A palmitoylation switch mechanism regulates Rac1 function and membrane organization

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The small GTPase Rac1 plays important roles in many processes, including cytoskeletal reorganization, cell migration, cell-cycle progression and gene expression. The initiation of Rac1 signalling requires at least two mechanisms: GTP loading via the guanosine triphosphate (GTP)/guanosine diphosphate (GDP) cycle, and targeting to cholesterol-rich liquid-ordered plasma membrane microdomains. Little is known about the molecular mechanisms governing this specific compartmentalization. We show that Rac1 can incorporate palmitate at cysteine 178 and that this post-translational modification targets Rac1 for stabilization at actin cytoskeleton-linked ordered membrane regions. Palmitoylation of Rac1 requires its prior prenylation and the intact C-terminal polybasic region and is regulated by the triproline-rich motif. Non-palmitoylated Rac1 shows decreased GTP loading and lower association with detergent-resistant (liquid-ordered) membranes (DRMs). Cells expressing no Rac1 or a palmitoylation-deficient mutant have an increased content of disordered membrane domains, and markers of ordered membranes isolated from Rac1-deficient cells do not correctly partition in DRMs. Importantly, cells lacking Rac1 palmitoylation show spreading and migration defects. These data identify palmitoylation as a mechanism for Rac1 function in actin cytoskeleton remodelling by controlling its membrane partitioning, which in turn regulates membrane organization.

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Introduction

Cell migration requires the spatio-temporal coordination of numerous signalling molecules. These include the Rho family of small GTPases, which are essential for cytoskeletal rearrangement (Lauffenburger and Horwitz, 1996; Ridley *et al.*, 2003; Raftopoulou and Hall, 2004; Vicente-Manzanares *et al.*, 2005; Wu *et al.*, 2009). Within this family, Rac1 regulates lamellipodia formation and membrane ruffling (Burridge and Wennerberg, 2004). Activated (guanosine triphosphate (GTP) loaded) Rac1 initiates various signalling cascades through interaction with effector molecules, one of the best characterized of which is p21-activated kinase (PAK; del Pozo *et al.*, 2000; Delorme *et al.*, 2007). GTP loading is regulated by GEFs (guanine nucleotide-exchange factors), which activate Rac1, and by GAPs (GTPase-activating proteins), which inactivate it (Etienne-Manneville and Hall, 2002).

Besides the interaction with regulatory proteins and effector molecules, a few Rho GTPases also form homodimers and oligomers (Zhang and Zheng, 1998; Zhang *et al.*, 2001). Similarly to the situation with other GTPases (Di Paolo *et al.*, 1999), self-assembly of Rac1 into large oligomers activates its intrinsic GAP activity, resulting in its down-regulation (Zhang *et al.*, 2001). However, the role of oligomerization in the regulation of Rac1 *in vivo* remains unclear.

In the cytosol, Rac1 remains soluble and bound to RhoGDI (guanosine diphosphate (GDP) dissociation inhibitor), preventing effector binding (del Pozo *et al.*, 2002). Upon stimulation with growth factors and cell attachment to the extracellular matrix, Rac1 associates with specific liquid-ordered (L_o) subdomains in the plasma membrane (PM; del Pozo *et al.*, 2000, 2002; Fujitani *et al.*, 2005; Grande-García *et al.*, 2005). These subdomains are cholesterol rich, and in membrane models are resistant to detergent extraction at 4°C (Brown, 2006; Simons and Gerl, 2010). However, it is unknown precisely how Rac1 translocates to these highly ordered detergent-resistant membranes (DRMs) and subsequently activates downstream effector proteins.

Rac1 targeting shares features in common with Ras GTPases, which require two signals to reach the PM. Like Rac proteins, Ras GTPases are isoprenylated in the CAAX sequence at the C-terminus. In the case of K-Ras, isoprenylation (farnesylation) is accompanied by the polybasic region as the second signal, and this combination correlates with exclusion from L_o PM domains (Magee and Marshall, 1999; Zacharias *et al.*, 2002; Abankwa *et al.*, 2007; Omerovic and Prior, 2009). In contrast, the second signal in H- and N-Ras consists of palmitoylation at cysteine residues, one in N-Ras (Cys181) and two in H-Ras (Cys181 and 184). In both proteins, combination of isoprenylation at Cys 186 and palmitoylation at Cys 181 permits localization to ordered membrane regions (Roy *et al.*, 2005). In Rac proteins, the CAAX motif is modified by geranyl geranylation, and the C-terminal polybasic region in Rac1 (KKRKRR) contributes to

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membrane localization (Michaelson *et al*, 2001). There is no Cys residue close to the CAAX prenylation site, which would predict a behaviour similar to K-Ras; however, like H-Ras, Rac1 associates with ordered membrane domains (Li *et al*, 2003; del Pozo *et al*, 2004), suggesting that Rac1 might be palmitoylated at upstream Cys residues.

Palmitoylation is the post-translational covalent linking of the 16-carbon fatty acid palmitate, mostly via a thioester bond. Unlike other fatty acid modifications, palmitoylation is reversible, and cycles of palmitoylation–depalmitoylation enable proteins to transiently associate with membranes, thereby regulating their sorting, localization and function (Huang and El-Husseini, 2005; Greaves and Chamberlain, 2007; Linder and Deschenes, 2007; Rocks *et al*, 2010). Moreover, palmitoylation promotes stable tethering of cytosolic proteins to intracellular membranes, controls endocytic trafficking and accounts for the lateral segregation of proteins into DRMs (Resh, 2006; Charollais and Van Der Goot, 2009; Levental *et al*, 2010).

Here, we demonstrate that Rac1 undergoes thioacylation with palmitic acid and show that this modification regulates Rac1 partitioning and stabilization into DRMs. Palmitoylation-mediated changes in Rac1 localization and activity induce actin cytoskeleton reorganization, which controls membrane organization, cell spreading and directional migration. These results define a critical role for palmitoylation in Rac1 function.

Results

Inhibition of palmitoylation alters Rac1 subcellular compartmentalization and GTP loading

2-Bromo-palmitate (2-Brp) is an effective inhibitor of protein palmitoylation *in vivo* (Webb *et al*, 2000). We incubated COS-7 cells expressing GFP-tagged Rac1 with 25 µM 2-Brp and analysed changes in subcellular distribution. Confirming the specificity of this inhibitor for palmitoylated proteins, 2-Brp had no effect on fluorescence distribution in cells expressing GFP alone (Figure 1A) or RhoA, a non-palmitoylated small GTPase that partially localizes to the PM (Supplementary Figure S1A). In contrast, 2-Brp triggered the relocation of wild-type (wt) GFP–Rac1 and a constitutively active form (GFP–V12Rac1) from the typical distribution (cytosol and PM, with nuclear accumulation) to the perinuclear area, partially inhibiting PM localization and excluding Rac1 from the nucleus (Figure 1A; Supplementary Movie S1). This effect was observed after short exposure to 2-Brp (30 min), contrasting with other palmitoylated proteins, whose subcellular distribution is altered only after several hours (Goodwin *et al*, 2005; Rocks *et al*, 2005). This difference might reflect different rates of palmitate turnover. Similar results were obtained with immunofluorescence of endogenous Rac1 in mouse embryonic fibroblasts (MEFs; Figure 1F). Normal GFP–Rac1 distribution was restored by washing out the 2-Brp and overnight cell recovery, suggesting a role for palmitoylation in Rac subcellular compartmentalization (Figure 1A).

