark filamentous actin. The symbols in the bottom left illustrate the confocal plane shown. Scale bar, 10 µm.
Figure 5 Endogenous RasGRF2 regulates Cdc42-mediated filopodia formation in HeLa cells. (a) Downregulation of endogenous RasGRF2 levels affects filopodia formation in HeLa cells. Top, representative confocal micrographs of HeLa cells co-transfected with pEGFP plus RasGRF2 siRNA, Cdc42 shRNA or Cdc42N17 and transfected with GFP-tagged versions of RasGRF1 and RasGRF2. Cells were starved and stained with phalloidin (red). Scale bar, 20 µm. Bottom, magnifications (×5) of the indicated areas. Filopodia numbers were compared between transfected versus untransfected cells in the same preparation. (b) Immunoblots showing the specificity of Cdc42 knockdown. Uncropped images of blots are shown in Supplementary Fig. S8.

DISCUSSION

We have identified RasGRF family GEFs as modulators of Cdc42 functions including tumour cell movement, invasion and colonization, critical aspects of metastatic dissemination. We unveil RasGRF family GEFs as regulators of the conversion between Rac1-dependent, elongated/mesenchymal-type movement and actomyosin-contractility-dependent, rounded-type movement in tumour cells. RasGRFs, through inhibiting Cdc42 activation, a key component of actomyosin-contractility-dependent tumour cell movement, can regulate the conversion from rounded to elongated modes of movement. These results provide a hitherto undescribed function for RasGRF family GEFs beyond their role in Ras activation. Although we cannot rule out Ras activation playing a role in the plasticity of tumour cell movement, our findings show that RasGRFs can regulate the transition between rounded and elongated modes of movement independently of their Ras-activating Cdc25 domain. RasGRFs directly bind to Cdc42 though their DH domains, but do not induce GDP/GTP exchange in this GTPase. The structural basis for this is unclear, but the homology between the DH domains of RasGRF1/2 and that of the archetypical Cdc42 GEF, Dbl, is below 50% and critical residues for Dbl exchange activity, such as E502, C592 and Q633, are not conserved in RasGRF1/2 (ref. 29). The selectivity of the interaction of RasGRFs with Cdc42 but not Rac1 and RhoA could be due to a steric clash with Trp 56 (Trp 58 in RhoA) that, as opposed to Cdc42 Phe 56, cannot accommodate some DH domains, as previously observed for other Rho GEFs (ref. 30).
We have also demonstrated that RasGRFs outcompete bona fide Cdc42 GEFs for binding to Cdc42 and, subsequently, activating Cdc42. Notably, RasGRFs are most effective at regulating Cdc42 functions under unstimulated conditions. Interestingly, stimuli that induce RasGRF activity on Ras, such as ionomycin and LPA (refs 19,20), behave differently with respect to Cdc42. RasGRF dampens Cdc42 activation by LPA, but not by ionomycin, and we have found that LPA potentiates RasGRF binding to Cdc42, whereas ionomycin does not. Thus, RasGRF expression and regulation could be critical for defining thresholds of Cdc42 activation. In contrast, the non-functional GAP (GTPase-activating protein) domain of IQGAP (IQ-motif-containing GAP protein) can enhance Cdc42 activation31. These data indicate that inactive GEF and GAP domains may be important regulators of Rho family GTPase activation. As the balance between Rac1 and Cdc42 activities is critical in the decision between rounded/amoeboid versus mesenchymal modes of cell movement, RasGRFs may be major determinants of these transitions. Interestingly, RasGRF2 is either hypermethylated or downregulated transcriptionally in primary tumours32,33. Similarly, RasGRF2 deficiency favours the development and dissemination of lymphomas in mouse models34; these properties are reminiscent of genes associated with tumour suppressor activities. In contrast, Dock10 is overexpressed in aggressive thyroid carcinomas exhibiting distant metastases35. These results indicate that the activation of Cdc42 resulting from the overexpression of exchange factors such as Dock10 and Dbl, as well as from the downregulation of negative regulators such as RasGRFs, may contribute to a more aggressive metastatic phenotype.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology

