Rac1 meets ubiquitin: New insights in Rac1 signalling
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Chapter 5

Caveolin-1 regulates the poly-ubiquitinylation and degradation of activated Rac1

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Summary
Extracellular stimuli such as growth factors or integrin engagement will induce activation of the Rho-like GTPase Rac1 by recruiting and activating Rac1 GEFs (Guanine nucleotide Exchange Factors). Active, GTP-bound Rac1 can interact with cognate effector proteins to trigger intracellular signalling. The inactivation of Rac1 is generally assumed to be dependent on its intrinsic GTP hydrolysis activity that can be stimulated by Rac1 GAPs (GTPase-activating proteins). We recently found that the Rac1 GTPase associates to the adapter protein Caveolin-1 (Cav1) and that Rac1 activity promotes Cav1 accumulation at Rac1-positive, peripheral adhesions. Using Cav1-deficient mouse fibroblasts as well as siRNA- and shRNA-mediated depletion of Cav1 expression in human epithelial and endothelial cells, we here show that loss of Cav1 induces an increase in the levels of total Rac1 protein and its activated, GTP-bound form. Cav1 controls Rac1 protein expression by regulating ubiquitinylation and degradation of activated Rac1 in an adhesion-dependent fashion. Finally, we show that Rac1-ubiquitinylation is not required for effector binding but rather regulates the dynamics of Rac1 at the periphery of the cell. These data extend the canonical model for Rac1 inactivation and uncover Cav1-regulated poly-ubiquitinylation as an additional mechanism to control Rac1 signaling.

Introduction
Directional cell migration is dependent on the spatiotemporal regulation of intracellular signalling events. These events control cytoskeletal dynamics, providing both driving force at the front of the cell and contraction at the back. The actin cytoskeleton is central to these processes and is regulated through signalling by Rho-like small GTPases such as RhoA, which stimulates myosin-based contractility, and CDC42 and Rac1, which promote actin polymerization and cell protrusion (Ridley et al., 2003; Vicente-Manzanares et al., 2005).

Like most other small GTPases, Rho family members act as molecular switches, cycling between an active, GTP-bound state and an inactive, GDP-bound state. These transitions are stimulated by guanine nucleotide exchange factors
(GEFs) and GTPase-activating proteins (GAPs), respectively (Bernards and Settleman, 2004; Bos et al., 2007; Rossman et al., 2005). Additionally, RhoGTPases cycle between the plasma membrane and the cytosol, where they are bound to RhoGDI (guanine nucleotide dissociation inhibitor) (DerMardirossian and Bokoch, 2005; Dovas and Couchman, 2005). GEF-mediated GTPase activation and dissociation from RhoGDI takes place at cellular membranes, as most GEFs associate with membranes through PH-domain-mediated binding to phosphatidylinositol lipids (Bos et al., 2007; Rossman et al., 2005). Although GTP-loading is critical for GTPase function, GTPase cycling, i.e. controlled GTPase inactivation, is considered equally important for efficient signal transduction. Accumulating evidence suggests that, in addition to GAP-stimulated GTP hydrolysis, other mechanisms exist that serve to turn off GTPase signalling.

Activation of endogenous Rac1 and RhoA by bacterial toxins such as CNF-1 (Cytotoxin Necrotizing Factor 1) leads after 4-6 hrs to proteasome-mediated degradation (Munro and Lemichez, 2005; Pop et al., 2004). This shows that cells possess intrinsic mechanisms to degrade activated GTPases, representing a third level of GTPase regulation, next to membrane translocation and GTP-loading and hydrolysis. Degradation of RhoA was shown to occur in a polarized fashion, and requires the E3 ubiquitin ligase Smurf1 (Wang et al., 2003). For Rac1, it has been shown that activated mutants are more susceptible to ubiquitinylation and degradation and that this pathway can be activated by receptor agonists, as demonstrated for HGF in epithelial MDCK cells (Doye et al., 2002; Lynch et al., 2006; Pop et al., 2004). However, the cellular mechanisms and proteins that control the degradation of activated Rac1 are unknown.

We have recently shown that Rac1 activity recruits Cav1 to Focal Adhesion (FAs) and that Rac1 associates to Cav1 in an adhesion-dependent fashion. Here we show that Cav1 is part of a signalling pathway that regulates the poly-ubiquitylation and degradation of Rac1. Cav1 regulates Rac1 protein levels by regulating the degradation of Rac1 in an adhesion-dependent fashion, controlling cell polarity, spreading and migration. Moreover, we show that a Rac1 mutant that cannot be ubiquitinylated displays altered dynamics at the peripheral ruffling
membranes. This suggests that Rac1 conjugation by ubiquitin plays a role in Rac1 dynamics. These data identify an endogenous signalling pathway that affects cell adhesion and motility by controlling Rac1 degradation. This represents an important extension of the canonical GDP/GTP cycle describing the regulation of Rac1 activation and inactivation.

Results

Rac1-localisation at FAs is independent of Cav1

In previous studies, we found that Rac1 binds to Cav1 and that Rac1 activation promotes the accumulation of Cav1 at FAs. To further investigate whether Rac1 acts upstream of Cav1 at FAs, the localisation of endogenous Rac1, β-Pix and pY118paxillin was studied by confocal microscopy in mouse embryonic fibroblasts (MEFs) genetically deficient for Cav1 (Cav1−/−).