Pull-down activity assays showed that 2-Brp decreased the levels of GTP-Rac (both GFP–Rac1 and endogenous Rac1; Figure 1B and F), but did not affect GFP–V12Rac1 (Supplementary Figure S4D). Incubation of Rac1 with the non-hydrolysable GTP analogue GTP-γ-S showed that

2-Brp does not prevent Rac1 binding to GTP, indicating that the loss of Rac1 activity was not due to changes in the nucleotide binding site.

To further explore the effect of palmitoylation on membrane partitioning of GFP–Rac1, we determined its distribution in soluble (S) and particulate (P; membrane-associated) fractions of 2-Brp-treated cells. Inhibition of palmitoylation did not affect the distribution of Rac1 in the particulate fraction (Figure 1C; Supplementary Figure S1C). These results suggest that palmitoylation regulates Rac1 subcellular distribution and activity, despite not significantly modifying protein hydrophobicity.

To investigate Rac1 palmitoylation in living cells, we labelled COS7 cells expressing GFP–Rac1 or GFP–V12Rac1 with [³H]-palmitic acid and immunoprecipitated with anti-GFP antibody. GFP-tagged Rac1 proteins specifically incorporated the radiolabel (Figure 1D), but labelling was abolished by 2-Brp treatment (Figure 1E). Palmitoylation of endogenous Rac1 was also detected in labelled MEFs (Figure 1F). The [³H]-palmitoyl group was liberated from GFP-immunoprecipitated Rac proteins by incubation with the nucleophile hydroxylamine (Figure 1E), confirming that the palmitate moiety attaches to Rac1 via a labile thioester bond.

Rac1 is an important mediator of the actions of growth factors; for example, it is required for the polymerization of actin to produce lamellipodia and edge ruffles stimulated by platelet-derived growth factor (PDGF; Nister *et al*, 1988; Ridley and Hall, 1992; Nobes *et al*, 1995). To test the association of Rac palmitoylation with its GTP loading and targeting in a biologically relevant setting, we analysed endogenous and GFP-tagged radiolabelled Rac1 in cells stimulated with PDGF (Figure 1F; Supplementary Figure S1D). In both cases, enhanced Rac1 palmitoylation induced by PDGF correlated with increased Rac1 GTP loading, suggesting a link between the palmitoylation state of Rac and its activation.

Identity of cysteine residues involved in Rac1 palmitoylation

The preferred cysteines for S-palmitoylation are usually close to transmembrane domains or membrane-associated domains of non-integral membrane proteins, though no conserved motif has been identified. These cysteines are commonly present near the amino or carboxy terminals (Alland *et al*, 1994; Resh, 1994). Carboxy-terminal sequences of some GTPases contain palmitoylatable cysteine residues upstream of the CAAX motif (Michaelson *et al*, 2001). In Rac1, a conserved Cys at position 178 is separated from the Cys of the CAAX box by a polybasic motif, and another Cys is present in the N-terminal region. To test whether Cys⁶ (N-terminus) or Cys¹⁷⁸ (C-terminus) are S-palmitoylation target sites, we introduced Cys to Ser substitutions (Figure 2A). Although substitution of Cys⁶ altered the nuclear and perinuclear distribution of Rac1 (Figure 2B), the mutant protein reached the PM and incorporated [³H]-palmitate to a similar extent as the wt form (Figure 2C and D). In contrast, the C178S mutant showed no labelling, which correlated with reduced PM localization (Figure 2C and D). Moreover, while the palmitoylatable C6S mutant was GTP loaded, the palmitoylation-deficient C178S Rac protein displayed significantly reduced GTP loading, although its capacity to bind GTP-γ-S was unaffected (Figure 2D and E). This lower

GTP loading was paralleled by reduced activation of PAK at the PM as measured by immunoblot with an antibody that specifically recognizes the phosphorylated Ser 141 in the active form of PAK (Figure 2C). These findings suggest that palmitoylation at Cys 178 favours Rac1 PM association and downstream signalling.

Low GTP loading of palmitoylation-deficient Rac1 correlates with decreased partitioning into liquid-ordered PM domains and increased oligomerization

Post-translational S-palmitoylation is a possible mechanism not only for segregating proteins in DRMs, but also for regulating their stability, intracellular trafficking and function

(Melkonian *et al*, 1999; Resh, 2006; Charollais and Van Der Goot, 2009). DRMs are biochemically defined as membrane microdomains that resist extraction with detergents such as Triton X-100 and Lubrol at 4°C (Brown and London, 1998). Although DRMs isolated by biochemical fractionation differ in some characteristics from L_o domains pre-existing in cell membranes, DRM fractionation remains the standard method for investigating the association of proteins with L_o domains (Brown, 2006). To test whether Rac palmitoylation is required for its stabilization in DRMs, we analysed the partitioning of palmitoylation-deficient C178S Rac1 on discontinuous sucrose gradients. Unlike wt Rac1, which co-fractionated with DRMs (Triton X-100-insoluble fractions: 8–10), only a limited amount of C178S Rac1 was detected in these fractions: most of the protein partitioned with the Triton X-100-soluble fractions at the bottom of the tube, similarly to the pattern observed for RhoGDI or tubulin (Figure 3A). This altered distribution correlated with a lower content of F-actin and active PAK (p-PAK) in DRMs of cells expressing C178S Rac1, and also with reduced recruitment of p-PAK to the PM (Figure 3A and C). Similar results were observed with endogenous Rac1 in MEFs: while 2-Brp did not alter partitioning between cytosol and membrane, it impaired co-fractionation of Rac1 with DRMs. Redistribution into DRMs was partially restored by washing out 2-Brp and overnight cell recovery (Figure 3B).

Although L_o domains are thought to be heterogeneous (Pike, 2003; Hancock, 2006), all contain high levels of the ganglioside GM1, which is widely used as a marker for these domains. To corroborate the previous results, we incubated cells with beads coated with GM1-binding cholera toxin subunit B (ChTxB) and analysed the clustering of Rac proteins (scheme in Figure 3D). Wt Rac1 colocalized with GM1, whereas colocalization of C178S Rac1 was negligible (Figure 3D).

This altered localization could reflect increased association of non-palmitoylated C178S Rac1 with RhoGDI, whose association with some GTPases is inhibited by their palmitoylation (Michaelson *et al*, 2001). To test this possibility, we used recombinant (His)₆-RhoGDI to pull down GFP-Rac1wt or GFP-C178S from COS7 cell lysates or endogenous Rac1 from control or 2-Brp-treated MEFs. Interaction between Rac1 and RhoGDI was similar in all cases, indicating that palmitoylation does not affect affinity of Rac1 for RhoGDI (Figure 3E). This finding was confirmed by pull down

or co-immunoprecipitation in COS7 cells co-expressing GFP-Rac1wt or GFP-C178S and myc-tagged RhoGDI (Supplementary Figure S3A).

During reversible palmitoylation, removal of palmitate can expose membrane-proximal Cys residues, influencing the oligomerization state of some proteins (Hsueh *et al*, 1997; Woehler *et al*, 2009). To evaluate whether this occurs with Rac1, we detected Rac1 proteins under non-reducing conditions. C178S Rac1 (GFP- or Cherry-tagged) formed stable oligomers, whereas palmitoylation-competent Rac1 (wt or C6S) mostly occurred as monomers. The amount of oligomeric wt Rac1 was increased by treatment with 2-Brp, suggesting that palmitoylation regulates the stability of Rac1 oligomers (Figure 3F).

