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SHORT REPORT

Rab5 is required in metastatic cancer cells for Caveolin-1-enhanced Rac1 activation, migration and invasion

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ABSTRACT

Rab5 is a small GTPase that regulates early endosome trafficking and other cellular processes, including cell adhesion and migration. Specifically, Rab5 promotes Rac1 activation and cancer cell migration, but little is known about the upstream regulators of Rab5. We have previously shown that the scaffolding protein Caveolin-1 (CAV1) promotes Rac1 activation and migration of cancer cells. Here, we hypothesized that CAV1 stimulates Rab5 activation, leading to increased Rac1 activity and cell migration. Expression of CAV1 in B16-F10 mouse melanoma and HT-29(US) human colon adenocarcinoma cells increased the GTP loading of Rab5, whereas shRNA-mediated targeting of endogenous CAV1 in MDA-MB-231 breast cancer cells decreased Rab5–GTP levels. Accordingly, shRNA-mediated downregulation of Rab5 decreased CAV1-mediated Rac1 activation, cell migration and invasion in B16-F10 and HT-29(US) cells. Expression of CAV1 was accompanied by increased recruitment of Tiam1, a Rac1 guanine nucleotide exchange factor (GEF), to Rab5-positive early endosomes. Using the inhibitor NSC23766, Tiam1 was shown to be required for Rac1 activation and cell migration induced by CAV1 and Rab5. Mechanistically, we provide evidence implicating p85α (also known as PIK3R1), a Rab5 GTPase-activating protein (GAP), in CAV1-dependent effects, by showing that CAV1 recruits p85α, precluding p85α-mediated Rab5 inactivation and increasing cell migration. In summary, these studies identify a novel CAV1–Rab5–Rac1 signaling axis, whereby CAV1 prevents Rab5 inactivation, leading to increased Rac1 activity and enhanced tumor cell migration and invasion.

KEY WORDS: Rab5, Small GTPases, Metastatic cell migration, Rac1, Caveolin-1, p85α

INTRODUCTION

Rab5 is a central regulator of early endosome dynamics that controls vesicle formation and fusion, homotypic fusion of early endosomes, early-to-late endosome maturation and motility along microtubules (Stenmark, 2009). As a small GTPase, Rab5 cycles between a GDP- (inactive) and GTP-bound (active) form. In addition to its canonical role in endocytosis, Rab5 has been implicated in other cellular processes, such as cell adhesion and migration (Torres and Stupack, 2011). Rab5 increases the rate of internalization and recycling of β1 integrins (Pellinen et al., 2006; Torres et al., 2010), stimulates focal adhesion disassembly (Mendoza et al., 2013; Palamidessi et al., 2013) and promotes the activation of Rac1, leading to cell migration (Palamidessi et al., 2008; Torres et al., 2010). Moreover, Rab5 is implicated in local actin rearrangement, by recruiting Tiam1, a Rac1 guanine nucleotide exchange factor (GEF), to early endosomes, leading to GTP loading of Rac1 and the formation of circular dorsal ruffles (Lanzetti et al., 2004; Palamidessi et al., 2008). Although the effectors and downstream signaling of Rab5 have been studied in detail, little is known about upstream regulators of this GTPase. In this respect, recent evidence suggests that there is a putative connection between Rab5-dependent trafficking at early endosomes and the scaffolding protein Caveolin-1 (CAV1) (Hagiwara et al., 2009), although the functional relevance of this relationship has not been addressed.

CAV1 is a 21-kDa protein that regulates numerous signaling cascades and that, depending on the cell model, acts as either a non-conventional tumor suppressor or a promoter of metastasis (Quest et al., 2008; Quest et al., 2013). CAV1 promotes the migration of both normal and tumor-derived cancer cells by mechanisms that appear to depend on the cellular context. In fibroblasts, CAV1-enhanced migration is associated with the accumulation of CAV1 at the rear of cells and the activation of RhoA (Grande-Garcia et al., 2007; Sun et al., 2007), whereas in metastatic cancer cells, migration is not dependent on either of these events (Urra et al., 2012). Rather, expression of CAV1 in MDA-MB-231 breast cancer and B16-F10 mouse melanoma cells is associated with increased turnover of focal adhesions and augmented Rac1–GTP levels by mechanisms that remain to be defined (Lobos-Gonzalez et al., 2013; Urra et al., 2012). Intriguingly, recent evidence has suggested that CAV1 interacts with Rab5 *in vitro* and that CAV1 promotes Rab5-dependent endocytosis (Hagiwara et al., 2009). Because both proteins are implicated in Rac1 activation and cell migration, we hypothesized that CAV1 promotes Rac1 GTP loading and migration of cancer cells *in vitro* by regulating Rab5. Expression of CAV1 in different metastatic cancer cells, including B16-F10 (murine melanoma), MDA-MB-231 (human breast adenocarcinoma) and HT-29(US) (human colon adenocarcinoma) led to increased Rab5 activation. Importantly, Rab5 was required for CAV1-driven cell migration and invasion, enhanced Tiam1 recruitment to early endosomes and Rac1 activation, as shown by the results of experiments involving