Note: Supplementary Information is available on the Nature Cell Biology website

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AUTHOR CONTRIBUTIONS

F.C. carried out all of the experiments with the exception of those shown in Figs 1 and 6, which were carried out by F.C. and V.S.-M. V.S.-M. and F.W. carried out the invasion assays and imaging of the invading cells. L.A.-I. carried out the in vitro GTPase binding experiments and the soft-agar colonies. F.C. and V.S.-M. also prepared the figures and carried out the statistical analyses. E.S. contributed in the lung colonization assays. F.C., V.S.-M., C.J.M. and P.C. conceived the study and C.J.M. and P.C. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Plasmids. Plasmids encoding RasGRF1 mutants have been described previously39. RasGRF2 mutants are cloned in-frame with an amino-terminal Flag epitope and have been described previously39. Plasmids encoding RasGRF1–GFP and RasGRF2–GFP were generated by replacing the Cdc25 domain of both GEFs with the sequence encoding GFP. The same approach was carried out with the ADH domain. Plasmids encoding GRF1DH–GFP and GRF2DH–GFP were generated by cloning the DH domains of RasGRF1 and RasGRF2 into pEFGP. All Cdc42 forms (wild type, N17 and QL) were subcloned in-frame in pCEFL–haemagglutinin (HA), pCEFL–AU3 and pCEFL–Flag vectors. Plasmids encoding onco-Vav1–GFP have been described previously37, pKeto-Super–sL-Cdc42 was generated to target nucleotides 515–537 of human Cdc42. The sequences of the oligonucleotides used are available on request.

siRNA transfections. The siRNA oligonucleotide (Dharmacon) sequences are as follows: RasGRF2 ON TARGET plus siRNA no. 7: 5′-CGAAAGAACUCCUAU-CAUU-3′; ON TARGET plus siRNA no. 8: 5′-UAGAAAGACCUCUAUAU-3′; ON TARGET plus siRNA no. 10: 5′-UAGCUAGCUAGUUGAUU-3′. Cells (2 × 10⁶) were plated in 60 mm dishes and transfected the next day with 20 nM SmartPools or individual oligonucleotides, with Optimem-I and Hiperfect (Dharmacon). Transfections were carried out at 24 h.

Cell culture. A375P and A375M2 cells were from R. Hynes (Howard Hughes Medical Institute, Massachusetts Institute of Technology, USA). COS-7, Hela, A375M2, WM266.6, WM1361 and SKMEL28 cells were grown in DMEM with 10% FCS. NIH3T3 cells were grown in DMEM with 10% CS. Jurkat cells were grown in RPMI with 10% FCS. COS-7 cells were transfected with diethylaminoethylose cellulose (DEAE)–dextran39. Hela and NIH3T3 cells were transfected with Lipofectamine 2000 reagent (Invitrogen) or with FuGene (Roche) for immunofluorescence microscopy studies. Jurkat and A375M2 cells were transfected with Lipofectamine 2000 reagent (Invitrogen). Before stimulation, cells were starved for 24 h, unless otherwise indicated. LPA, tumour-necrosis factor-α (TNF-α), epidermal growth factor (EGF) and ionomycin (Sigma) were used for 5 min at 10 µM, 100 nM, 50 ng ml⁻¹ and 1 µM, respectively. V27632 (Tocris) was used at 10 μM for 16 h.

Cell culture on thick layers of collagen. For cell movement assays, fibrillar bovine dermal collagen was prepared at a 1.7 mg ml⁻¹ dilution in DMEM according to the manufacturer’s protocol (Cohesion). Cells were seeded in 12-well plates on top of collagen (700 µl/well) in medium containing 10% serum, and were allowed to adhere for 24 h when the medium was changed to 1% serum for 16–24 h.

Invasion assays. Cells were suspended in serum-free collagen 1 at 2.3 mg ml⁻¹ to a final concentration of 10,000 cells per/100 µl and processed as previously described. Samples were run in quadruplicate and averaged in a high-content microscope (INCELL 3000). Invasion index = % number of invading cells at 50 µm/number of cells at 3 µm. For 3D imaging of invaded cells, sequential z sections of embedded GFP-labelled cells were obtained using a Zeiss 710 confocal microscope. 3D reconstructions of invaded cells were made using Volocity software (Improvision Inc). Invading cells were considered to have their centroid at 40 µm or above. To evaluate shape, a cell was considered elongated when its longest dimension was twice that of the shortest and when it showed at least one protrusion.