The role of DRMs as platforms for protein sorting and signalling has mainly been characterized in the PM; however, recent studies propose that nuclear lipids are organized into microdomains with similar characteristics to PM L_o regions (Cascianelli *et al*, 2008). Nuclear FRAP assays to determine whether the absence of palmitoylation affected entry of Rac1 to the nucleus showed that whereas Rac1wt readily entered the nucleus after bleaching, signal recovery was impaired for the C178S mutant (Supplementary Figure S3B). Thus, palmitoylation on Cys 178 is also required for association of Rac1 with the nuclear membrane.

C-terminal prenylation and the polybasic region are required but not sufficient for Rac1 palmitoylation

Palmitoylation sites are often close to other lipid-modifiable sites, polybasic motifs or hydrophobic sequences, and this context is required for palmitoylation (Resh, 1994; Kleuss and Krause, 2003; Mann and Beachy, 2004). We tested the dependence of Rac1 palmitoylation on geranyl-geranyl modification and the C-terminal polybasic region, two signals known to be required for Rac1 membrane targeting. For this analysis, mutations were introduced into the constitutively active V12Rac1 background, in order to study effects of these mutations on membrane targeting separately from potential effects on GTP loading. Basic residues in the polybasic region were replaced with glutamines (V12-6Q Rac1) or the prenylation site was mutated (V12-SAAX), and we also studied the C178S mutation (V12-C178S). Neither V12-6Q nor V12-SAAX incorporated palmitic acid efficiently, suggesting that both the geranyl-geranyl modification and the polybasic motif are

Figure 1 Rac1 incorporates [³H]-palmitic acid and its localization and activity are altered by inhibition of palmitoylation. (A) COS7 cells expressing GFP, GFP-Rac1 or GFP-V12Rac1 were incubated with 25 μM 2-Brp or DMSO (control) for 30 min. Cell recovery was analysed 24 h after 2-Brp washes out. GFP fluorescence was detected by confocal microscopy after excitation at 488 nm (bar, 25 μm). (B) GST-PBD pull-down assay of Rac1 activity. Incubation with GTP-γ-S was included to confirm that 2-Brp does not interfere with nucleotide binding. The chart shows quantification of GTP-Rac band intensities relative to total Rac1. Values are means ± s.e.m. (n = 3). (C) Subcellular fractionation of 2-Brp-treated cells. Lysates of GFP-Rac1-expressing COS7 cells treated with vehicle or 2-Brp were separated into soluble (S) or particulate (P) fractions, which were analysed by western blot for GFP-Rac1 content. Caveolin-1 and RhoGDI were detected as fraction-specific markers. (D) Autoradiograph showing [³H]-palmitic acid incorporation by GFP-tagged Rac1 proteins expressed in COS7 cells (top panel). Cells were incubated with radiolabel for 4 h, and Rac1 proteins were immunoprecipitated with anti-GFP. Content of GFP-tagged proteins in immunoprecipitates was confirmed by western blot (bottom panel). (E) For acyl thioester analyses, gels prepared as in (A) were incubated with either Tris (1 M, pH 7.0) or hydroxylamine (1 M, pH 7.0) for 24 h before autoradiography. Where indicated, cells were treated with 2-Brp during the last 30 min of radiolabelling. (F) Effect of PDGF and 2-Brp on the subcellular distribution and palmitoylation of endogenous Rac1. Mouse embryonic fibroblasts (MEFs) were stimulated with 20 ng/ml PDGF during the last 15 min of [³H]-palmitic acid labelling or treated with 2-Brp as previously indicated. Palmitoylation was quantified relative to the control condition (left chart). Changes in Rac1 subcellular distribution were analysed by confocal microscopy (bar, 25 μm). Effective stimulation by PDGF was confirmed by GST-PBD pull-down assay of Rac1 activity. The chart on the right shows quantification of GTP-Rac band intensities relative to total Rac1. Values are means ± s.e.m. (n = 3) *P < 0.05, **P < 0.01 (see also Supplementary Figure S1 and Supplementary Movie S1).

Figure 2 Rac1-GTP binding depends on palmitoylation at cysteine 178. (A) Comparison of amino- and carboxy-terminal sequences of Rac1 with other human Ras and Rho GTPases. CAAX prenylation motifs are in blue. Polybasic residues are underlined. Putative or known palmitoylated cysteines are in red (carboxy-terminal sequences) or green (amino-terminal sequences). The positions of introduced mutations are shaded grey. A scheme of the cysteine substitution mutants of GFP-tagged Rac1 is shown below. (B) Confocal images showing the subcellular localization of GFP-tagged single (C6S or C178S) and double (C6S + C178S) Rac1 mutants (bar, 25 μ m). (C) Western blot of plasma membrane-enriched fractions of cells expressing the indicated GFP-tagged Rac1 constructs. Blots were probed with antibodies to GFP (Rac1), the phosphorylated (active) form of the Rac1 effector PAK (p-PAK) and total PAK. Results are representative of three independent experiments. (D) [3 H]-palmitic acid incorporation by wt and mutant GFP-tagged Rac1 proteins. iNOS (1–94 aa) was used as a positive control. (E) GST-PBD pull-down assay in cells expressing wt and mutant GFP-tagged Rac1 proteins. Bands were quantified relative to total Rac1. Values are means $\pm$ s.e.m. ($n = 3$) ** $P < 0.01$. Incubation with GTP- γ S was included to confirm that the C178S mutation does not affect the potential of Rac1 to bind nucleotides.

required for Rac1 palmitoylation (Figure 4A). Analysis of the colocalization of V12Rac1 mutants with different subcellular organelles showed that the absence of the polybasic region (V12-6Q) resulted in exclusion from the nucleus and PM, and accumulation in perinuclear areas (Figure 4B; Lanning *et al*, 2004). V12-6Q Rac1 colocalized with markers of ER (calreti-

culin) and Golgi (β -COP), but not the early endocytic marker EEA1. Mutation of the prenylation site (V12-SAAX) also excluded Rac1 from the PM pool; but unlike the V12-6Q mutant, V12-SAAX accumulated in the nucleus, and did not colocalize with any subcellular markers tested (Figure 4B). V12-C178S also did not colocalize with subcellular markers,

and retained the ability to reach the cell periphery, although to a lesser extent than V12Rac1.

Deletion mutants containing only the region flanking the palmitoylation site are often more efficiently palmitoylated (Liu *et al*, 1997; Galbiati *et al*, 1999). We therefore transfected COS7 cells with constructs encoding the final 50 amino acids of the Rac1 sequence (Figure 4C). Both GFP-50aaCt, corresponding to the wt Rac1 carboxy-terminal, and GFP-50aaC178SCt accumulated in the nucleus, with some signal in cytosol and PM. Neither GFP-50aaCt nor GFP-50aaC178SCt incorporated palmitate efficiently (Figure 4D). These results indicate that sequences upstream of the last 50aa are needed for Rac1 palmitoylation, probably by ensuring correct conformational exposure of Cys 178.