As endogenous Rac1 (co)localises with pY118 paxillin at peripheral sites of adhesion in Cav1−/− MEFs, Rac1 targeting towards FAs appears independent of Cav1 (Fig. 1A). Likewise, Rac1 localisation at the ends of actin stress fibers was also unaffected in Cav1-siRNA-transfected Hela cells (Fig.1B) (Beardsley et al., 2005). Finally, the interaction of Rac1 with β-Pix, which we previously found to drive Rac1 targeting to FAs, as well as their co-localisation at FAs, was unaffected in Cav1−/− MEFs (Fig. 1C,D) (ten Klooster et al., 2006). These data indicate that Rac1 acts upstream of Cav1 at a step downstream of β-Pix-mediated targeting of Rac1 to FAs.

Loss of Cav1 promotes Rac1-mediated cell spreading

We further analysed the effects of loss of Cav1 expression on cell spreading and tested the role for Rac1 signaling under these conditions. Similar to Cav1−/− MEFs, HeLa cells transfected with Cav1-siRNA showed a loss of polarity with an apparent increase in cell spreading (Fig. 2A). To study this in more detail we performed several experiments using Cav1-deficient MEFs as well as HeLa cells transfected with the Cav1 siRNA. Real-time analysis of cell spreading of wt and Cav1−/− MEFs on fibronectin-coated ECIS electrodes (ten Klooster et al., 2006)
showed that, in line with the data of Cav1-siRNA transfected HeLa cells (Fig 2A), depletion of Cav1 increases cell spreading, as recorded within the first hour after seeding the cells (Fig. 2B).

Figure 1. Rac1-localisation at FAs is independent of Cav1 (A) Immunostainings for endogenous Rac1 in wild-type (wt) and Cav1-/- MEFs show localisation of Rac1 at FAs, marked by pY118 paxillin, even in the absence of Cav1 (arrows). (B) HeLa cells, transfected with the Cav1 siRNA (asterisk) were immunostained for endogenous Rac1 and analysed for its localisation to F-actin marked sites of adhesion. The cell on the right, in which Cav1 expression is not reduced, serves as a positive control. Bar, 20 μm. These stainings show that Cav1 is not required for the targeting of activated Rac1 to FAs. (C) Rac1 C-terminal interaction with β-PIX was analysed in the absence or presence of Cav1 with lysates from wild-type (wt) and Cav1-/- MEFs. The association of Rac1 to PIX is independent of Cav1. (D) Immunostainings for endogenous Rac1 in wild-type (wt) and Cav1-/- MEFs were analysed for co-localisation to β-PIX-marked FAs (arrows). Bar, 10 μm.

Complementary phase-contrast imaging of the Cav1-/- MEFs, seeded for one hour on fibronectin, showed increased cell spreading as compared to the wt cells (Fig. 2C). This was similar to the effects seen in HeLa cells transfected with Cav1-siRNA, which showed, next to an increase in cell surface area, also impaired chemotaxis, likely as a result of the loss in polarity (Fig. 2D).
Figure 2. Loss of Cav1 promotes Rac1-mediated cell spreading (A) Immunostainings for F-actin underscore the cell spreading and loss of polarity of Cav1 siRNA-transfected HeLa cells on fibronectin-coated glass cover slips. Bar, 20μm. The lower, polarized cell in which Cav1 expression (green) is not reduced, serves as a positive control. (B) Cell spreading of wild-type (wt) and Cav1⁻/⁻ MEFs in the presence or absence of the Rac1 inhibitor EHT1864 (50μM) was analysed by ECIS on fibronectin-coated gold electrodes and depicted as normalised resistance (graph in left panel). Right: phase-contrast images of Cav1⁻/⁻ MEFs spread on fibronectin for 30 minutes or 3 hours in the presence or absence of EHT1864 (50μM). Bar, 20μm. These data show that the increased spreading in the Cav1⁻/⁻ MEFs is Rac1-dependent. (C) Phase-contrast images of wild type (wt) and Cav1⁻/⁻ MEFs seeded for 1 hour on fibronectin-coated glass slides show the increased spreading in Cav1⁻/⁻ MEFs. Bar, 40μm. (D) Analysis of cell spreading of control and Cav1 siRNA-transfected HeLa cells seeded on fibronectin for 1 hr as quantified by measuring the cell area (left panel), and analysed for chemotaxis towards 10% FCS in a transwell migration assay (right panel) (*, p<0.05). (E) Cell spreading of HeLa cells following ectopic expression of GFP-Cav1 was analysed on fibronectin-coated gold electrodes by ECIS and results are presented as normalised resistance. (F) HeLa cells treated with siRNA to Cav1 were analysed for cell spreading using ECIS in the presence or absence of the Rac1-GEF inhibitor NSC23766. (G) Cell spreading was compared between control or Rac1 siRNA-transfected HeLa cells and quantified by real time analysis on fibronectin-coated gold electrodes by ECIS. Data are depicted as normalised resistance. Expression analysis by Western blotting, confirming reduced Rac1 levels, is shown on the right.