shRNA-targeting of Rab5. Particularly, CAV1-dependent activation of Rab5 was associated with sequestration of p85α (also known as PIK3R1), a Rab5 GTPase-activating protein (GAP), thereby precluding Rab5 inactivation. Thus, here, we identify a novel CAV1–Rab5–Rac1 signaling axis, which is required for CAV1-enhanced metastatic cancer cell migration.

RESULTS AND DISCUSSION

CAV1 promotes Rab5 activation in metastatic cancer cells

CAV1 was recently shown to promote the migration of MDA-MB-231 and B16-F10 cells *in vitro* (Urra et al., 2012). To extend these findings to additional models of metastatic cancer cells, we evaluated the effect of CAV1 on the migration of human colon adenocarcinoma HT-29(US) cells, a metastatic derivative of the commercially available HT29 (ATCC), which we previously characterized (Bender et al., 2000; Torres et al., 2007). As anticipated, expression of CAV1 stimulated the migration of HT-29(US) cells in wound healing and Boyden Chamber assays (Fig. 1A,B). Therefore, in subsequent experiments, we evaluated the effect of CAV1 on Rab5 activation in all three metastatic cancer cell lines [HT-29(US), B16-F10 and MDA-MB-231 cells], using the Rab5 binding domain (R5BD) pull-down assay described previously (Torres et al., 2008). Expression of CAV1 in both B16-F10 and HT-29(US) cells increased Rab5–GTP levels (Fig. 1C,D), whereas shRNA-mediated knockdown of endogenous CAV1 in MDA-MB-231 cells decreased Rab5–GTP levels (Fig. 1E). These data indicate that the presence of CAV1 promotes Rab5 activation in metastatic cancer cells.

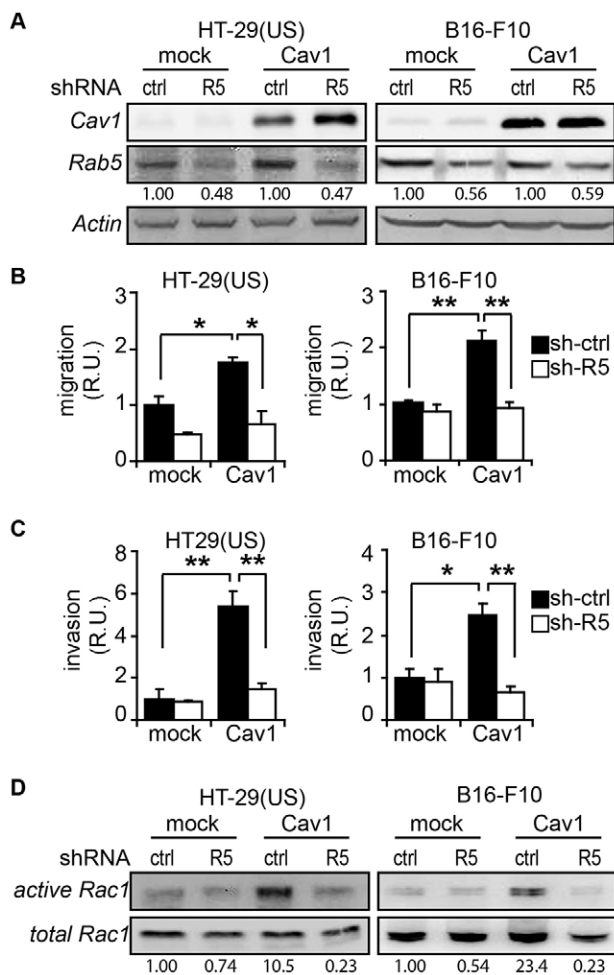### Rab5 is required for CAV1-dependent Rac1 activation, cell migration and invasion