Protein S-palmitoylation is reversible (Smotrys and Linder, 2004), but the regulation of palmitate turnover is poorly understood. FKBP12, a member of the prolyl isomerase (PI) superfamily, promotes depalmitoylation of H-Ras in a manner dependent on proline 179 (Ahearn *et al*, 2011). To study the action of PIs on steady-state palmitoylation of Rac1, we treated MEFs with the PI inhibitor FK506 and analysed Rac1 subcellular localization, activity and palmitoylation. FK506 promoted lamellipodia formation, correlating with enhanced Rac1 GTP loading and palmitic acid incorporation (Supplementary Figure S2A). PI activity requires a proline-rich target sequence (Lu *et al*, 2007), and the Rac1 palmitoylation site is immediately followed by three prolines (179–181). Deletion of these residues increased palmitoylation and GTP loading (Supplementary Figure S2B), demonstrating that these prolines are important for the regulation of Rac1 palmitoylation.

All Rac isoforms (Rac1, 2 and 3) contain an invariant cysteine residue at position 178 but vary in the amino-acid composition of the downstream sequence (Supplementary Figure S6A). The three Rac isoforms show clear differences in overall distribution in COS-7: unlike Rac1, Rac2 and 3 accumulated in the perinuclear region, overlapping with the Golgi marker GM130 (Supplementary Figure S6B and C) and showed little targeting to DRMs (Supplementary Figure S6D). For palmitoylation analysis, cells expressing the three Rac isoforms were labelled with [³H]-palmitate and immunoprecipitates were analysed by SDS-PAGE/autoradiography and immunoblotting (Supplementary Figure S6E). Neither Rac2 nor 3 incorporated palmitic acid efficiently, confirming that the sequence between Cys 178 and the prenylation site determines the efficiency of Rac palmitoylation.

Rac1 palmitoylation regulates the organization of membrane domains

The membrane raft hypothesis proposed that interactions between cholesterol and sphingolipids generate a lipid platform (Brown and London, 2000; Simons and Toomre, 2000; Simons and Gerl, 2010). More recent findings, however, suggest that L_o domains are formed or stabilized through specific protein associations (Douglass and Vale, 2005; Plowman *et al*, 2005). For example, the cortical actin cytoskeleton, a lattice of filaments that underlies and attaches to the PM, imparts an ordering effect on bilayer lipids (Yanagida *et al*, 2007; Kusumi *et al*, 2010). Given the role of Rac1 in actin cytoskeletal organization, we tested the influence of Rac1 palmitoylation on PM organization. Through high-resolution imaging of COS7 cells (TIRFm; total internal reflection fluor-

escence microscopy), we detected GFP-Rac1wt localized to elongated, worm-like PM regions that were GM1 positive and sensitive to methyl- β -cyclodextrin (Figure 5A–C; Supplementary Movie S6). These GM1-positive PM structures were also detected in non-transfected cells (Figure 5F), although they were shorter than in cells expressing GFP-Rac1wt, supporting a role for Rac1 in the rearrangement and stabilization of PM regions. In contrast, worm-like lipid structures were scarce in the PM of cells expressing GFP-C178SRac1; instead, GFP-C178SRac1 was mostly organized in small amorphous pond-like agglomerations with weak GM1 staining, suggesting non-uniform attachment to substrate (Figure 5A and E). The edges of these contained rapidly retracting finger-like processes (Supplementary Movies S4 and S5). Similar results were obtained in immortalized MEFs (not shown) and in primary Rac1-deficient MEFs reconstituted with Rac1wt (Supplementary Figure S5B).

To evaluate whether the elongated (worm-like) and compact (pond-like) PM regions correspond to different cytoskeletal PM compartments, we studied their colocalization with the actin marker lifeact (Riedl *et al*, 2008). The worm-like localization of wt Rac1 coincided extensively with filamentous actin. In contrast, F-actin colocalized with non-palmitoylated Rac1 only at the borders of the pond-like regions (Figure 5B). In COS7 cells expressing constitutively active Rac1 forms (V12Rac1 or V12-C178S Rac1), TIRFm revealed further differences in actin staining. While V12Rac1 mostly localized to the cell edges, where it colocalized with F-actin, V12C178S covered the ventral cell surface, nicely staining actin fiber tips (Supplementary Figure S4B).

Rac1 activates Arp2/3-mediated actin polymerization and branching of actin filaments (Machesky and Way, 1998; Kunda *et al*, 2003; ten Klooster *et al*, 2006). To test directly whether Arp2/3-mediated actin polymerization is implicated in the generation and stabilization of Rac1-positive worm-like PM structures, we silenced expression of p21-Arc (Arp (actin-related protein) complex) or Arp 3, subunits of the Arp2/3 complex. Silencing of p21-Arc or Arp 3 abolished most of the elongated worm-like Rac1 staining, replacing it with the pattern seen in cells expressing C178SRac1 (Figure 5D, lower panels; Supplementary Figure S7). Cytochalasin D had the same effect (not shown). These results suggest that actin polymerization is required for the membrane organization mediated by palmitoylated Rac1.

To gain more insight into the effect of Rac1 palmitoylation on membrane organization, we examined membrane fluidity *in vivo* in cells expressing wt or C178SRac1 by labelling with the fluorescent probe Laurdan. Comparison of the generalized polarization (GP) of membrane distribution demonstrated that while the remaining ordered regions were more rigid, C178SRac1 reduced the coverage by ordered domains (Figure 6A). These differences were not due to differences in cholesterol content (Figure 6B). These results suggest that Rac1 stabilizes ordered membrane domains. Consistently, analysis of sucrose gradients in Rac1^{-/-} cells revealed impaired flotation of two well-known DRM markers, caveolin1 and GM1, an effect partially rescued by reintroducing either Rac1wt or C178SRac1, with GM1 recovery into DRMs slightly better in Rac1 wt reconstituted cells (Figure 6C). *In-vivo* fluidity measurement with Laurdan in Rac1^{-/-} and reconstituted cells was technically challenging due to sequential adenoviral and lentiviral infections, colour incompatibil-

ities and the short window of cell viability. Nonetheless, the evidence from these experiments suggests that the membranes of Rac1 null cells contain L_0 domains that are not detected in DRMs, and which cover a smaller area but are more rigid than L_0 domains in Rac1 wt cells (Figure 6D), similarly to the situation in C178SRac1-expressing COS7

cells. Rac1 reconstitution in Rac1^{-/-} cells rescued the wt phenotype, while C178SRac1 rescued it partially. Moreover, cells that efficiently lost Rac1 after adeno-Cre infection were less viable after lentiviral expression of C178SRac1 (not shown), suggesting that the observed results overestimate the rescue effect.

Loss of Rac1 palmitoylation reduces spreading and delays wound closure

To investigate the functional consequences of Rac1 palmitoylation, we performed scratch wound-healing assays with cells expressing wt and C178SRac1. Cells expressing C178SRac1 showed slower wound closure (Figure 7A) were rounder (Figure 7B), and migrated with less directionality (Figure 7C), suggesting that Rac1 palmitoylation controls cell motility.

Constitutively active Rac1, but not Rho or Cdc42, induces spreading and increases integrin-dependent adhesion of T cells to immobilized fibronectin (D'Souza-Schorey *et al*, 1998). To test the effect of Rac1 palmitoylation on these processes, we transfected Jurkat T cells with V12Rac1 or V12C178S. When plated on fibronectin, V12Rac1 cells spread and produced lamellipodia, whereas cells expressing V12C178S retained a contracted morphology similar to suspended cells (Supplementary Figure S4A).

