

ORIGINAL ARTICLE

The interaction between caveolin-1 and Rho-GTPases promotes metastasis by controlling the expression of alpha5-integrin and the activation of Src, Ras and Erk

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Proteins containing a caveolin-binding domain (CBD), such as the Rho-GTPases, can interact with caveolin-1 (Cav1) through its caveolin scaffold domain. Rho-GTPases are important regulators of p130^{Cas}, which is crucial for both normal cell migration and Src kinase-mediated metastasis of cancer cells. However, although Rho-GTPases (particularly RhoC) and Cav1 have been linked to cancer progression and metastasis, the underlying molecular mechanisms are largely unknown. To investigate the function of Cav1–Rho-GTPase interaction in metastasis, we disrupted Cav1–Rho-GTPase binding in melanoma and mammary epithelial tumor cells by overexpressing CBD, and examined the loss-of-function of RhoC in metastatic cancer cells. Cancer cells overexpressing CBD or lacking RhoC had reduced p130^{Cas} phosphorylation and Rac1 activation, resulting in an inhibition of migration and invasion *in vitro*. The activity of Src and the activation of its downstream targets FAK, Pyk2, Ras and extracellular signal-regulated kinase (Erk)1/2 were also impaired. A reduction in α 5-integrin expression, which is required for binding to fibronectin and thus cell migration and survival, was observed in CBD-expressing cells and cells lacking RhoC. As a result of these defects, CBD-expressing melanoma cells had a reduced ability to metastasize in recipient mice, and impaired extravasation and survival in secondary sites in chicken embryos. Our data indicate that interaction between Cav1 and Rho-GTPases (most likely RhoC but not RhoA) promotes metastasis by stimulating α 5-integrin expression and regulating the Src-dependent activation of p130^{Cas}/Rac1, FAK/Pyk2 and Ras/Erk1/2 signaling cascades.

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Introduction

The scaffold protein p130^{Cas}, Rho-GTPases and caveolin-1 (Cav1) are among the key signaling molecules mediating actin cytoskeleton remodeling (Hall 1998; Meenderink *et al.*, 2010; Fecchi *et al.*, 2011). This remodeling depends on Rac1 activation, which is controlled by phosphorylated p130^{Cas} (Marti *et al.*, 1997; Ohta *et al.*, 1999; Brabek *et al.*, 2005). Phosphorylation of p130^{Cas} is thus a pivotal step in cell movement and metastasis. In normal and cancer cells, p130^{Cas} is phosphorylated by Src kinase, thus Src also regulates the cytoskeleton (Sakai *et al.*, 1994). Src-mediated p130^{Cas} phosphorylation drives Rac1 activation at the cell's leading edge (Timpson *et al.*, 2001; Sharma and Mayer 2008), thereby promoting cell motility.

Rho-GTPases have been linked to cancer cell migration/invasion, and elevated levels of RhoC have been frequently associated with metastasis (Sahai and Marshall 2002; Narumiya *et al.*, 2009). Our previous work showed that RhoC^{+/+}-PyMT and RhoC^{-/-}-PyMT mice develop primary tumors at the same frequency but RhoC^{-/-}-PyMT mice exhibit significantly fewer metastatic lesions in the lungs (Hakem *et al.*, 2005). *In vitro*, mammary tumor cells from RhoC^{-/-}-PyMT animals show greatly impaired migration/invasion abilities. However, the underlying mechanism by which RhoC functions in metastasis is largely unknown. At the molecular level, small Rho-GTPases are required not only for cytoskeletal remodeling but also for Ras-induced transformation (Yamamoto *et al.*, 1999; Jaffe and Hall 2002). Ras activates various downstream mediators important for cell migration, proliferation and survival (Bonni *et al.*, 1999; Yip *et al.*, 2007). Rho-GTPases are also linked to integrins, which are critical for cell adhesion, migration and survival.

Inhibition of integrin signaling in melanoma cells suppresses the Rho/ROCK pathway, thus blocking cell invasion and metastasis (Kidera *et al.*, 2010).