Because Rab5 has been implicated in cell migration in a variety of normal and cancer cell lines (Mendoza et al., 2013; Pellinen et al., 2006; Spaargaren and Bos, 1999; Torres et al., 2010), we hypothesized that CAV1-dependent cell migration might require Rab5 expression and activity. To this end, endogenous Rab5 was knocked down by using shRNA in both mock- and CAV1-transfected HT-29(US) and B16-F10 cells (Fig. 2A). In HT-29(US) cells, Rab5 expression was reduced by 52% in mock and 53% in CAV1-expressing cells, as compared with cells treated with control shRNA. In B16-F10 cells, Rab5 levels were reduced by 44% in mock and 41% in CAV1 cells, as compared with cells treated with control shRNA (Fig. 2A). Moreover, shRNA-mediated loss of Rab5 was associated with a substantial loss of active Rab5–GTP (data not shown). As shown previously [Fig. 1A; (Urra et al., 2012)], CAV1 expression stimulated the migration of HT-29(US) and B16-F10 cells, and treatment with control shRNA did not affect CAV1-enhanced cell migration, as observed in both Boyden Chamber (Fig. 2B) and wound healing assays (data not shown). Intriguingly, CAV1-dependent cell migration was impaired in cells treated with shRNA against Rab5 (Fig. 2B).

Previous studies showed that CAV1-induced cell migration and Rac1 activation are accompanied by increased melanoma metastasis *in vivo* (Lobos-Gonzalez et al., 2013). Here, we evaluated the requirement for Rab5 in CAV1-dependent cell invasion in Matrigel assays. As expected, CAV1 promoted invasion of HT-29(US) and B16-F10 cells expressing control shRNA, whereas shRNA-mediated targeting of Rab5 abolished this effect (Fig. 2C). Because Rac1 is an important GTPase involved in cell migration and is known to be activated by CAV1
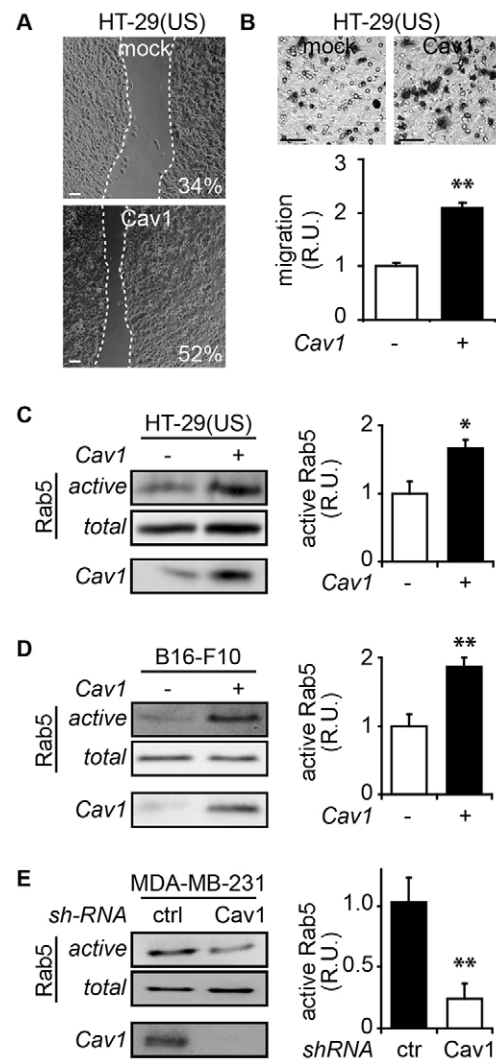
Fig. 1. CAV1 promotes Rab5 activation in metastatic cells. (A) HT-29(US) cells stably transfected with either pLacIOP (M1, mock) or pLacIOP-caveolin-1 (C14, Cav1) were described previously (Bender et al., 2000). Cells were grown to confluence, monolayers were wounded and cells were allowed to migrate for 24 h. Representative phase-contrast images are shown, and the numbers within panels indicate the mean percentage of wound closure from three independent experiments. (B) HT-29(US)(M1) and HT-29(US)(C14) cells were allowed to migrate for 2 h in Transwell chambers coated with 2 µg/ml fibronectin. Cells that migrated were visualized by staining with Crystal Violet. Upper panels, representative images; lower panel, quantification of cell migration, shown as the mean±s.e.m. (three independent experiments). R.U., relative units. Scale bars: 50 µm. (C–E) Rab5–GTP levels were measured by using the GST–R5BD pull-down assay. Representative western blot images are shown for HT-29(US)(M1) and HT-29(US)(C14) cells (C), B16-F10(mock) and B16-F10(Cav1) cells (D), and MDA-MB-231(shRNA-control) and MDA-MB-231(shRNA-Cav1) cells (E). Transfection of B16-F10 cells with CAV1 and shRNA-mediated downregulation of endogenous CAV1 in MDA-MB-231 cells were described previously (Urra et al., 2012). Graphs show densitometric quantification of each experiment as the mean±s.e.m. (three independent experiments); *$P<0.05$; **$P<0.01$.