Rac1^{-/-} MEFs, obtained from conditionally gene-targeted Rac1 mice, have a spindle-like, contracted morphology (Figure 7D; Guo *et al*, 2006). Reintroduction of wt Rac1 into these cells induced the typical spread morphology of wt fibroblasts, whereas C178SRac1 did not rescue the phenotype (Figure 7D and E). Defective cell spreading by Rac1^{-/-} MEFs was associated with disrupted F-actin and vinculin immunostaining (Supplementary Figure S5A), which correlated with defective lamellipodia formation and migration (Figure 7E; Supplementary Movies S2 and S3). These phenotypes were rescued by wt Rac1 but not by C178SRac1. These data strongly suggest that palmitoylation is critical for Rac1 function.

Discussion

In this study, we demonstrate that Rac1 undergoes palmitoylation. This modification regulates Rac1 oligomerization state and activity and its partitioning and stabilization into ordered cholesterol-rich membrane domains (Figure 8). Palmitoylation of Rac1 at Cys 178 requires prior prenylation at Cys 189 and the presence of the polybasic C-terminal region. Palmitoylation-mediated changes in Rac1 localization and GTP loading modulate the capacity of Rac1 to bind effectors in the PM, and signalling pathways activated through these interactions trigger actin polymerization, which would favour coalescence of pre-existing L_o domains into larger structures.

Proteomic analysis confirms that Rac1 is a strong candidate for palmitoylation (Kang *et al*, 2008). An earlier study based on chemical labelling concluded that Rac1 is not

palmitoylated (Roberts *et al*, 2008); however, the published results in fact indicate low level palmitoylation that was affected by mutation of C178, albeit against a high background. We found similar results using this technique (Supplementary Figure S1C), but we conducted our main analysis by the more sensitive method of [³H]-palmitate radiolabelling (Drisdell and Green, 2004). This approach provided clear evidence of palmitoylation. Although palmitoylation levels are low compared with other characterized palmitoylated proteins, they are similar to those reported for R-Ras, a GTPase also involved in cell adhesion and spreading (Furuhjelm and Peranen, 2003). Moreover, signal loss upon hydroxylamine treatment demonstrates that Rac1 incorporates palmitate through a thioester linkage, and the sensitivity to 2-Brp supports a role for palmitoylation in the regulation of Rac1. The low palmitoylation signal might reflect rapid turnover (Rocks *et al*, 2010), which would be consistent with the dynamic regulation of Rac1 activity.

Although there is no known consensus sequence for palmitoylation, it in many cases requires prior lipid modifications such as prenylation or N-myristoylation (Resh, 2006). Our data confirm that Rac1 palmitoylation requires not only geranyl geranylation but also the presence of the C-terminal polybasic region. These two features are essential for proper Rac1 PM targeting and interaction with multiple lipids (Ueyama *et al*, 2005; Chae *et al*, 2008). Analysis of the atomic structure of Rac1 reveals that Cys 178 is positioned immediately carboxy-terminal to the C5 α -helix (Hirshberg *et al*, 1997), indicating that palmitoylation at this residue might extend the region of Rac1 that binds to membranes. Our results demonstrate that the triproline motif (179–181) also limits the steady-state palmitoylation of Rac1, likely through a *cis-trans* isomerization mechanism. Cys 178 is conserved in all Rac isoforms; however, Rac2 and 3 are not efficiently palmitoylated. These observations suggest that the sequence between Cys 178 and the prenylation site in Rac1 favours palmitoylation.

Our results cannot discriminate the cellular site of Rac1 palmitoylation. In addition to the classical compartments (ER, Golgi complex and PM) where protein palmitoylation was first reported, the incorporation of palmitic acid into Rac1 might take place in specific vesicles in which activation of Rac1-mediated pathways has been reported (Palamidessi *et al*, 2008). Such a situation has been described for H-Ras, where the palmitoyl moiety is essential for localization to recycling endosomes (Misaki *et al*, 2010). Enzymatic protein

Figure 3 Absence of palmitoylation impairs recruitment of Rac1 to detergent-resistant membrane fractions and favours its oligomerization. (A) Western blots of sucrose density gradient fractions of cells expressing GFP-tagged wt or C178S Rac1. Red boxes denote caveolin-enriched DRM fractions (8–10). Fractions were analysed for the distribution of Rac1 (GFP), Cav1 RhoGDI, F-actin, tubulin and p-PAK. GM1 was detected with horseradish peroxidase-tagged cholera toxin B subunit. The chart shows the amounts of wt and C178S Rac1 in each fraction, relative to the total amount; mean $\pm$ s.e.m. ($n = 3$). (B) Recruitment of endogenous Rac1 to DRMs depends on palmitoylation. Lysates of 2-Brp-treated MEFs were separated into soluble (S) and particulate (P) fractions (left) or centrifuged to equilibrium on sucrose density gradients (right). Fractions were analysed by western blot for the content of Rac1. Caveolin-1 and RhoGDI were detected as fraction-specific markers. (C) Confocal images showing p-PAK colocalization (yellow) in cells expressing wt or C178S Rac1 (bar, 50 μ m). Plasma membrane regions are enlarged in the right panel. (D) Localized clustering of GM1-containing DRMs promoted by cholera toxin-coated beads. The top panel shows the experimental scheme. COS7 cells expressing GFP-wt or GFP-C178S Rac1 were incubated with 5 μ m beads coated with the GM1 marker Alexa 647-cholera toxin B. Representative confocal images show intense GFP signal around beads (bar, 10 μ m). Plots show pixel intensities for Rac proteins (green) and GM1 (red) along the lines drawn through the indicated beads. (E) Regulation of Rac1-RhoGDI binding by palmitoylation. Lysates of 2-Brp-treated MEFs (upper panel) or COS7 expressing the indicated GFP-tagged Rac1 proteins (lower panel) were pulled down with recombinant (His)₆-tagged RhoGDI. Pull downs were analysed by western blot. (F) Oligomerization of palmitoylation-deficient Rac1. GFP- or pCherry-tagged wt, C6S or C178S Rac1 was loaded onto non-reducing polyacrylamide gels and analysed by western blot. Oligomerized Rac1 was detected in untreated cells expressing C178S Rac1 or cells expressing wt Rac1 treated with 2-Brp (see also Supplementary Figure S2).

Figure 4 Rac1 palmitoylation requires prenylation and the polybasic region. (A) Incorporation of [^3H]-palmitic acid by wtRac1, constitutively active Rac1 (V12Rac1), or derived constructs mutated in cysteine 178 (V12-C178S), the polybasic region (V12-6Q) or the prenylation site (V12-SAAX). Arrow indicates palmitoylated Rac1. (B) Rac1 colocalization of Rac1 proteins with markers of subcellular trafficking. COS7 cells were transfected with V12Rac1, V12-6Q, V12-SAAX and V12-C178S. Confocal images show single staining of Rac1 mutants (GFP) in green (left), subcellular localization markers (the early endosome marker EEA1, the Cis-Golgi marker β -cop, and the endoplasmic reticulum marker calreticulin) in red, and colocalization of Rac1 proteins with all the markers in yellow (middle) (bar, 25 μm). Intensity profiles across the cell diameter are shown for the four Rac1 proteins. (C) Scheme of GFP-tagged fragments corresponding to the C-terminal (Ct) 50 aa of Rac1; the position of the mutation corresponding to C178 is indicated. Images show distribution of the GFP fusions in COS7 cells (bar, 25 μm). (D) Incorporation of [^3H]-palmitic by cells expressing the Ct Rac1 constructs. H-Ras was used as a positive control.

palmitoylation occurs via the action of palmitoyl transferases (PATs), which contain an Asp-His-His-Cys (DHHC) motif (Mitchell *et al*, 2006). At least 23 distinct mammalian PATs are localized to diverse tissues and subcellular locations (Ohno *et al*, 2006). However, the substrate specificity of these enzymes is very broad (Fukata *et al*, 2006; Mitchell *et al*, 2006). Further research is needed to determine whether

Rac1 palmitoylation is mediated by PAT activity or non-enzymatically depending on the local concentration of palmitoyl-CoA (Bano *et al*, 1998; Mollner *et al*, 1998).