To confirm that these effects were linked to the Cav1 protein, we also performed the reverse experiment and transfected HeLa cells with GFP-Cav1. Spreading analysis on fibronectin-coated ECIS electrodes showed that expression of GFP-Cav1 reduced cell spreading (Fig. 2E), in good agreement with the above data showing that loss of Cav1 promotes spreading.
To establish the relevance of Rac1 in the effects of loss of Cav1 on cells spreading, we pre-treated MEFs or HeLa cells with the Rac1 inhibitor EHT1864 (50μM) (Shutes et al., 2007) or the Rac GEF inhibitor NSC23766 (100 μM), respectively, prior to cell spreading. The EHT inhibitor completely impaired the enhanced cell spreading in Cav1-/- MEFs (Fig. 2B). Similarly, the NSC compound blocked the increased spreading of HeLa cells, transfected with Cav1 siRNA (Fig. 2F). These data support the notion that the enhanced spreading that follows the loss of Cav1 expression is due to increased Rac1 activity. In line with this idea, siRNA-mediated reduction of Rac1 expression also impaired cell spreading, as measured by ECIS (Fig. 2G). Thus, as Rac1 targeting to FAs is driven by β-Pix (ten Klooster et al., 2006), subsequent recruitment of Cav1 could serve to negatively regulate Rac1 signaling to allow cell polarization.

**Cav1 regulates Rac1 activity and protein expression levels**

Since Cav1 depletion promotes Rac1-dependent cell spreading, we analysed Rac1-GTP-loading as a measure for Rac1 activity in control and Cav1 siRNA-treated HeLa cells, using the Pak-Crib pull-down assay (ten Klooster et al., 2006). In line with published data with Cav1 -/- MEFs (Grande-Garcia et al., 2007), loss of Cav1 is accompanied by increased Rac1GTP loading (Fig. 3A). Surprisingly however, also the levels of total Rac1 protein were increased in siRNA-Cav1 transfected HeLa cells (Fig. 3A). To further corroborate this result, we analysed Rac1 levels in Cav1 -/- MEFs in more detail. Also in these cells, we found that the levels of total Rac1 as well as its GTP-bound form were increased as compared to the levels of total and activated Rac1 in the wild type MEFs (Fig. 3B).

The Pak-Crib pull-down is performed in NP40-containing lysis buffer. To exclude that our interpretation was obscured as a result of a change in solubility of a fraction of endogenous Rac1, Rac1 protein levels were analysed in control and Cav1 siRNA-treated HeLa cells that were lysed directly in SDS-sample buffer (Fig. 3C,D).
Figure 3. Cav1 regulates Rac1 activity and protein expression levels. (A) Rac1 activation was assayed by biotinylated Pak-Crib peptide-based pull-down with lysates of control and Cav1 siRNA transfected HeLa cells. Bar diagram depicts the relative increase in Rac1 expression and activation levels in Cav1 siRNA treated cells compared to control cells, as determined by quantification of Western blots (n=3). Loss of Cav1 is associated with approximately 2-fold increase in total and activated Rac1. (B) Rac1 activation and expression was assayed by biotinylated Pak-Crib peptide-based pulldown in lysates of wild type (wt) and Cav1-/- MEFs. Bar diagram depicts the relative increase in Rac1 expression and activation levels in Cav1-depleted Cav1-/- MEFs compared to parental cells, as determined by quantification of blots (n=3). Like siRNA-based depletion of Cav1, loss of Cav1 in Cav1-/- MEFs shows approximately 2-fold increase in total and activated levels of Rac1. (C) SDS lysates of control and Cav1 siRNA-transfected cells were analysed for protein levels of Rac1, CDC42 and RhoA, and Rac1 levels in SDS-lysatess were also determined in Cav1 siRNA-transfected HUVEC and in Cav1-/- MEFs. (D) Total cell lysates of HeLa cells were analysed for Rac1 and Cav1 expression at 24hrs, 48hrs and 72hrs after transfection with control or Cav1 siRNA, showing a time-dependent decrease of Cav1 levels and a concomitant increase in Rac1 levels. (E) Rac1 expression levels analysed in HeLa cells, 48hrs after shRNA-based depletion of Cav1, using two different Cav1 shRNAs. Both Cav1 shRNAs showed an approximate 90% loss of endogenous Cav1, which correlated with a 2-fold increase of Rac1 expression levels as determined by quantification of the blots (n=3). (F) Relative mRNA expression levels of Rac1 and RhoA normalised to the β-glucuronidase housekeeping gene in control or Cav1 siRNA-transfected HeLa cells. Data are means of two independent experiments and show that Cav1 siRNA does not affect Rac1 or RhoA mRNA levels.