(Lobos-Gonzalez et al., 2013; Urra et al., 2012) and Rab5 (Palamidessi et al., 2008; Torres et al., 2010), we sought to evaluate whether Rab5 is required for CAV1-induced activation of Rac1. As expected, the expression of CAV1 increased Rac1–GTP levels in HT-29(US) and B16-F10 cells treated with

Fig. 2. Rab5 is required for CAV1-driven Rac1 activation, cell migration and invasion. (A) HT-29(US) and B16-F10 cells stably transfected with pLacIOP (M1, mock) or pLacIOP-caveolin-1 (C14, Cav-1) were treated with either control (ctrl) or Rab5-specific (R5) shRNA constructs [shRNA-Rab5 sequence F10 for HT29(US) cells; shRNA-Rab5 sequence F8 for B16-F10 cells]. Whole-cell lysates were prepared and proteins were analyzed by western blotting for Rab5, CAV1 and actin. Representative images are shown for HT29(US) (left panels) and B16-F10 cells (right panels). Residual Rab5 levels were quantified by scanning densitometry and normalized to actin (numerical data are shown below panels). Mean residual Rab5 levels were calculated from three independent measurements and are summarized as follows (±s.e.m.): HT-29(US)(M1/shRNA-Rab5), 0.48±0.24; HT-29(US)(C14/shRNA-Rab5), 0.47±0.19; B16-F10(mock/shRNA-Rab5), 0.56±0.13 and B16-F10(Cav1/shRNA-Rab5), 0.59±0.10. (B) HT-29(US) and B16-F10 cells were allowed to migrate for 2 h in Transwell chambers coated with 2 μg/ml fibronectin. Migrated cells were visualized by staining with Crystal Violet. R.U., relative units. Data show the mean±s.e.m. (three independent experiments). (C) HT-29(US) and B16-F10 cells described in A were allowed to invade in Matrigel chambers for 24 h. Data represent the quantification of invasion (mean±s.e.m.; three independent experiments); *$P<0.05$; **$P<0.01$. (D) Rac1–GTP levels were measured with the GST–protein-binding-domain (PBD) pull-down assay. Measurements were performed in HT-29(US) (left panel) and B16-F10 cells (right panel), as described in A. Representative images are shown, and numbers below the panels indicate the relative levels of Rac1–GTP, obtained by scanning densitometry analysis and normalized to total Rac1. Data show the mean (three independent experiments) and are summarized as follows (±s.e.m.): HT-29(US)(M1/shRNA-ctrl), 1.00±0.6; HT-29(US)(M1/shRNA-Rab5), 0.74±0.6; HT-29(US)(C14/shRNA-ctrl), 10.5±1.4; HT-29(US)(C14/shRNA-Rab5), 0.23±0.1; B16-F10(mock/shRNA-ctrl), 1.00±0.5; B16-F10(mock/shRNA-Rab5), 0.54±0.3; B16-F10(Cav1/shRNA-ctrl), 23.4±10.3 and B16-F1(Cav-1/shRNA-Rab5), 0.23±0.1.

control shRNA, but not in cells treated with shRNA against Rab5 (Fig. 2D). Taken together, these observations demonstrate that Rab5 is required for CAV1-dependent Rac1 activation, cell migration and invasion.

Tiam1 is required downstream of CAV1 and Rab5 to activate Rac1 and cell migration