Lung colonization assay. WM266.6 cells stably expressing GFP or monomeric red fluorescent protein (mRFP) were transiently transfected with Mock or siRNA no. 7 against RasGRF2 plus mRFP or GFP respectively. At 48 h post-transfection, cells were trypsinized, mixed in equal numbers and injected into the tail vein of nude mice. An aliquot of each injection was seeded separately and the actual proportion of GFP- and mRFP-expressing cells was evaluated by immunofluorescence microscopy. Mice were euthanized after 2 or 20 h, and lungs were examined for GFP- or mRFP-expressing cells.

Time-lapse phase-contrast microscopy. Multi-site microscopy of cells was carried out in 24-well plates containing thick layers of collagen, in a humidified CO₂ chamber using a Diaphot inverted microscope (Nikon) with a motorized stage (Prior Scientific), controlled by Simple PCI software (Compix). For evaluation of the percentage of elongated cells, a cell was considered elongated when its longest dimension was twice that of the shortest and when it showed at least one protrusion.

Antibodies. Mouse monoclonal anti-HA (1:1,000), rabbit polyclonal anti-H-Ras, -panRasGRF, -RasG2, -Rap1, -RhoA, -Cdc42, -ERK2 (1:1,000), -Cdc42, -Vav1, -Ost and -Sox1 (1:1,000) were from Santa Cruz Laboratories. Rabbit monoclonal anti-Pak1, -phospho-Pak, -phospho-MLC2 and -tubulin (1:1,000) were from Cell Signaling. Rabbit polyclonal anti-TC21 (1:1,000) was supplied by X. R. Bustelo (Salamanca, Spain). Mouse monoclonal anti-Cdc42 (1:1,000) was from BD. Mouse monoclonal anti-Flag (1:2,000) and Phalloidin–tetramethyl rhodamine isothiocyanate (TRITC) were from Sigma. Mouse monoclonal anti-AU5 (1:1,000) was from Covance. Rabbit monoclonal anti-GFP (1:1,000) was from Invitrogen. Antibodies were used at a 1:500 dilution except when noted otherwise.

Co-immunoprecipitation assays. Cells were lysed in 50 mM Tris–HCl at pH 7.5, 100 mM NaCl, 1 mM sodium orthovanadate, 10 mM MgCl₂, 10 mM NaF, 10% NP-40, 10% glycerol, 1 mM phenylmethyl sulphonyl fluoride, 10 µg ml⁻¹ aprotinin and 10 µg ml⁻¹ leupeptin. Lysates were cleared by centrifugation and subjected to specific immunoprecipitation with 1 µg ml⁻¹ of the appropriate antibody and 20 µl of protein G Sepharose beads for 2–4 h at 4 °C. Immunoprecipitates were washed four times with lysis buffer and immunocomplexes were separated using SDS–PAGE. Mouse brains were homogenized before cell lysis.

Ras–GTP loading assays. Ras–GTP loading assays were carried out as described previously39. Quantifications show GT-Pase/GTP total GT-Pase levels, relative to levels found in control cells, from a representative result of three independent experiments.

Cdc42, RhoA and Rac1–GTP loading assays. For analyses of Cdc42, RhoA and Rac1 activity, pulldown assays were carried out as described previously39. Ras– and Cdc42–GTP were affinity purified using glutathione S-transferase (GST)–Pak1–CBD (Cdc42/Rac binding domain), and Rho–GTP was affinity purified with GST–Rho-kinetin–RBD (Rho binding domain). Quantifications show GT-Pase/GTP total GT-Pase levels, relative to levels found in control cells, from a representative result of three independent experiments.

In vitro pulldown assays. Bacterially produced histidine (His)–tagged HA–RasGRF2 DH–PH and His-tagged DH–PH were purified following the manufacturer’s instructions (Protopo Ni-IDA 150 packed columns; Macherey–Nagel). GST–Rho family GT-Pases bound to glutathione-Sepharose 4B beads were loaded with the DH protein. After incubation for 2 h at 4 °C, the beads were collected, washed twice with cold PBS, 1% NP-40/PBS and twice with lysis buffer. Where indicated, different amounts of His–HA–RasGRF2–DH–PH were added and after 2 h of incubation at 4 °C, the beads were collected and washed as before. Pellets were resuspended in 2× Laemmli buffer, boiled for 5 min and separated by 10% SDS–PAGE.