Also under these conditions, Rac1 protein levels were found to be increased upon depletion of Cav1 by Cav1-siRNA, which shows that this effect concerns the total pool of cellular Rac1. Rac1 protein expression is also increased in SDS-sample buffer lysates from siRNA-Cav1 transfected HUVEC, as well as in Cav1-/- MEFs, which furthermore shows that the increased expression of Rac1 upon loss of Cav1 is not cell-type specific (Fig. 3C). Moreover, two independent Cav1-shRNAs, which were taken along as a control for possible off-target effects of the Cav1-siRNA, were also found to increase Rac1 protein expression upon silencing of Cav1 (Fig. 3E). Unlike Rac1, the protein expression levels of RhoA or CDC42 were not
afected by loss of Cav1 (Fig. 3C). Of note, although Cav1 can associate to RhoA (Chapter 3;Fig 1a), RhoA-GTP levels have been described to decrease in the absence of Cav1 (Grande-Garcia et al., 2007) which further supports the notion that the Cav1-dependent regulation of RhoA markedly contrasts with Cav1 regulation of Rac1. Finally, since this data suggests that Cav1 affects Rac1 signaling output by regulating its protein expression level, we analyzed whether depletion of Cav1 increases mRNA levels of Rac1. RQ-PCR on cDNA from control and Cav1 siRNA-transfected cells revealed no changes in the mRNA levels of either Rac1 or RhoA (Fig. 3F).

**Cav1 regulates Rac1 ubiquitinylation**

Since the increase in Rac1 protein expression following loss of Cav1 could not be explained by increased mRNA synthesis, we analyzed potential modulation of Rac1 degradation as an alternative mechanism. It has been shown previously that activated Rac1 can be ubiquitinylated and degraded by the proteasome (Boyer et al., 2006; Doye et al., 2002; Lerm et al., 1999; Visvikis et al., 2008). To test whether Cav1 plays a role in the ubiquitinylation and degradation of Rac1, control- and Cav1-siRNA transfected cells were co-transfected with His-tagged ubiquitin, to isolate endogenous ubiquitinated Rac1 by a cobalt-beads-based approach (Fig. 4A). SiRNA-mediated depletion of Cav1 induced a 4-fold accumulation of mono-ubiquitinated, endogenous Rac1, which migrates at an apparent Mw of 30kD on SDS-PAGE. In contrast, the levels of poly-ubiquitinated Rac1 were reduced to approximately 25% in the absence of Cav1. Thus, Cav1 regulates poly-ubiquitinylation and likely also the consequent degradation of Rac1, which may explain the increase in Rac1 protein levels upon loss of Cav1 expression. As Rac1 degradation is driven by the proteasome, we tested if treatment of wt and Cav1⁻⁻ MEFs with proteasome inhibitors would level the increase in Rac1 protein levels in Cav1-depleted cells. However, these experiments were inconclusive since 4 hrs of treatment with a proteasome inhibitor is toxic to the cells, precluding further analysis.
Since integrins mediate Rac1 activation (Price et al., 1998) and adhesion regulates the Rac1-Cav1 interaction, we tested whether loss of integrin function would also affect the ubiquitinylation of Rac1. We found that in cells kept in suspension for two hrs mono- as well as poly-ubiquitinylation of endogenous Rac1 was inhibited, relative to its ubiquitinylation in adherent cells (Fig. 4B). Thus, integrins regulate Rac1 activation (Price et al., 1998) and ubiquitinylation.

**Figure 4. Cav1 regulates Rac1 ubiquitinylation**

(A) Endogenous, ubiquitinated Rac1 was isolated by cobalt-bead-based pull-down from lysates of control and Cav1 siRNA-transfected HeLa cells co-transfected with 6xHis-myc-tagged ubiquitin. Two exposures of the Western blot are shown to underscore the increase in mono-ubiquitinated endogenous Rac1 (short exposure, left) and the reduction in poly-ubiquitinated Rac1 (long exposure, right) in the cells transfected with the Cav1 siRNA. The relative differences in mono- and poly-ubiquitinylation were quantified by densitometry (n=3) and are indicated in the bar graphs. In addition, the sizes of the various ubiquitinated forms of Rac1 (Rac1 ~22kD, Rac1-Ub ~30 kD; Rac1-Ub2 ~38 kD; Rac1-Ub3 ~46 kD and Rac1-Ub4 ~54 kD) are indicated. (B) Analysis of adhesion-dependent mono-ubiquitination of endogenous Rac1 in HeLa cells. Prior to lysis and isolation of ubiquitinated proteins, cells transfected with 6xHis-myc-tagged ubiquitin were either seeded on fibronectin for 2 hrs or kept in suspension. Western blotting following isolation of ubiquitinated proteins was used to detect mono-ubiquitinated Rac1 (short exposure, left) and poly-ubiquitinated Rac1 (long exposure, right) as detected by immuno-staining with a Rac1 antibody.