The scaffold protein Cav1 appears to act either as a tumor suppressor or as an oncogene but its precise role in metastasis remains unclear (Goetz *et al.*, 2008; Quest *et al.*, 2008). Although Cav1^{-/-} mice do not develop spontaneous tumors, these animals did develop malignancies when either treated with a carcinogen (Capozza *et al.*, 2003) or crossed with a tumor-prone strain such as the MMTV–PyMT (mouse mammary tumor virus–polyoma middle-T antigen) mouse (Williams *et al.*, 2003, 2004). On the other hand, the TRAMP mouse model (transgenic adenocarcinoma of mouse prostate), which develops spontaneous prostate carcinomas, exhibited greatly reduced metastatic spread when Cav1 was genetically deleted (Williams *et al.*, 2005). In tumor-prone mice, loss of Cav1 gene expression dramatically accelerated the development of multifocal dysplastic mammary lesions (Williams *et al.*, 2003). However, increased Cav1 expression correlated with metastasis in later neoplastic stages during hepatocellular carcinogenesis (Cokakli *et al.*, 2009). Also, increased Cav1 expression have been observed in melanoma, lung adenocarcinoma, prostate cancer and renal cell carcinoma in humans (Thompson *et al.*, 1999; Li *et al.*, 2001; Ho *et al.*, 2002; Campbell *et al.*, 2008; Felicetti *et al.*, 2009). These conflicting observations highlight the lack of understanding of the diverse biological role of Cav1 in cancer.

At the biochemical level, Cav1 co-fractionates with many signaling molecules, including Rho-GTPases (Smart *et al.*, 1999; Couet *et al.*, 2001). Rho-GTPases bind to Cav1 through their caveolin-binding domains (CBDs) (Couet *et al.*, 1997). *In vitro*, an isolated CBD peptide derived from a G-protein was shown to interact directly with the caveolin scaffold domain of Cav1 and competitively inhibits the binding of the G-protein to Cav1 (Couet *et al.*, 1997). This makes the CBD a useful tool for examining the effects of disrupting the Cav1–Rho-GTPase interaction on cell migration, invasion and metastasis.

In this study, we show that the disruption of Cav1–Rho-GTPase interaction prevents the activation of Rac1 mediated by Src-dependent p130^{Cas} phosphorylation, as well as Src-dependent activation of Ras and extracellular signal-regulated kinase (Erk)1/2. Importantly, we demonstrate that Cav1–Rho-GTPase interaction is crucial for α 5-integrin expression. *In vivo*, reducing the interaction of Cav1 with Rho-GTPases results in decreased extravasation and reduced survival of metastatic cells. Our data implicate the Cav1-mediated control of Rho-GTPase signaling as an important mechanism promoting metastasis.

Results

Cav1–Rho-GTPase interaction regulates p130^{Cas} phosphorylation

Various clones of the B16 melanoma cell line exhibit diverse metastatic potential and express different levels of RhoC. The B16 F10 clone is the most invasive and

shows higher levels of RhoC and phosphorylated p130^{Cas} (ph-p130^{Cas}) than the weakly invasive F0 clone (Clark *et al.*, 2000). We assessed the levels of Cav1 expression in F0, F1 and F10 B16 clones, and evaluated the Src-dependent phosphorylation of p130^{Cas} at Y410 and Y165. Both Cav1 protein and ph-p130^{Cas} levels were increased in F1 and F10 cells compared with F0 cells (Figure 1a). To determine whether Cav1 was required for p130^{Cas} phosphorylation, we silenced Cav1 expression in F10 cells and found that Cav1 indeed regulates p130^{Cas} phosphorylation (Figure 1b). To define RhoC's role in p130^{Cas} phosphorylation, we generated stable F10 clones expressing small interfering RNA (siRNA) or short hairpin RNA against RhoC (siRhoC, shRhoC). These cells also exhibited lower levels of ph-p130^{Cas} but Cav1 expression was not altered (Figure 1c). To determine if this effect held true in other tumor cells, we established a cancer cell-line (designated as PyMT) from explanted MMTV–PyMT mammary epithelial tumor cells. RhoC knockdown in PyMT cells also decreased ph-p130^{Cas} without affecting Cav1 (Figure 1c). Notably, the knockdown of RhoA did not affect ph-p130^{Cas} levels (Supplementary Figure S1a). These data show that both Cav1 and RhoC regulate ph-p130^{Cas} in cancer cells, and that RhoC's function in this context cannot be compensated for by other Rho-GTPases.