Although the non-palmitoylated C178S Rac1 mutant can reach the PM, the fractionation data show that it interacts more weakly than wt Rac1 with L_0 microdomains. Similar findings have been reported for the Rho family member TC10,

Figure 5 Rac1 palmitoylation state regulates plasma membrane domain organization. (A) Absence of Rac1 palmitoylation induces PM restructuring. Images show TIRF microscopy of live COS7 cells expressing GFP-tagged wt or C178S Rac1 (bar, 25 μ m). Magnified views of boxed areas show the distinct PM structures observed (arrows). (B) TIRF microscopy analysis of the colocalization of GFP-tagged wt or C178S Rac1 with the actin marker mRFP-Ruby-Lifeact (bar, 10 μ m). (C) Effect of cholesterol depletion on wt Rac1-dependent PM structures. Cells were treated with 10 mM methyl- β -cyclodextrin before TIRF microscopy. (D) Differential Rac1-regulated PM organization is driven by distinct actin polymerization assembly. TIRF microscopy analysis of the colocalization of GFP-tagged wt Rac1 with mRFP-Ruby-Lifeact in cells expressing siRNAs against p21Arc, a member of the Arp2/3 complex (bar, 10 μ m). (E) TIRF microscopy analysis of the colocalization of Rac1 membrane structures with liquid-ordered domains. Live COS7 cells expressing GFP-tagged wt or C178S Rac1 were incubated with 2.5 μ g/ml cholera toxin B-Alexa Fluor 594, a ligand of the liquid-ordered domain marker GM1, with solid arrows indicating 'worm'-like structures (Rac1 wt) and dashed arrows indicating 'pond'-like structures (C178S). (F) Distribution of GM1 in non-transfected COS7 cells measured by TIRF microscopy (see also Supplementary Figures S4B and S5 and Supplementary Movies S4–S6).

which can localize to PM in the absence of palmitoylation but is excluded from cholesterol-enriched PM compartments (Watson *et al*, 2003). The FRAP analysis shows that non-palmitoylated Rac1 is also defective for incorporation into the nuclear membrane, where microdomains similar to L_o PM

domains have been identified (Cascianelli *et al*, 2008). This suggests that palmitoylation might also control the nuclear trafficking of Rac1. Nuclear Rac1 has been shown to play an important role in the regulation of cell proliferation (Michaelson *et al*, 2008; Woodcock *et al*, 2010).

Figure 6 Rac1 regulates membrane fluidity. (A) COS7 cells expressing mCherry-wtRac1 or the palmitoylation-deficient mutant mCherry-C178SRac1 were labelled with 5 μM Laurdan for 30 min at 37°C. mCherry confocal image (inset) and two-photon Laurdan images were acquired sequentially. Laurdan images were converted into GP images (see Materials and methods). GP images are pseudo-coloured with high GP (ordered membranes) in yellow and low GP (fluid membranes) in blue. The right panels show GP images masked for the mCherry-Rac1 channel and the GP colour scale image merged with the mCherry intensity channel. Plots show mean GP and relative abundance (percentage of coverage) for fluid and ordered membranes. Error bars are standard deviations of two independent experiments with a total of 35 cells per condition. (B) Measurement of cholesterol levels in COS1 cells expressing mCherry-wtRac1 or the palmitoylation-deficient mutant mCherry-C178SRac1. (C) Effect of loss of Rac1 on sucrose gradient partitioning of caveolin 1 and GM1. Western blots of sucrose density gradient fractions of wt and Rac1^{-/-} MEFs and Rac1^{-/-} MEFs reconstituted with wt or C178S Rac1. Fractions were analysed for the distribution of Rac1 and Cav1. GM1 was detected with horseradish peroxidase-tagged cholera toxin B subunit. Red boxes denote DRM fractions (7–10). Note the absence of GM1 and Cav1 from DRM fractions of Rac1^{-/-} cells and the partial recovery upon reexpression of wt or C178S Rac1. (D) Wt and Rac1^{-/-} MEFs and Rac1^{-/-} MEFs reconstituted with wt or C178S Rac1 were labelled with 5 μM Laurdan for 30 min at 37°C. GP images are pseudo-coloured with high GP (ordered membranes) in red and low GP (fluid membranes) in blue. The panels show GP images (left inset) masked for the mCherry-Rac1 channel (right inset) and the GP colour scale image merged with the mCherry intensity channel. Plots (right) show mean GP and relative abundance (percentage of coverage) for fluid and ordered membranes.

Figure 7 Role of Rac1 palmitoylation in cell motility. (A) Wound closure assays with COS7 cells expressing GFP-wt or GFP-C178S Rac1. The graph plots relative wound area ($n = 3$) (bar, 100 μm). (B) Morphology of migrating MEFs expressing GFP-wt or GFP-C178S Rac1. Actin and Hoechst staining show cell positions. Inserts in the merged images highlight the different morphologies (bar, 100 μm). (C) Cells expressing GFP-wt or C178S Rac1 were plated at low density on FN and migration recorded by time-lapse video microscopy (bar, 50 μm). Lines show representative migration tracks. The chart shows net distance as a function of total distance travelled (directionality index). Values are means $\pm$ s.e.m. of 50 cells per condition from two independent experiments. (D) Identical studies were performed with conditionally Rac1^{-/-} MEFs. The Rac1 allele was deleted by treating Rac1 loxp/loxp MEFs with adeno-Cre. Rac1 expression was rescued by transfection with pCherry-wtRac1 or the C178S mutant. Representative confocal images show cell morphologies and distributions of reexpressed Rac1 proteins (bar, 20 μm). (E) Migration of reconstituted cells on FN (bar, 50 μm). Charts show average velocity ($\mu\text{m}/\text{min}$) and directionality index. Values are means $\pm$ s.e.m. of 25 cells per condition from two independent experiments (see also Supplementary Figures S4A and S5B and Supplementary Movies S2 and S3).