Immunoblotting. Total lysates and affinity precipitates were fractionated by SDS–PAGE and transferred to nitrocellulose filters. Immunocomplexes were visualized by enhanced chemiluminescence detection (GE Healthcare) using horseradish-peroxidase-conjugated secondary antibodies (BioRad).

Confocal immunofluorescence microscopy. Cultured cells were washed twice in PBS, fixed with ice-cold 3.7% formaldehyde in PBS for 10 min and washed with ice-cold PBS. They were rinsed in PBS with 0.05% Tween 20 (Sigma–Aldrich), incubated for 1 h with the primary antibodies, washed and incubated for 45 min with the appropriate secondary antibodies conjugated to fluorescein isothiocyanate (FITC), Texas Red or cyanine 5 (Cy5). Coverslips were mounted in Vectashield (Vector Labs) and sealed. Confocal microscopy was carried out with an LSM10 microscope (Zeiss), using excitation wavelengths of 488 nm (for FITC), 543 nm (for Texas Red) and 633 nm (for Cy5).

Focus formation assays. Focus formation assays were carried out as described previously39, except that cells were transfected using Lipofectamine reagent (Invitrogen). NIH3T3 cells were transfected with 2 µg of onco-DbI or onco-Vav1 plus 1 µg of the co-transfected complementary DNAs as indicated. For soft-agar colony assays, NIH3T3 cells were resuspended in 0.2% low-melting-point agarose, and plated onto solidified DMEM 0.4% agarose. After incubating for 20–30 days, colonies >100 µm in diameter were counted. Five independent experiments were carried out for each assay.

Statistical analyses. Statistical analyses were carried out using Prism 4.0 software (GraphPad). Student’s t-test was used to generate the P values.

Figure S1 RasGRF2 depletion affects the morphology of melanoma cells. (a) Micrographs showing the morphologies of WM266.4, WM1361 and SKMEL28 melanoma cell lines transfected with scrambled control siRNAs (mock) or with siRNA #7 against RasGRF2, cultured in a thick layer of collagen. Scale bar: 100 μm. Bar charts show the percentage of elongated cells after transfection of 2 siRNAs against RasGRF2 (#7, 10) (500/cells per experiment n=3. Error bars indicate SE. p values: * p< 0.05  by Student’s t test). (b) Down-regulation of RasGRF2 increases phosphorylation of MLC2. Total lysates of WM266.4, WM1361 and SKMEL28 cells transfected as in (a) were monitored for phosphorylated MLC2 and the indicated proteins by immunoblotting. Bar charts show the fold increase in p-MLC2 relative to mock transfected cells (n=3). Error bars indicate SE. p values: * p< 0.05; ** p<0.01  by Student’s t test.
Figure S2 (a) Diagram depicting RasGRF constructs used in the study. RasGRF2 constructs present an N-terminal Flag epitope that permits their detection by immunoblotting. GFP-tagged constructs of RasGRF1/2 have their Cdc25 domain replaced by GFP. The DH domain of RasGRF1 (DH1) and RasGRF2 (DH2) was also epitoped with GFP. Notice that a common region (*) corresponding to aminoacids 450-484 in RasGRF1, is deleted in both ΔDH and ΔPH2 mutants, whereas such region is present in the DH-GFP constructs. (b) RasGRF2 affects the morphology of A375M2 melanoma cells. Pictures show the morphologies of A375M2 cells: control, control treated with the ROCK inhibitor RI: Y27632, stably trasfected with RasGRF2: wild type, Cdc25 or ΔDH cultured in a thick layer of collagen. Red arrows show cells with typical elongated morphology. Green arrows show rounded cells. Scale bar: 100 μm.
**Figure S3** (a) Statistics for Rac1 and Rho activation levels in A375M2 and HeLa cells after depletion (si) or over-expression of RasGRF2, in cells exponentially growing or serum-starved for 24 h. n=3, error bars indicate SE. Student’s t test reveals no significance relative to the corresponding controls.  

(b) Statistics for Cdc42 activation levels in A375M2, Jurkat and HeLa cells after depletion (si) or over-expression of RasGRF2, in exponentially growing or 24 h-starved cells. n=5, error bars indicate SE. p values, *p< 0.05 by Student’s t test.