**Rac1 ubiquitinylation regulates peripheral Rac1 dynamics**

Previous studies, with activation of endogenous Rac1 by CNF1 toxin or using expression of activated Rac1 mutants, have clearly demonstrated the positive correlation between Rac1 activation, its ubiquitinylation and its consequent degradation (Doye et al., 2002; Pop et al., 2004; Visvikis et al., 2008).
Figure 5. Ubiquitinylation is not required for Rac1 targeting

(A) Ubiquitinylation of HA-tagged Rac1Q61L or Rac1Q61LK147R was analysed as in Fig. 4. Immunoblots were stained for HA to detect ubiquitinated Rac1. (B) Effector binding of Rac1Q61L and Rac1Q61LK147R was assayed with a Pak-Crib peptide-based pulldown. Ubiquitinylation of activated Rac1 is not required for binding to the Pak-Crib domain. (C) Confocal imaging of immunostainings showing that Rac1Q61LK147R localises to β-Pix-positive FAs (upper panels) and to membrane ruffles as identified by F-actin (lower panels, arrows). Bar, 10 μm. (D) HeLa cells transfected with myc-tagged Rac1Q61LK147R were stained for (co)localisation of Rac1Q61LK147R and endogenous Cav1 as seen in the upper-right cell. The lower left cell is untransfected and shows Cav1 primarily at the rear of the cell. The zoom underscores the colocalisation of Rac1Q61LK147R and Cav1 at peripheral sites of adhesion. Bar, 20 μm. (E) Still images and corresponding kymograph analysis, derived from the indicated line (t (time), 25 sec, d (distance), 1 μm) obtained by live-cell imaging of YFP-tagged Rac1Q61L and mCherry-tagged Rac1Q61LK147. (F) Still images of live cell analysis of mCherry wt Rac1 and mCherry K147R wt Rac1. K147R wt Rac1 that can not be ubiquitinated localized more prominently to peripheral ruffles, compared to the wt Rac1. Bar, 1 μm.

However, the relevance of ubiquitinylation for Rac1 localization or signaling has not been previously investigated. Whereas constitutively active Rac1Q61L shows extensive poly-ubiquitinylation, an activated mutant in which lysine 147 is mutated (Rac1Q61LK147R) (Visvikis et al., 2008) is not ubiquitinated at all (Fig. 5A). Activated Rac1 and the K147 mutant bind equally well to the CRIB
domain of PAK, and localise readily to β-Pix-positive FAs and membrane ruffles (Fig. 5B,C). Interestingly, Rac1- induced recruitment of Cav1 to peripheral adhesions is not driven by (mono-)ubiquitylation of Rac1, as Rac1Q61LK147R still promotes Cav1 localisation at such adhesions (Fig. 5D).

These data therefore suggest that neither the targeting of Rac1, nor its interaction with the effector protein PAK requires its ubiquitylation. In line with this idea, expression of the Rac1Q61LK147R protein, like Rac1Q61L, stimulated peripheral membrane ruffling. However, live cell-analysis of these peripheral membranes showed that the Rac1Q61LK147R protein markedly accumulates at the periphery of the cell, as compared to the Rac1Q61L protein (Fig. 5E, Supplementary movies 1,2). Moreover, activated Rac1Q61L was seen in regions near and in ruffles, whereas the Rac1Q61LK147 protein localized to a more stable and narrow region near the peripheral membrane. Similarly, live-cell analysis of a mCherry-Rac1wt K147R mutant showed that this protein also localised more prominently at peripheral ruffles, compared to mCherry Rac1 wt (Fig. 5F). These data suggest that ubiquitylation of Rac1 neither impairs its targeting to the periphery of the cell, nor affects Rac1 binding to effector proteins, but is important for Rac1 dynamics in peripheral membrane ruffles.

**Discussion**

The cycling of Rho-like GTPases, regulated by GEFs and GAPs, is generally assumed to be the principal mechanism by which cells ensure proper activation and inactivation of these GTPases at the correct time and location (Bos et al., 2007; Rossman et al., 2005). However, there is increasing evidence that regulation of RhoGTPase signalling by ubiquitylation and degradation occurs as well. Whereas the initial studies in this field were mainly based on the use of the RhoGTPase-activating toxin CNF1, the identification of the RhoA ubiquitin-ligase Smurf1 has generated novel insights in the role of GTPase ubiquitylation in the regulation of cell polarity. Smurf1 is recruited to cellular protrusions by the a-typical protein kinase zeta, and locally regulates RhoA levels (Wang et al., 2003), cell protrusion as well as cell migration (Sahai et al., 2007). This supports the concept that localized
degradation may represent one of the mechanisms to regulate polarized GTPase activation (Kraynov et al., 2000) and ultimately directional cell migration.

In contrast to RhoA, cellular components regulating the degradation of Rac1 have not yet been identified. Previous studies, based on CNF1-induced activation and degradation of Rac1 have underscored the relevance of the hypervariable domain in its C-terminus for degradation (Lanning et al., 2004; Pop et al., 2004). In line with the high sequence diversity in this region between, otherwise very homologous, Rac isoforms it was shown that CNF1-induced degradation is isoform-specific and can in fact be transferred by exchanging the hypervariable domain of Rac1 (Pop et al., 2004). Yet, the site for ubiquitinylation in Rac1, Lys 147, does not reside in the C-terminus but is located more upstream in the protein (Visvikis et al., 2008). Thus, the C-terminal domain of Rac1 mediates a function and/or interaction that allows ubiquitinylation further upstream in the protein. Our current findings suggest that the interaction between Rac1 and Cav1, mediated by the Rac1 hypervariable C-terminal domain, is part of an adhesion-regulated mechanism that controls the Rac1 ubiquitinylation and degradation.