The localization of several GTPases in ordered membrane domains depends on the GDP/GTP loading state (Prior *et al*, 2001) and binding to RhoGDI (Michaelson *et al*, 2001). The

C178S mutation does not affect GTP- γ -S binding (Figure 2E; Knaus *et al*, 1998); moreover, although C178SRac1 mostly occurs in the GDP-bound form the binding

Figure 8 Working model: A palmitoylation switch mechanism in the regulation of Rac1. (A) Scheme of the three carboxy-terminal motifs of Rac1 involved in association with membranes. A polybasic domain is flanked by two post-translational modifications, prenylation at Cys 189 (blue) and palmitoylation at Cys 178 (red). (B) Proposed model of three-signal membrane targeting mechanism for Rac1 regulation. Palmitoylation would favour signaling downstream of Rac1 by stabilizing its localization to pre-existing L_o domains. L_o -localized Rac1 would initiate spatially confined branched-actin polymerization, which in turn would promote the expansion or coalescence of L_o domains, a process essential for cell migration. Cycles of palmitoylation and depalmitoylation of Rac1 would thus provide a rapid and spatially restricted mechanism for the control of Rac1-dependent cell functions.

to RhoGDI is not modified. The reduced GTP loading thus might be due to an inability of palmitoylation-deficient Rac1 to associate with effectors in L_o regions that stabilize the GTP-bound form. This impaired association might be favoured by the higher tendency of C178S Rac1 to form oligomers, which downregulates Rac1 activity. Self-association of Rac1 was originally demonstrated in gel filtration experiments (Zhang *et al*, 2001), but it is not known how Rac1 oligomers form inside the cell and what function they have. Wong *et al* (2008) recently demonstrated that self-association of Rac1 requires

the C-terminal polybasic region and the prenylation signal, and that forced localization of Rac1 to L_o domains (by NH₂-terminal myristoylation) does not promote formation of Rac1 oligomers. The more efficient oligomerization of C178S Rac1 thus might reflect the inability of this mutant to partition in L_o domains, but could also indicate that the absence of palmitoylation in this position alters the orientation of the protein in the membrane. Oligomerization might maintain a pool of inactive membrane-associated Rac1 that could be rapidly mobilized.

The release of Rac1 from this inactive membrane pool by palmitoylation might render it accessible to its effector PAK (Chenette *et al*, 2006; Parrini *et al*, 2009). Our results indicate that Rac1 palmitoylation promotes activation of PAK at the PM. Altered signalling via PAK or other effectors in the absence of Rac1 palmitoylation could explain the associated migratory defects. Interestingly, mutation of the palmitoylation site in a constitutively active form of Rac (Rac1-Q61L-C178S) was reported to reduce soft agar colony formation (Roberts *et al*, 2008), which is consistent with the low activity and impaired signalling we observed for this mutation.

Early descriptions of the membrane raft model proposed that interactions between cholesterol and sphingolipids generate a lipid platform with which specific proteins associate. Later refinements propose that formation of L_o domains also involves membrane-associated proteins (Douglass and Vale, 2005; Plowman *et al*, 2005). For example, membrane domains are regulated by the cortical actin cytoskeleton, a filament lattice that underlies and attaches to the PM (Chichili and Rodgers, 2009). RhoGTPases regulate the actin cytoskeleton by stimulating actin polymerization, and we now propose that Rac1 might influence membrane organization through this activity via a mechanism dependent on palmitoylation. In TIRFm experiments, inhibition of actin polymerization with Cyt D or siRNAs against p21-Arc and Arp 3 reproduced the F-actin and Rac1 distribution seen in cells expressing C178SRac1. Moreover, Rac1-deficient cells, which have severely disrupted actin cytoskeleton, lacked biochemically defined rafts (DRMs), an effect partly rescued by reexpression of Rac1 (Figure 6C). Although GM1 recovery into DRMs was slightly better in Rac1 wt reconstituted Rac1^{-/-} cells, the palmitoylation-deficient Rac1 also recovered DRMs. A similar result was obtained by inhibiting palmitoylation of endogenous Rac1 with 2-Brp, which removed detectable Rac1 from DRMs while leaving the domains intact (Figure 3B). However, it is highly unlikely that biochemical fractionation is sensitive enough to detect transient changes in membrane organization mediated by Rac1 palmitoylation. Supporting this, *in-vivo* fluidity measurement with Laurdan in Rac1 null cells reveals the existence of small liquid-ordered (L_o) domains that are not detectable in DRMs, which would correspond to larger (more stable, clustered) L_o domains. Indeed, Triton X-100 treatment at 4°C by itself promotes the coalescence of L_o domains (Heerklotz, 2002; de Almeida *et al*, 2003). Technical difficulties prevented a completely satisfactory Laurdan analysis of reconstituted Rac1-null cells. However, the results were similar to those obtained by overexpressing wt or C178S Rac1 in COS7 cells, which express low levels of endogenous Rac1. These analyses thus validate the conclusion that the Rac1 palmitoylation state influences lipid membrane organization, promoting the formation of larger L_o domains. We propose that small L_o domains exist independently of Rac1 and of its palmitoylation state, and that these domains are not detected as DRMs; non-palmitoylated Rac1 is able to localize to these domains, but palmitoylation favours this association, increasing its extent. Rac1 effectors also localize to these sites (Goetz *et al*, 2011), and Rac1-induced actin polymerization would trigger expansion or coalescence of these small L_o domains into larger ones, which would be detectable as DRMs. Thus, the DRM technique, though informative, cannot detect regulation at the level of small L_o domains, and needs to be

combined with *in-vivo* approaches able to detect transient, dynamic changes in smaller regions. Supporting this hypothesis, smaller scale experiments that do not require 100% reexpression (spreading and directional migration) confirmed that C178S Rac1 could not rescue the wt phenotype.

This conclusion is also supported by the TIRF microscopy analysis, which yielded detailed information about how Rac1 influences the spatio-temporal organization of membrane lipids, providing evidence that Rac1 palmitoylation restructures and stabilizes L_o membrane microdomains (Figure 5; Supplementary Figure S5B). This is further supported by the observation that the number of disordered membrane regions is greater in Rac1^{-/-} cells or cells expressing palmitoylation-deficient Rac1. However, the ordered domains in these cells have a higher GP value in Laurdan two-photon analysis than those in cells expressing wt Rac1. The lower GP value in cells expressing wt Rac1 might indicate that cholesterol PM levels are limiting and hence not sufficient to maintain the same degree of order detected in cells expressing palmitoylation-deficient Rac1. Similar levels of cholesterol in both cell types support this hypothesis; and in other conditions where the number of ordered domains increases, GP values of these domains decrease (M Calvo, C Rentero and C Enrich, unpublished observations). Higher GP values in cells expressing palmitoylation-deficient Rac1 might also reflect spreading defects and subsequent reduced surface area in these cells, since the relative amount of cholesterol per surface area would be higher.

A role for Rac1 in regulating membrane organization is also supported by the low level of palmitoylation. Since palmitoylation is the modification that best correlates with protein partitioning into ordered domains (Zacharias *et al*, 2002; Levental *et al*, 2010), heavy palmitoylation would be expected in a protein that is stably localized to ordered domains. In contrast, weak and transient palmitoylation (as we observe for Rac1) is more appropriate for a protein whose role is to dynamically regulate PM organization, and that would therefore need to cycle between ordered and non-ordered domains.

Rac1 partitioning into L_o domains is critical for a positive feedback loop for sustained Rac1 activation through recruitment of a protein complex containing coronin 1A, PAK, RhoGDI and ArhGEF7 (Castro-Castro *et al*, 2011; Symons, 2011). Therefore, increased partitioning of palmitoylated Rac1 into L_o domains could contribute to the amplification of Rac1 activation, and this could also explain, at least in part, the apparent discrepancy between the large effect of the C178S mutation on GTP binding (~80% less than Rac1 wt) and the mild effect on PM localization (~20–30% less).