(c) Statistics for Pak1 phosphorylation levels in A365M2 cells under the same conditions. n=5, error bars indicate SE. p values, ***p< 0.001 by Student’s t test relative to the corresponding controls.

(d) Ectopic expression of Flag-tagged murine RasGRF2 wild-type and ΔCdc25 but not the ΔDH mutant reverts Cdc42 activation and phospho-MLC2 levels resulting from the down-regulation of endogenous RasGRF2 by siRNA #7, in HeLa and A375M2 cells, blots correspond to A375M2 cells. n = 5, error bars indicate SE. p values, * p< 0.05 by Student’s t test relative to the values in control cells transfected with siRNA#7. Right panels: representative western blots for the indicated proteins.
Figure S4 GDP-bound Cdc42 preferential associates with RasGRF GEFs. (a) Sos1 does not bind to Cdc42, neither Rho or Rac1 bind to RasGRF2. Lysates from HeLa and Jurkat cells were immunoprecipitated for Sos1, RhoA and Rac1 and immunoprecipitates were probed for the indicated proteins. (b) RasGRFs bind to GDP-bound Cdc42. COS-7 cells were transfected with plasmids encoding HA-RasGRF1 or Flag-RasGRF2, and, respectively, Flag- or AU5-epitoped Cdc42 constructs: Cdc42 N17, Cdc42 wt or Cdc42 QL. RasGRFs or Cdc42s were specifically immunoprecipitated with anti-HA, anti-Flag or anti-AU5 antibodies, as indicated and associated proteins were revealed by immunoblotting. (c) Top panel: Agonist-induced Ras activation in HeLa cells, starved (st) or after 2 min stimulation with the indicated stimuli. FS=foetal serum. Lower panel: agonist-induced binding of RasGRF2 to Cdc42. The association of the endogenous proteins was analyzed in agonist-stimulated HeLa cells. (d) RasGRFs bind to GDP-bound Cdc42 in vitro. The indicated, bacterially-purified, GST-Rho GTPases nucleotide free (NF), GDP- or GTPγS- loaded, were incubated with lysates of cells expressing the indicated GFP-tagged GEFs and RasGRF 1 and 2 DH domains (DH1 and DH2 respectively) and association was determined by immunoblotting. (e) RasGRFs DH domain is sufficient to inhibit Cdc42 activation. Cdc42 activation was assayed in growing COS-7 cells transfected with HA-Cdc42 together with vectors encoding for RasGRF1 and 2 or their respective DH domains (DH1 and DH2). (f) RasGRF DH but not Cdc25 domain is required to inhibit Cdc42 activation and Pak1 phosphorylation. Cdc42 activation and Pak1 phosphorylation were analyzed in cells transfected with HA-Cdc42 and oncoDbl, plus RasGRF1, and its ΔCdc25 and ΔDH mutants. Bar charts show the fold increase in p-MLC2 relative to control cells (n=3). Error bars indicate SE. * P< 0.05; *** P<0.001 by Student’s t-test relative to the levels of Dbl-transfected cells.
Figure S5 (a) Bona fide Cdc42 GEFs associate preferentially with dominant negative Cdc42. COS-7 cells were transfected with oncoDbl or Dock10DHR2 as shown. Where indicated, Flag-epitoped Cdc42 wt, Cdc42 N17 or Cdc42 QL was included. GEF-Cdc42 interaction were analyzed in anti-Flag immunoprecipitates. (b) RasGRFs outcompete Ost for binding to Cdc42. COS-7 cells were transfected with AU5-Cdc42 N17 plus Ost in addition to Ras GRFs. Cdc42 was anti-AU5 immunoprecipitated and associated proteins were revealed by immunoblotting. (c) RasGRF1 DH but not Cdc25 domain is required to inhibit Dbl/Cdc42 association. Cells were transfected with AU5-Cdc42 N17 and oncoDbl, plus RasGRF1 or its Cdc25 and DH deletion mutants. Cdc42 was anti-AU5 immunoprecipitated and associated proteins were revealed by immunoblotting. (d) Purified RasGRF1 DH-PH domain displaces Dbl from binding to Cdc42 in vitro. Dbl was bound in vitro to purified GST-Cdc42 N17 and incubated by increasing concentrations of a purified peptide spanning RasGRF1 DH-PH domains. Proteins associated to Cdc42 were determined by immunoblotting.
**Figure S6** RasGRF GEFs do not affect Rac1-dependent cellular events. 