Based on these observations, we propose a signaling cascade whereby CAV1 activates Rab5 and then Rac1 to promote migration and invasion of cancer cells. Rac1 is known to be activated at early endosomes in a Rab5-dependent manner. There, Rab5 activation promotes the recruitment of the Rac1-GEF Tiam1, leading to increased Rac1 GTP loading and actin reorganization (Palamidessi et al., 2008). Hence, it was tempting to speculate that CAV1 increased the recruitment of Tiam1 to early endosomes by promoting Rab5 activation. To test this possibility, we first evaluated the requirement for Tiam1 in Rac1 activation by CAV1 using inhibitors. Treatment of HT-29(US) cells with the Tiam1 inhibitor NSC23766 (Gao et al., 2004) prevented the activation of Rac1 by CAV1 and prevented cell migration, indicating that Tiam1 is the GEF involved in the activation of Rac1 by CAV1 in these cell lines (Fig. 3A). Thereafter, we evaluated the recruitment of Tiam1 to Rab5-positive early endosomes by using confocal microscopy. As shown in Fig. 3B, the expression of CAV1 was associated with a moderate, but significant increase in the colocalization of Tiam1 with GFP–Rab5-positive early endosomes. To further investigate the sequence of events implicated in CAV1-driven cell migration, constitutively active Rab5 (GFP–Rab5/Q79L) was expressed in CAV1-deficient cells. As expected, expression of active Rab5 was sufficient to recapitulate the CAV1-driven effects on cell migration. Alternatively, expression of inactive Rab5 (GFP–Rab5/S34N) abolished CAV1-driven cell migration (Fig. 3C). Likewise, as shown for CAV1-dependent cell migration (Fig. 3A), Rab5/Q79L-driven cell migration required functional Tiam1, because treatment with the inhibitor NSC23766 prevented cell migration and Rac1 activation (Fig. 3C; data not shown). Therefore, we conclude that Rab5 activity is essential for CAV1-driven cell migration, and that Tiam1 is required downstream of CAV1 and Rab5 to activate Rac1.

Sequestration of p85α by CAV1 promotes Rab5 activation and cell migration

Finally, we investigated the molecular mechanisms implicated in Rab5 activation by CAV1. Rab5 GTP loading depends on targeting to early endosome membranes. Hence, Rab5 activation by CAV1 could be explained by increased stabilization of the protein on early endosomes. However, we did not detect any differences in Rab5 recruitment to early endosomes of B16-F10 cells lacking or expressing CAV1, as judged by analysis of the colocalization of Rab5 with the early endosome marker EEA1 (data not shown). Therefore, Rab5 activation by CAV1 cannot be explained by physical relocalization, suggesting that additional molecular players are involved in the CAV1-dependent activation of Rab5. Among currently known regulators of Rab5 activity, p85α, the regulatory subunit of PI3K, is particularly interesting because it is a Rab5-GAP proposed not only to inactivate Rab5 (Chamberlain et al., 2004), but also to prevent Rab5-dependent cell migration (Torres et al., 2008). Most importantly, p85α was recently shown to interact in a complex with CAV1 (Faulstich et al., 2013). Thus, we
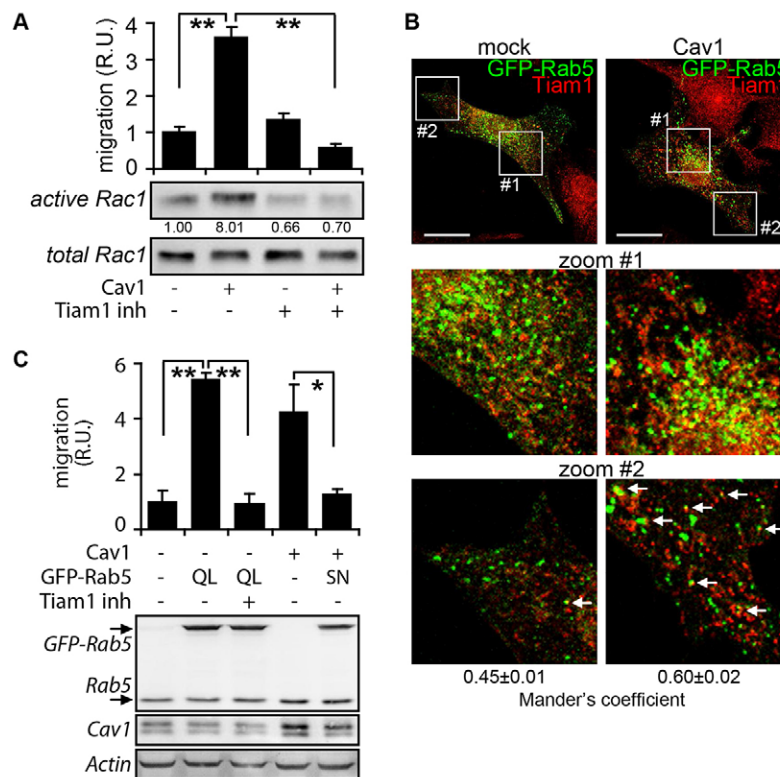
Fig. 3. CAV1 promotes the recruitment of Tiam1 to Rab5-positive early endosomes, and Tiam1 is required for CAV1- and Rab5-driven activation of Rac1 and cell migration. (A) Lower panel, HT-29(US) cells stably transfected with pLacIOP (M1, −) or pLacIOP-caveolin-1 (C14, +) were treated with either vehicle (DMSO, −) or 10 μM NSC23766 [Tiam1 inhibitor (inh), +] for 30 min, and Rac1–GTP levels were measured by using the GST–protein-binding-domain (PBD) pull-down assay. Representative images from three independent experiments are shown. Rac1–GTP levels were quantified by scanning densitometry and normalized to total Rac1 (numerical data are shown), and are summarized as follows: HT-29(US)(M1)/DMSO, 1.00±0.1; HT-29(US)(C14)/DMSO, 8.01±5.2; HT-29(US)(M1)/NSC23766, 0.66±0.4 and HT-29(US)(C14)/NSC23766, 0.70±0.5 (mean±s.e.m.; three independent measurements). Upper panel, cells were allowed to migrate for 4 h in Transwell chambers coated with 2 μg/ml fibronectin, in the presence of either vehicle (−) or 10 μM NSC23766 (+). Migrated cells were visualized by staining with Crystal Violet. R.U., relative units. Data represent the mean±s.e.m. (three independent experiments). (B) B16-F10 cells stably transfected with pLacIOP (mock) or pLacIOP-caveolin-1 (Cav1) were transfected with pEGFP-Rab5-wild-type and grown on glass coverslips for 24 h. Samples were fixed and stained with anti-Tiam1 antibody (red). Samples were analyzed by confocal microscopy, and representative images are shown. Arrows, colocalization of Rab5 and Tiam1 (Mander's coefficient is shown below each panel; mean±s.e.m.; n=3, P<0.01). Lower panels, magnifications of boxed areas. Scale bars: 10 μm. (C) HT-29(US) cells stably transfected with pLacIOP (−) or pLacIOP-caveolin-1 (+) were transfected with either pEGFP-C1, pEGFP-Rab5/Q79L or pEGFP-Rab/S34N for 24 h. Cells were further treated with either vehicle (−) or 10 μM NSC23766 (+) for 30 min and used for migration assays (upper panel) or western blotting (lower panel). Upper panel, cell migration was measured as in A; data show the mean±s.e.m. (three independent experiments); *P<0.05; **P<0.01. Lower panel, samples were analyzed by western blotting and representative images from three independent experiments are shown.