To explore whether Cav1–Rho-GTPase interaction was important for p130^{Cas} phosphorylation, we exploited the fact that the presence of the isolated CBD (Supplementary Figures S1b and c) can inhibit Cav1–Rho-GTPase interaction (Couet *et al.*, 1997). We generated F10 cells stably overexpressing the wild-type CBD (CBD^{wt}) or a non-functional mutant form of CBD (CBD^{mut}) (Supplementary Figure S1c). We then assessed the ability of CBD^{wt} to inhibit Cav1–Rho-GTPase interaction by pulling down GTPases with GST–Rhotekin–RBD, followed by immunoblotting for Cav1. In CBD^{wt}-expressing cells, we found that considerably less Cav1 was bound to active Rho-GTPases in comparison with CBD^{mut}-expressing cells. This indicated that the CBD^{wt} indeed disrupts the Cav1–Rho-GTPase interaction (Figure 1d). To evaluate the functional consequences of CBD^{wt} expression, we expressed either CBD^{wt} or CBD^{mut} in-frame with green fluorescent protein (GFP) (Supplementary Figure S1c) in F10 cells. Cells expressing CBD^{wt}–GFP showed reduced ph-p130^{Cas} similar to cells expressing CBD^{wt} alone, whereas CBD^{mut}–GFP expression had no effect on ph-p130^{Cas} levels (Figure 1e, Supplementary Figure S1d). Thus, the inhibition of Cav1–Rho-GTPase interaction by CBD^{wt} had the same effect on ph-p130^{Cas} as the lack of Cav1 or RhoC expression. These results indicate that the interaction of Cav1 with Rho-GTPases is critical for normal p130^{Cas} phosphorylation. As the lack of Cav1 or RhoC alone almost completely abolished p130^{Cas} phosphorylation, we consider RhoC as the main GTPase that binds to Cav1 and elicits p130^{Cas} signaling in metastatic tumor cells.

Cav1–Rho-GTPase interaction is required for cellular migration/invasion

Activation of Rac1 is important for both migration and cytoskeletal remodeling in many different cell types and

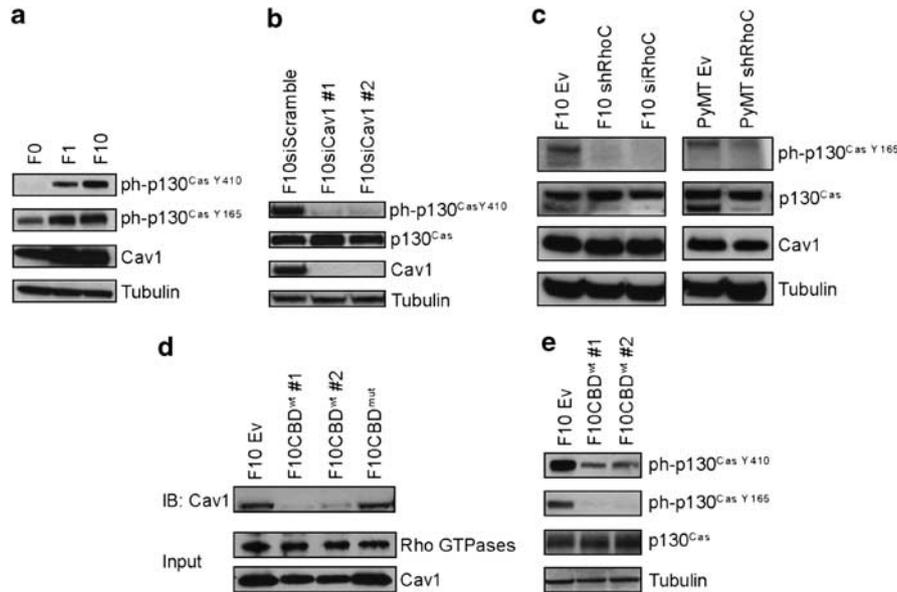


Figure 1 RhoC and Cav1 regulate p130^{Cas} phosphorylation. (a) Correlation between Cav1 and ph-p130^{Cas}. The indicated clones of B16 melanoma cells were evaluated by immunoblotting (IB) for levels of Cav1 and p130^{Cas} phosphorylation at Y410 or Y165. (b) Transient silencing (two independent clones #1, #2) of Cav1 expression reduces p130^{Cas} phosphorylation. (c) RhoC controls ph-p130^{Cas} levels. F10 cells stably expressing siRhoC or shRhoC were analyzed by IB to detect ph-p130^{Cas} and Cav1. The ph-p130^{Cas} levels are also reduced in PyMT cells expressing shRhoC. (d) CBD^{wt} inhibits the binding of Rho-GTPases to Cav1. Active Rho-GTPases were pulled-down with agarose beads GST-Rhotekin-RBD-conjugated (RBD: Rho Binding Domain) and Cav1 was detected by IB. (e) CBD^{wt} expression reduces ph-p130^{Cas}. The levels of p130^{Cas} and p130^{Cas} phosphorylated at Y410 or Y165 were assessed by IB in F10Ev (control F10 cells expressing empty vector) and two independent clones of F10 cells overexpressing CBD^{wt} (F10CBD^{wt} #1, #2).