Our findings show that Rac1 function is regulated by palmitoylation in the PM, through a mechanism requiring prior prenylation and the polybasic region (Figure 8A). Palmitoylation favours stabilization of Rac1 in L_o domains, triggering downstream activation of effectors such as PAK, F-actin polymerization mediated by Arp2–3, and formation of lamellipodia. Rac1-mediated actin polymerization would induce the expansion or coalescence of ordered microdomains into larger domains, thus promoting membrane order (Figure 8B). Interestingly, Rac1-induced actin polymerization is essential for the recruitment of the coronin 1A/ArhGEF7 complex (Castro-Castro *et al*, 2011; Symons, 2011), thus amplifying Rac1 activation. We thus propose that Rac1 can

act as a membrane organizer through a mechanism dependent on the actin cytoskeleton. Depalmitoylation, by promoting oligomerization, might be a mechanism for maintaining a membrane pool of GDP-bound Rac1, primed for rapid activation. Palmitoylation of Rac1 influences the morphology and dynamics of cell membranes, and might underlie processes characterized by continual rearrangement of PM architecture, such as directional migration. The identification of Rac palmitoylation as a determinant of Rac1 function opens the way to further studies into the implication of Rac palmitoylation in different membrane compartments and cell contexts.

Materials and methods

Reagents

Antibodies used were anti-Rac1 mAb (Upstate Biotechnology); anti- γ -tubulin mAb (Sigma-Aldrich); anti-PAK1/2/3[pS¹⁴¹] pAb and antiphospho-Akt mAb (pS473: Biosource); anti-caveolin1 pAb and anti-EEA1, anti-p21-Arc and anti-GM130 mAbs (BD Biosciences); anti-RhoGDI pAb (Santa Cruz Biotechnology); anti-Akt (Cell Signaling Technology); anti-GFP mAb (Clontech); and rabbit anti-GFP antiserum (a gift from I Rodríguez-Crespo, Complutense University, Madrid). Alexa fluor 647 or 594 conjugated phalloidin and Alexa 594 conjugated secondary antibodies were from Molecular Probes, and peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were from Jackson ImmunoResearch Laboratories. PDGF-BB was from Peprotech. [9, 10-³H]-palmitic acid (40–60 Ci/mmol) and ProteinA/G-Sepharose were from GE Healthcare. A 'smartpool' set of four siRNAs against *p21-Arc* (sense sequences: GAUGAGAGCCUAUUUACAA, UGCCAAACCUGCAAACAAA, GAGACUGGACUGAGACUUU, GGUGAGAAAGAAAUGUAUA) and a control non-targeting siRNA (control #1) were obtained from Dharmacon. Alternative siRNAs against *p21-Arc* and Arp 3 (Actin-related protein 3) (flexitubes with material numbers SI00299138, SI02664298, SI02664305 and SI02652272) were purchased from Qiagen. All other chemicals were of the highest grade available and were obtained from regular commercial sources.

Metabolic labelling with [³H]-palmitate and immunoprecipitation

Twenty-four hour after transfection, COS-7 or MEF monolayers were starved for 1 h with DMEM lacking FBS. [9, 10-³H]-palmitic acid (250 μ Ci) was dried under nitrogen and resuspended in 10 μ l DMSO and made up to 1 ml in DMEM containing 0.5% de-fatted BSA (Sigma). Cells were labelled for 4 h, washed with PBS, scraped and lysed in RIPA buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate, pH 7.35) in the presence of protease inhibitors. After centrifugation at 6000 g for 10 min at 4°C, the supernatant was incubated with 10 μ l rabbit anti-GFP antibody for 4 h at 4°C. ProteinA-Sepharose beads (25 μ l; GE Healthcare) were added to each sample and incubation continued for 2 h at 4°C. Immunoprecipitates were centrifuged at 16 000 g for 10 min, and after removal of the supernatant were washed twice in 0.5 ml ice-cold RIPA buffer. Samples were separated by SDS-PAGE, and the gels were treated with Enhance solution (NAMP 100, GE Healthcare) to improve the radioactive signal. Gels were dried in a Bio-Rad vacuum apparatus and exposed to photographic film for at least 4 weeks at –80°C.

Protein palmitoylation was also analysed by labelling with 1-biotinamido-4-4-(maleimidomethyl)cyclohexanecarboxamido)-butane (Biotin-BMCC), as described in Drisdell and Green (2004). For further details, see Supplementary Materials and Methods.

Time-lapse video microscopy of cell trajectories and wound healing

Motility of cells expressing wt or C178S Rac1 constructs was recorded by capturing phase-contrast images at 8 min intervals for 10 h. To determine cell trajectories, the centroids of cell nuclei were tracked with the track objects function in MetaMorph. For wound-healing assay, confluent monolayers were scraped with a 0.1–2 μ l pipette tip, and wound closure was monitored by capturing images at 10 min intervals over 24 h.

PM isolation

PMs were isolated by the protocol of Smart *et al* (1995). For further details, see Supplementary Materials and Methods.

Rac GTPase assay

Rac activity was determined by pull-down assay as described in del Pozo *et al* (2000). For further details, see Supplementary Materials and Methods.

Preparation of DRM fractions

DRMs were purified as described in Navarro-Lerida *et al* (2002). For further details, see Supplementary Materials and Methods.

Bead binding assay

Latex-beads were coated with cholera toxin B (CTxB) or fibronectin (FN) as previously described (del Pozo *et al*, 2004).

Microscopy and image analysis

Images were acquired with a Leica SP5 or a Leica SPE confocal microscope. For FRAP, a pre-bleach event was acquired with the Leica SP5, followed by a bleach event induced by stimulation at 488 nm. Fluorescence during recovery was monitored at 10 s intervals, and was normalized to the pre-bleach intensity.

TIRFm was used at a penetration depth of 90 nm, allowing observation of events at or near to the PM. For TIRFm movies, frames were acquired every 0.3 s with a Peltier cooled (–75°C) Andor iXON EMCCD camera.

Laurdan microscopy

Live cells grown on glass coverslips were stained for 30 min at 37°C with 5 μ M Laurdan (Invitrogen) in complete medium, and imaged after two rinses in medium as described previously (Gaus *et al*, 2003; Rentero *et al*, 2008). Laurdan imaging was performed with a TCS SP5 inverted confocal microscope (Leica, Germany) equipped with a near infrared laser (Mai Tai Broad Band 710–990 nm) and an APO $\times$ 63 glycerol immersion objective (1.3 NA). Confocal mCherry signal (excitation 561 nm; emission 570–620 nm) was recorded, followed by two-photon Laurdan images, which were acquired at 800 nm excitation, with emission ranges collected simultaneously at 400–460 and 470–530 nm. To calibrate the relative sensitivity of the two channels, a correcting factor (G) was obtained by imaging a standard solution of 5 μ M Laurdan in DMSO at room temperature under the same imaging conditions as experiments. Laurdan intensity images were converted into GP images (Gaus *et al*, 2006), with each pixel calculated in ImageJ from the two Laurdan intensity images according to the equation:

$$GP = (I_{(400-460)} - G \times I_{(470-530)}) / (I_{(400-460)} + G \times I_{(470-530)}).$$

GP distributions were obtained from the histogram values of mCherry-masked GP images and non-linear fitted to one or two Gauss distributions using a custom-built macro in ImageJ. GP images were pseudo-coloured and merged with the mCherry intensity channel.

Statistics

Statistical significance was determined by two-tailed paired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: IN-L performed most of the experiments, analysed data and wrote the first draft of the manuscript. SS assisted in biochemistry experiments and cloning. MC, CR and CE performed and analysed the Laurdan two-photon microscopy

experiments. YZ provided the Rac1 loxp/loxp primary MEFs. CE and YZ contributed discussion. MADP conceived the project, directed the work, analysed data, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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