(a) RasGRF GEFs prevent NIH3T3 soft agar colony formation induced by onc-Dbl. Bars represent the number of colonies, mean +/- s.d of 5 independent experiments, expressed as a percentage relative to those induced by onc-Dbl alone (ns p > 0.05; **p < 0.01; (*** p < 0.001 by Student’s t-test).

(b) RasGRF GEFs do not affect NIH3T3 transformation by onco-Vav. NIH3T3 cells were transfected with onco-Vav plus the indicated cDNAs. Bars represent the number of transformed foci, mean +/- s.d of 5 independent experiments, expressed as a percentage relative to those induced by oncoVav alone (ns p > 0.05, Student’s t-test).

(c) RasGRF GEFs do not affect NIH3T3 lamellipodia formation induced by Vav. Representative confocal micrographs of NIH3T3 cells transfected with oncoVav, GFP plus the indicated cDNAs. Cells were stained with Phalloidin (red) to mark filamentous actin. Symbol on the lower, right hand corner shows the confocal plane displayed. Bar: 10 μm.
**Supplementary Information**

**Figure S7** RasGRF2 regulates invasion and lung colonization by melanoma cells. (a) RasGRF GEFs impair Dock10-induced filopodia formation in NIH3T3 cells. Graph shows the number of filopodia per cell upon transfection with the indicated constructs. Bars represent the average +/- SE of at least 25 cells. (b) RasGRF2 levels modulate filopodia-formation in HeLa cells. Graph shows the number of filopodia per cell upon transfection with the indicated constructs after 24 h starvation. Cells transfected with either RasGRF2 siRNA or Cdc42 shRNA were co-transfected with pEGFP (5% of the total DNA transfected) to detect transfected cells. Bars represent either RasGRF2 siRNA or Cdc42 shRNA were co-transfected with pEGFP-transfected cells (***  p<0.01; Student’s t-test). (c-e) Down-regulation of endogenous RasGRF2 levels in the indicated melanoma cell lines by two RasGRF2 siRNAs (#7, 10). (d) Depletion of RasGRF2 results in increased phosphorylation of MLC2 in a Cdc42-dependent fashion. WM266.4 cells were transfected as indicated with siRNA #7 against RasGRF2 plus vector (c) or increasing concentrations of a siRNA against Cdc42. The levels of the indicated proteins were revealed by immunoblotting. Bar charts show the fold increase in p-MLC2 relative to control cells transfected with siRNA (n=3). Error bars indicate SE. ** p<0.01; Student’s t-test. (e) Depletion of RasGRF2 results in increased phosphorylation of MLC2 in a Cdc42-dependent fashion. Top panel: as in (b) but Cdc42 down-regulation was achieved both by siRNA interference and by the use of Cdc42 N17 dominant negative mutant (DN). Bar charts show the fold increase in p-MLC2 relative to control cells (n=3). Error bars indicate SE. ** p<0.01 by Student’s t-test. (f-g) Inter-cellular comparison of invasive indexes among the melanoma cell lines used. Absolute invasion index into collagen-I matrix of A375M2, SKMEL28, WM1361 and WM266.4 cells (n=3, error bars +SE). Student’s t-test was used to generate p values: *p<0.05.
Figure S8 Uncropped, full scans of the films showing the key experiments displayed in the main figures.
Supplementary Movies

**Movie S1** Time-lapse video-microscopy using a 10x magnification objective of A375P cells plated on top of collagen 48 h post Mock transfection.

**Movie S2** Time-lapse video-microscopy using a 10x magnification objective of A375P cells seeded on top of a collagen matrix 48h post-transfection with RasGRF2 OT7 siRNA.

**Movies S3** Time-lapse video-microscopy using a 10x magnification objective of empty vector A375M2 stable cell line plated on top of collagen.

**Movies S4, S5, S6** Time-lapse video-microscopy using a 10x magnification objective of RasGRF2WT A375M2 stable cell line (Movie 4), RasGRFΔCdc25 (Movie 5) and RasGRFΔDH (Movie 6) plated on top of collagen. Compare to Movies S3.