In polarized cells, most of the Cav1 localizes to the rear (Fig. 2 A) near areas of elevated contractility. This polarized distribution is in good agreement with its positive regulatory role in RhoA signalling (Grande-Garcia et al., 2007; Joshi et al., 2008). Moreover, we found that loss of Cav1 does not alter the protein levels of RhoA, the activity of which is critical for myosin-based contractility at the rear end of migrating cells. Given the role of Cav1 in Rac1 degradation, the concept of localized GTPase degradation in polarized cells that has been proposed for RhoA (Wang et al., 2003), may also apply to Rac1. Rac1 activity is high in the leading edge of polarized cells, away from the majority of Cav1 protein, but our data suggest that a portion of Cav1 is recruited to leading-edge FAs as a result of local activation of Rac1. The high levels of Cav1 at the rear end of polarized cells may serve to maintain Rac1 activity at a low level, locally preventing protrusive activity (Worthylake and Burridge, 2003).

The finding that reduction of Cav1 expression results in the accumulation of (activated) non-ubiquitinated Rac1 and of mono-ubiquitinated Rac1, but not of
poly-ubiquitinated Rac1, suggests which Cav1 plays a specific role in the regulation of poly-ubiquitinylation and subsequent degradation of Rac1. For some proteins, such as Ras and the EGF-receptor, mono-ubiquitinylation is a trigger for internalization and signal transduction, e.g. at endosomes (Haglund et al., 2003; Jura et al., 2006; Mukherjee et al., 2006). It is currently unclear at what subcellular location Rac1 ubiquitinylation and degradation occurs. Cav1 has been implicated in integrin-dependent internalization of Rac1-containing membrane domains which, is accompanied by a loss of Rac1-effector interactions (del Pozo et al., 2004; del Pozo et al., 2005). Since loss of Cav1 does not impair mono-ubiquitinylation of Rac1, this step may occur prior to Cav1-mediated internalization and subsequent poly-ubiquitinylation. Whether mono-ubiquitinylation of Rac1 mediates Rac1 internalization is currently subject of investigation.

The cycling of Rho-like GTPases, regulated by GEFs and GAPs, is generally assumed to control localized activation and inactivation of these GTPases (Bos et al., 2007). Our current findings indicate that in the absence of Cav1, the levels of activated, endogenous Rac1 remain elevated over prolonged periods of time (i.e. 48 hrs). In line with this observation, interfering with this pathway by reducing Cav1 results in the accumulation of activated Rac1 and induction of a Rac1 phenotype i.e. increased cell spreading, loss of polarity and consequently reduced directional migration. This suggests that endogenous Rac1-GAP activity is not sufficient to counteract this accumulation of GTP-loaded Rac1, indicating that the degradation of activated Rac1 represents an important complementary mechanism to control Rac1 signaling. Interestingly, a similar finding was recently reported for RhoA and its ubiquitinylation by a Cullin3/BACURD complex, as siRNA-mediated knockdown of Cul3 not only increased the levels of total RhoA, but also of its activated, GTP-bound form (Chen et al., 2009).
Figure 6. A model for the regulation of Rac1 activation and inactivation. Integrin-ligand interactions promote Rac1 activation (1), which, together with β-Pix, induce the accumulation of Rac1 at peripheral adhesions. Rac1 in activation is balanced by (2) GAP-stimulated GTP hydrolysis, as well as by a Rac1-ubiquitinylation pathway (3), which is regulated by Cav1 (4). Loss of Cav1 is associated with reduced poly-ubiquitinylation of Rac1, and with an accumulation of mono-ubiquitinated Rac1, as well as activated and total Rac1.

In conclusion, our previous and current data suggest the following model (Fig. 6). In polarized cells, integrin-mediated adhesion recruits the GEF β-PIX to leading edge FAs (ten Klooster et al., 2006), followed by PIX-mediated recruitment and local activation of Rac1 at FAs. Rac1 activity recruits Cav1 to FAs, and integrin activation promotes the Rac1-Cav1 association. Cav1 subsequently controls efficient termination of Rac1 signalling by regulating Rac1 poly-ubiquitinylation and degradation. Future experiments will be aimed at defining the subcellular localisation of the different steps in the Rac1 ubiquitinylation pathway as well as the identification of the relevant E3 ligase. The polarized distribution of Cav1 and its role in Rac1 ubiquitinylation provide further support for the concept of localized protein degradation in polarized cells.

Materials and Methods

Antibodies and reagents The following antibodies were used: anti-Caveolin-1 (610493), anti-Caveolin-2 (610684), anti-pY14-Caveolin-1 (610059), anti-Paxillin (610619) (all from BD bioscience) anti-Rac1 (clone 23A8) and anti-beta-pix (AB3829) (both from Millipore), anti-RhoA (sc-418) and JIP2A (Santa Cruz), anti-pY118Paxillin (44-722G) (Invitrogen), anti-GFP (JL-8; Clon tech), anti-c-myc (Zymed). F-actin was stained with rhodamine-labeled phalloidin (Invitrogen). The Rac1 inhibitors EHT1864 and NSC23766 were obtained from Sigma and Calbiochem, respectively. Confocal imaging of immunostained samples as well as live cell imaging was done using a ZEISS LSM510 Meta system in combination with ZEISS Zen software for analysis and processing. All data are representative for at 3 or
more experiments, unless otherwise indicated. Movies are representative for at least 3-10 cells, analysed individually.