evaluated the participation of p85α as a Rab5–GAP in CAV1-dependent Rab5 activation and cell migration. Accordingly, p85α was present in a complex with CAV1 in both HT-29(US) and B16-F10 cells, as shown by co-immunoprecipitation assays (Fig. 4A,B). Likewise, CAV1 co-immunoprecipitated with ectopically expressed mCherry–p85α in B16-F10 cells (Fig. 4B). Next, we tested whether the expression of p85α interfered with the activation of Rab5 by CAV1. As expected (Fig. 1), CAV1 induced a 2.2-fold increase in Rab5–GTP levels, and ectopically expressed p85α substantially reduced the ability of CAV1 to promote Rab5 GTP loading (Fig. 4C). Accordingly, expression of p85α prevented CAV1-dependent cell migration in both B16-F10 and MDA-MB-231 cells (Fig. 4D,E). Therefore, we conclude that sequestration of p85α in a complex with CAV1 stimulates the activation of Rab5 and enhances cell migration.

Taken together, our data point towards the existence of a novel CAV1–Rab5–Rac1 axis implicated in the regulation of cancer cell migration and invasion. Interestingly, expression of all

three proteins is known to be deregulated in cancer, and their overexpression is associated with poor patient prognosis (Engers et al., 2007; Liu et al., 2013; Meije et al., 2002; Yang et al., 1999; Yang et al., 2011). Moreover, this novel mechanism is dependent on the Rac1-GEF Tiam1, which is also known to be overexpressed in cancer (Chen et al., 2012; Zhao et al., 2011). Tiam1 was previously shown to be recruited to Rab5-positive early endosomes (Palamidessi et al., 2008), and our data show that basal colocalization of GFP–Rab5 and endogenous Tiam1 was further increased by the presence of CAV1. Mechanistically, CAV1 recruits the Rab5-GAP p85α to a complex that precludes Rab5 inactivation by p85α. In summary, the data provided here uncover an unanticipated connection linking the CAV1–Rab5–Rac1 axis, which is regulated by Tiam1 and p85α, to augmented cancer cell migration, invasion and metastasis.