depends on ph-p130^{Cas} (Klemke *et al.*, 1998; Heasman and Ridley 2008). We therefore assessed the effects of CBD^{wt} expression or RhoC depletion on Rac1 activation, actin cytoskeleton organization and migration/invasion *in vitro*. We assayed the levels of active (GTP-bound) Rac1 in F10 cells expressing the control empty vector (Ev), CBD^{wt}, CBD^{mut} or siRhoC, we found that active Rac1 was dramatically reduced in F10CBD^{wt} and F10siRhoC cells compared with F10CBD^{mut} and F10Ev cells (Figure 2a). Consistent with the loss of Rac1 activation, rhodamine-phalloidin staining revealed that the actin cytoskeleton of F10CBD^{wt} cells was clearly altered compared with F10Ev cells (Figure 2b). The CBD^{wt}-expressing cells displayed reduced F-actin stress fibers but abundant fragmented F-actin (puncta) characteristic of defects in actin polymerization or stability. This finding suggested that, in the absence of Cav1–Rho-GTPase interaction, tumor cells might not be able to sufficiently remodel their cytoskeletons to permit migration/invasion. Indeed, F10CBD^{wt} and F10siRhoC cells both exhibited severely impaired migration as well as invasion (Figures 2c and d and Supplementary Figure S2). These data show that cells in which Cav1–Rho-GTPase interaction is disrupted exhibit reduced Rac1 activation that is associated with defective cytoskeletal remodeling and reduced migration/invasion capacity.

Cav1–Rho-GTPase interaction and RhoC are important for Src, Ras and Erk activation

Elevated Src expression is usually associated with increased metastatic capability (Wheeler *et al.*, 2009).

As our earlier results showed that Cav1 controls p130^{Cas} phosphorylation (Figure 1b), and Src kinase is known to phosphorylate p130^{Cas} (Sakai *et al.*, 1994), we evaluated the impact of loss of Cav1 expression on Src activation. F10shCav1 cells showed a significant impairment of SrcY416 phosphorylation compared with F10Ev cells (Figure 3a). As RhoC depletion had a strong effect on ph-p130^{Cas} levels (Figure 1c), we examined Src activation in F10siRhoC and PyMTshRhoC cells and found that RhoC expression was indeed crucial for Src activation in both cell lines (Figure 3b). These data are consistent with previous reports showing that various Rho-GTPases are involved in recruiting Src to the plasma membrane, and that this event may be important for Src activation (Fincham *et al.*, 1996; Timpson *et al.*, 2001; Yamana *et al.*, 2006). Importantly, depletion of RhoC alone was sufficient to almost completely abolish Src activation in metastatic cancer cells.

We next assessed whether CBD^{wt}-mediated disruption of Cav1–Rho-GTPase interaction could prevent Src activation. F10CBD^{wt} cells did show a decrease in Src activation, although to a lesser extent than in cells lacking Cav1 or RhoC (Figure 3c). Furthermore, consistent with the decreased Src activation in F10CBD^{wt} cells, the levels of activated FAK and Pyk2, two direct downstream targets of Src, were reduced upon RhoC knock-down or CBD^{wt} expression (Figure 3c). Importantly, the expression in F10 cells of a dominant-negative form of Src (Src-DNRF) showed the same suppressive effect on FAK and Pyk2 phosphorylation. As FAK and Pyk2 are involved in cell migration/invasion and adhesion (Ilic *et al.*, 1995; Sieg *et al.*, 1998; Schaller 2010), the altered