**Cell culture** HeLa cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM; BioWhittaker) containing 10% heat inactivated FCS (Bodinco), 2mM L-glutamine and Penicillin/streptomycin (all purchased from PAA Cell Culture Company). Cells were passed by trypsinization. HUVEC were obtained from Lonza and cultured in EGM2 medium (Lonza) prepared according to the manufacturer’s instructions. Cav1 +/- knockout (ATCC-CRL-2752) and parental wt mouse embryonic fibroblasts (ATCC-CRL-2753) were obtained from ATCC and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% heat inactivated FCS (Bodinco), 2mM L-glutamine and Penicillin/streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

**Cell transfection and DNA constructs** HeLa cells were transiently transfected with FuGene (Roche) as described by the manufacturer. Briefly; 1 μg DNA was mixed with 6 μl of FuGene in 100μl Optimem (519850260) (Invitrogen) and incubated for 30 min at RT, after which the transfection mix was applied to 50% confluent cells. The following constructs were used: GFP-Cav1 (kind gift from M.A. Schwartz, University of Virginia, Charlottesville VA, USA). GFP-Cav1Y14F was constructed by site-directed mutagenesis (Stratagene). 6xHis-myc-tagged ubiquitin expression plasmid, originally from R.R. Kopito (Ward et al.,1995) was kindly provided by J. Bertoglio (Inserm U749, France). Myc-Cav1 was kindly provided by D. Anderson (Machleidt et al., 2000). pCMV myc-tagged Rac1Q61L was subcloned into pCruz HA-tagged expression vector (sc-5045), after which Rac1Q61L K147R was generated by site-directed mutagenesis (Stratagene). In parallel, Rac1Q61L and Rac1Q61L K147R were subsequently cloned in pE-YFP-(C1) and m-Cherry (C1)-tagged constructs. The same protocol has been used for wt Rac1. CNF1 was isolated as previously described (Schmidt et al., 1997). Cells were incubated with 500ng/ml purified GST-CNFI toxin, as indicated.

**Design and transfection of siRNA oligos** HeLa cells (4x10^5) were passed by trypsinisation in a 10cm dish, neutralised with complete IMDM, and cultured o/o in Opti-mem® 1 medium (Invitrogen). After 18 hrs, siRNA transfection mix containing siRNA primers (87μl of 20μM) and Oligofectamine (87μl) was prepared in Opti-mem® 1 medium according manufacturer’s protocol (Invitrogen) and incubated for 30’ at RT. During this incubation the Opti-mem® 1 medium on the cells was replaced for IMDM complete medium. Transfection medium was removed 24 hrs after transfection by replacing the culture medium with complete IMDM. The following chemically synthesized, double-stranded siRNAs, with 19-nt duplex RNA and 2nt 3’dTdT , purchased from Eurogentec in non-modified and desalted form, were used: Caveolin-1 siRNA primers AAUCUCAUCAGGAACUCGC (5’-3’) and GAGCUUCCUGAUUGAGAUU (5’-3’) (Beardsley et al., 2005). Rac1 siRNA primers GAGGAAGAGAAAAUGCCU (5’-3’) and CAGGCAUUUUCUCUUCCUC (5’-3’) (Noritake et al., 2004). As a nonspecific control in this study, we used siRNA against a luciferase reporter gene: CGUACGCGGAAUACUUCGA (5’-3’) and GCAUGGCGCUUAGAAGCGU (5’-3’).

**Lentiviral shRNAi silencing** Lentiviral shRNA constructs from the TRC/Sigma Mission library (Root et al., 2006) were obtained from Sigma-Aldrich (St. Louis, MI). The human CAV1-specific constructs used were: TRCN0000007999-8002 (called A6-A9) and TRCN0000011218 (called A5). The SHC002 scrambled shRNA construct (Sigma-Aldrich) was used as a negative control. All shRNA constructs were in the pLKO.1 vector backbone. shRNA-expressing lentiviral particles were prepared by transfection of HEK293T cells with pLKO.1 shRNA plasmid, together with the pMD2.G, pMDLg/RRE, and pRSV-Rev plasmids. The following cDNA constructs were used: Myc-Cav1 (kind gift from M.A. Schwartz, University of Virginia, Charlottesville VA, USA). GFP-Cav1Y14F was constructed by site-directed mutagenesis (Stratagene). 6xHis-myc-tagged ubiquitin expression plasmid, originally from R.R. Kopito (Ward et al.,1995) was kindly provided by J. Bertoglio (Inserm U749, France). Myc-Cav1 was kindly provided by D. Anderson (Machleidt et al., 2000). pCMV myc-tagged Rac1Q61L was subcloned into pCruz HA-tagged expression vector (sc-5045), after which Rac1Q61L K147R was generated by site-directed mutagenesis (Stratagene). In parallel, Rac1Q61L and Rac1Q61L K147R were subsequently cloned in pE-YFP-(C1) and m-Cherry (C1)-tagged constructs. The same protocol has been used for wt Rac1. CNF1 was isolated as previously described (Schmidt et al., 1997). Cells were incubated with 500ng/ml purified GST-CNFI toxin, as indicated.