Previous work suggested that Src-dependent phosphorylation of CAV1 on tyrosine 14 is relevant to CAV1-dependent cell migration (Urra et al., 2012). Accordingly, we observed that
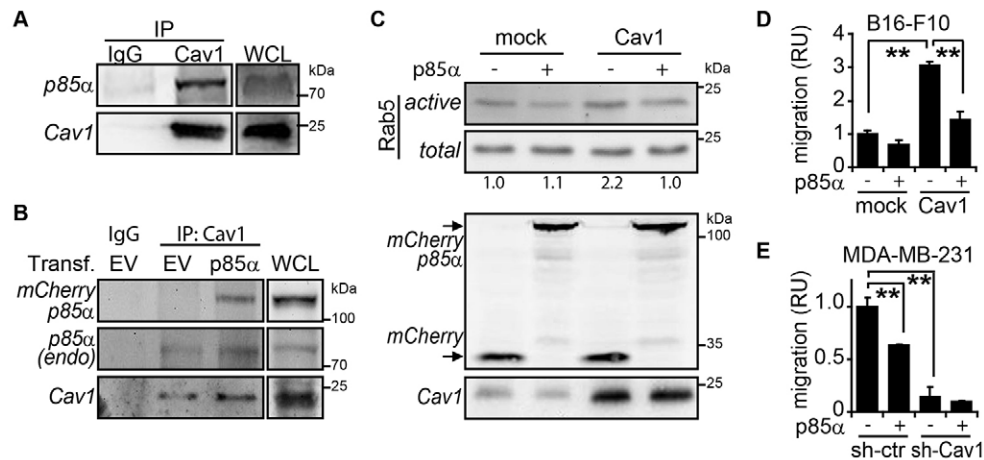
Fig. 4. CAV1 promotes Rab5 activation by recruitment of p85α. (A) Whole-cell lysates (WCL) were prepared from subconfluent cultures of HT-29(US) cells, and CAV1 was immunoprecipitated (IP) with a polyclonal antibody. Samples were analyzed by western blotting with the monoclonal antibodies anti-p85α and anti-caveolin-1. For comparison, 50 µg of whole-cell lysate was analyzed, and immunoprecipitation with an irrelevant IgG was included as a control. A representative image from two independent experiments is shown. (B) B16-F10 cells were transfected with either mCherry (empty vector, EV) or mCherry-p85α (p85α) for 24 h. Subsequently, whole-cell lysates were prepared and CAV1 was immunoprecipitated with a polyclonal antibody. Samples were analyzed by western blotting with the monoclonal antibodies anti-mCherry (for detection of mCherry-p85α), anti-p85α (for detection of endogenous p85α) and anti-caveolin-1 (for detection of CAV1). For comparison, 50 µg of whole-cell lysate was analyzed, and control immunoprecipitation experiments with an irrelevant IgG were included. Representative images from two independent experiments are shown. (C,D) B16-F10 cells stably transfected with pLacIOP (mock) or pLacIOP-caveolin-1 (Cav1) were transfected with either mCherry (−) or mCherry–p85α (+) for 24 h and used for subsequent analysis of Rab5–GTP levels (C) and cell migration (D). (C) Rab5–GTP levels were measured by using the GST–R5BD pull-down assay. Representative western blot images are shown. Rab5–GTP levels were quantified by scanning densitometry and normalized to total Rab5 (numerical data is shown). Rab5–GTP levels are shown as the mean of four independent experiments, and are summarized as follows (±s.e.m.): B16-F10 (mock/mCherry-p85α), 1.1±0.8; B16-F10(Cav1/mCherry), 2.2±0.7 and B16-F10(Cav1/mCherry-p85α), 1.0±0.3. As a control, 50 µg of whole-cell lysates were analyzed by western blotting with the monoclonal antibodies anti-mCherry and anti-caveolin-1. (D) Cell migration was measured in Transwell chambers coated with 2 µg/ml fibronectin. Migrated cells were visualized by staining with Crystal Violet. R.U., relative units. Data show the mean±s.e.m. (three independent experiments). (E) MDA-MB-231 cells treated with shRNA-control (sh-ctr) or shRNA-caveolin-1 (sh-Cav1) were transfected with either mCherry (−) or mCherry–p85α (+) for 24 h, and cell migration was measured in Transwell chambers, as described in D. Data show the mean±s.e.m. (three independent experiments); **$P<0.01$.

treatment of B16-F10 cells with the Src family kinase inhibitor PP2 reduced CAV1 phosphorylation and cell migration. Consistent with those findings, activation of Rab5 by CAV1 was also reduced by PP2, although inhibition was only partial (data not shown). These data suggest that mechanisms in addition to tyrosine phosphorylation of CAV1 are required for the CAV1-dependent activation of Rab5. Future studies are required to unravel the nature of these intriguing additional possibilities.