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**RNA extraction, Reverse Transcription and RQ-PCR** Total cellular RNA from control and Cav1 knockdown cells was extracted with RNasebe (Bioconnect), according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA according to the European Against Cancer (EAC) guidelines (Gabert et al., 2003). Primers for Rac1 and RHOA were designed using Primer Express 1.5 (Applied Biosystems, Foster City, CA) and Oligo 6 (Molecular Biology Insights Inc, Cascade, CO) on the
basis of published gene sequences (http://www.ncbi.nlm.nih.gov/). Amplicons spanned an intron of at least 500 base pairs. Primers were synthesized by Eurogentec (Liege, Belgium). Primer combination for RAC1: forward primer 5'-CCTGATGCAGGCCATCAAG-3'; reverse primer 5'-AGTGGGATATATCCTCCAGAAAATG-3'. Primer combination for RHOA: forward primer 5'-GGACCTCGGATTGCTCGCT-3'; reverse primer 5'-CCATCACCACAAATCCACCTAGTT-3'. Primer combination for β-galactosidase (GUS): forward primer 5'-GAAAATATGTGGTTGGAGAGCTCATT-3'; reverse primer 5'-CCGGATGAGATCCCTTTTAT-3'. RQ-PCR was performed in an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster city, CA). Reactions were carried out in 25 μl containing 12.5 μl SYBR GREEN PCR Master Mix (Applied Biosystems), 300 nM forward and reverse primer, 5 μl cDNA (100ng RNA equivalents) and started with 10 minutes at 95°C followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. The specificity of all PCR products was determined by melt curve analysis. To correct for differences in the amount of total RNA input and for RT-efficiency, the quantity of the RAC1 and RHOA transcripts was normalized to the amount of GUS gene transcripts.

**Pull-down assays**

Peptide pull-down assays were performed as described previously (ten Klooster et al., 2006). In short, each assay was performed with 5 μg of indicated biotin-labelled peptide, 25 μl streptavidin-coated beads (Sigma-Aldrich) in NP-40 lysisbuffer (50mM Tris-HCl, 150mM NaCl, 10mM β-mercaptoethanol). Cells were subsequently allowed to migrate for 4 hrs towards IMDM with 10% FCS. Cells in the upper compartment were removed with a cotton swab and the migrated cells was determined by manual counting of fluorescent stained nuclei.

**Cell migration**

Endogenous, ubiquitinated Rac1 was detected by western-blotting for Rac1. Western-blotting was performed as described previously (Kanters et al., 2008) using antibodies to Rac1 (Abcam). Beads were washed 5 times with urea buffer and resuspended in SDS sample buffer. 25 μl of prewashed, blocked (1 hr RT with 200 μg/ml BSA) Talon beads (Clontech) at RT for 1 hr while rotating. Beads were washed 5 times with urea buffer and resuspended in SDS sample buffer. Endogenous, ubiquitinated Rac1 was detected by western-blotting for Rac1.

**Peptide synthesis**

Peptides were synthesized on a peptide synthesiser (Syro II) with Fmoc solid phase chemistry. Peptides encoded a biotinylated protein transduction domain (Biotin-YARAARQARAG) (Ho et al., 2001) followed by the 10 amino acids proceeding the CAAX domain for all used RhoGTPase peptides. The sequences of the Rac1 (P-A) and the Rac1 (RKR-AAA) mutants are respectively: CAAAVKKKRKK and CPPPVKKAAXAK. The biotinylated protein transduction domain for the Cavtratin peptide (Gratton et al., 2003) was followed by 20 amino acids corresponding to the scaffolding domain (amino acids 82-101) of Cav1: DGIWKAFTFTTFTKVYFYKR.

**Rac1 ubiquitylation assay**

To detect endogenous, ubiquitinated Rac1 in HeLa cells, cells were transfected with 6×His-myc-tagged Ubiquitin. Twenty-four hrs after transfection, cells were washed with PBS (containing Mg²⁺ and Ca²⁺) at RT and lysed for 5 min in Urea buffer (20mM Tris–HCl, pH 7.5, 200 mM NaCl, 10 mM Imidazol, 0.1% Triton X-100 in 8M urea). Cells were scraped, collected and incubated for 5 min at 37°C and centrifuged 5 min at 14000 x rpm, after which the supernatant was incubated with 25 μl of prewashed, blocked (1 hr RT with 200 μg/ml BSA) Talon beads (Clontech) at RT for 1 hr while rotating. Beads were washed 5 times with urea buffer and resuspended in SDS sample buffer. Endogenous, ubiquitinated Rac1 was detected by western-blotting for Rac1.

**Cell migration**

Serum-starved control and Cav1 siRNA transfected HeLa cells were seeded 48 hrs after transfection in fibronectin-coated 6.5 mm, 5-µm pore Transwell (Corning Cell) by measuring the impedance for up to 4 hrs.

**References**