MATERIALS AND METHODS
Materials
Mouse monoclonal anti-caveolin-1 and mouse monoclonal anti-Rac1 antibodies were from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-actin antibody was from R&D Systems (Minneapolis, MN). Mouse monoclonal anti-Rab5 and rabbit polyclonal anti-Tiam1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit-IgG and goat anti-mouse-IgG antibodies coupled to horseradish peroxidase were from Bio-Rad Laboratories (Hercules, CA). Alexa-Fluor-568-conjugated secondary antibody and Geneticin (G418 sulfate) were from Invitrogen (Carlsbad, CA). Cell medium and antibiotics were from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT). Glutathione–Sepharose-4B was from GE Healthcare (Piscataway, NJ). The chemiluminescent substrate (EZ-ECL) and Protein-A/G beads were from Pierce Chemical (Rockford, IL). The Rab5 lentiviral short hairpin RNAs (shRNA) were from Open Biosystems (Huntsville, AL). The Rac1 inhibitor NSC23766 was from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids
The plasmids pLacIOP and pLacIOP-caveolin-1 (Bender et al., 2000) and the pEGFP-C1 plasmids encoding wild-type Rab5, Rab5/S34N (with

a high affinity for GDP) and Rab5/Q79L (GTPase-deficient) were described previously (Mendoza et al., 2013). The pCDH-mCherry and pCDH-mCherry-p85α constructs were generated as follows. mCherry and full-length bovine p85α (Torres et al., 2008) were shuttled between XbaI/NheI and NheI/EcoRI of the pCDH lentiviral expression vector (System Biosciences), respectively.

Cell culture
HT-29(US), MDA-MB-231 and B16-F10 cells were cultured in high-glucose DMEM, DMEM-F12 and RPMI respectively, supplemented with 10% FBS and antibiotics. Stable transfection of caveolin-1 in HT-29(US) and B16-F10 cells, and stable knockdown of endogenous caveolin-1 in MDA-MB-231 cells were described previously (Bender et al., 2000; Torres et al., 2007; Urra et al., 2012). Rab5 was downregulated as described previously (Torres et al., 2010). Control cells were infected with a lentivirus encoding a nonspecific shRNA sequence (plasmid 1864; Addgene, Cambridge, MA). Rab5 downregulation in HT-29(US) and B16-F10 cells was performed by using the shRNA sequence #F10 (Open Biosystems) for HT-29(US) cells, and sequence #F8 (Open Biosystems) for B16-F10. Stable cell lines were selected and maintained in culture medium containing 2 µg/ml puromycin.

Immunofluorescence
Immunofluorescence was performed as described previously (Mendoza et al., 2013). Tiam1 was detected with a polyclonal antibody (Santa Cruz Biotechnology) followed by an Alexa-Fluor-568-conjugated secondary antibody. Samples were analyzed with a confocal microscope (Carl Zeiss LSM-Pascal 5).

Rab5–GTP and Rac1–GTP pull-down assay
Rab5–GTP and Rac1–GTP pull-down assays were performed as described previously (Torres et al., 2008; Urra et al., 2012).

Migration and invasion assays

Cell migration was evaluated in wound healing and Boyden Chamber assays (Transwell Costar, 6.5-mm diameter, 8-µm pore size), whereas invasion was evaluated in Matrigel (BD Biosciences, 354480), as reported previously (Mendoza et al., 2013; Urra et al., 2012).

Image analysis

Confocal images were analyzed for colocalization using the ImageJ software. To this end, the threshold was adjusted in both channels, green for GFP–Rab5 and red for Tiam1, and colocalization was measured with the JACoP plugin. Mander's coefficients were calculated with respect to GFP–Rab5. At least ten images per experiment were measured.

Immunoprecipitation

Immunoprecipitation was performed as described previously (Torres et al., 2008). Cell extracts were prepared in a buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40 and protease inhibitors. Supernatants obtained after centrifugation (at 13,000 g for 2 min at 4℃) were used for immunoprecipitation assays (500 µg of total protein per assay) with antibody-coated Protein-A–Sepharose beads. CAV1 was immunoprecipitated for 2 h with mouse monoclonal anti-CAV1.

Statistical analysis

Where pertinent, results were compared using unpaired t-tests with the GraphPad Prism 5 software (San Diego, CA). Values averaged from at least three independent experiments were compared. A P-value of <0.05 was considered significant.

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