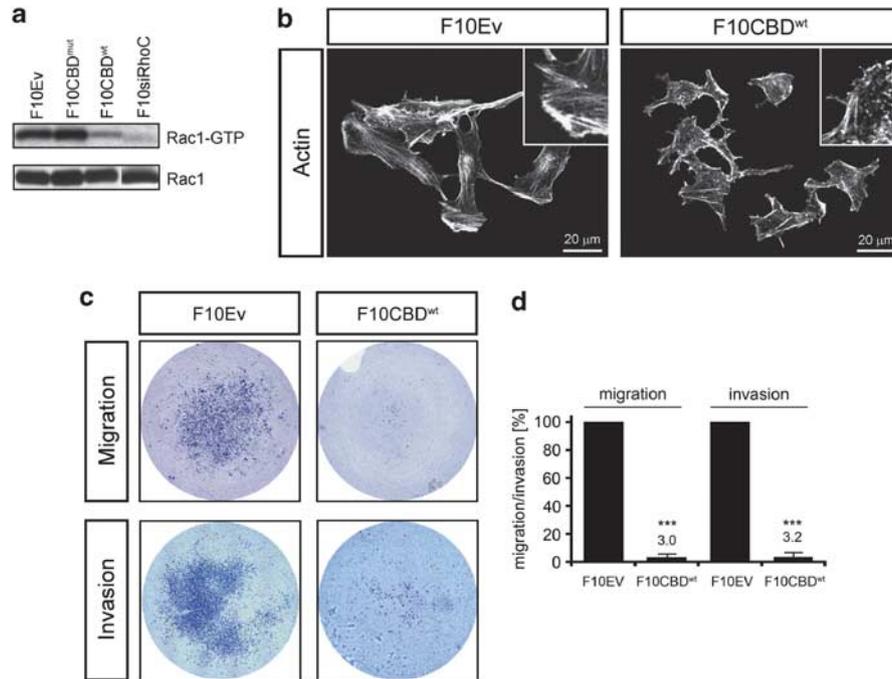


Figure 2 Effects of disrupting the Cav1-Rho-GTPase interaction. (a) Decreased Rac1 activation in the presence of CBD^{wt} or absence of RhoC. Active Rac1 (Rac1-GTP) was pulled down with GST-PAK-PDB-conjugated agarose beads (PAK: p21-activated kinase 1; PDB: p21-binding domain) from extracts of F10 cells expressing Ev, CBD^{mut}, CBD^{wt}, siRhoC. Rac1 was detected by IB. (b) CBD^{wt} expression alters cytoskeletal remodeling. Rhodamine-phalloidin stainings of F10CBD^{wt} cells show abundant F-actin fragments and reduced F-actin stress fibers compared with F10Ev cells. Insets, 2.5-fold magnification of a representative cell area. (c, d) CBD^{wt} expression impairs migration/invasion. Top: F10Ev cells are able to migrate on collagen IV-coated filters; F10CBD^{wt} cells are unable to do so. Bottom: F10CBD^{wt} cells show severe impairment to invade Matrigel. Quantification was performed by using ImagePro software (Media Cybernetics, Inc., Bethesda, MD, USA)—‘filter-area’ occupied by cells is presented in percentage of migration/invasion, *** = statistically significant (two-tailed *t*-test with s.e.m.), *P* < 0.001.

regulation of Rac1, Src, FAK/Pyk2 that follows the disruption of Cav1-Rho-GTPase interaction or the reduction of RhoC expression explains the defects in migration/invasion observed *in vitro*.

Erk1/2 activation downstream of Src promotes cancer cell migration/invasion (Krueger *et al.*, 2001). To determine whether RhoC depletion or CBD^{wt} expression affected Erk1/2 activation, we assayed phospho-Erk1/2 levels in F10 CBD^{wt} and F10siRhoC cells. Erk1/2 activation was drastically reduced in the absence of RhoC or in the presence of the CBD^{wt}, to an even greater extent than in F10SrcDNRF cells (Figure 3d). Src acts through Ras to elicit Erk1/2 activation (Gardner *et al.*, 1993), and a large body of evidence indicates that Ras is intimately associated with integrin function required for cell adhesion, migration and growth (Zhang *et al.*, 1996; Kinbara *et al.*, 2003). Therefore, we first assayed Ras activation in F10CBD^{wt} and F10shRhoC cells and found that disruption of Cav1-Rho-GTPase interaction, or RhoC silencing, markedly reduced the levels of Ras-GTP (Figure 3e). Next, we examined α 5-integrin expression in F10CBD^{wt}, F10CBD^{mut}, F10shRhoC and F10shRhoA. We found that control F10 cells expressed high levels of α 5-integrin, a marker that correlates with a highly malignant phenotype in melanoma (Natali *et al.*, 1995; Keely *et al.*, 1998). We detected similar levels of α 5-integrin in F10CBD^{mut} cells but found a drastic reduction of α 5-integrin expression in CBD^{wt}-expressing

cells and in shRhoC cells (Figure 3f). Remarkably, F10shRhoA cells showed only a slight reduction on α 5-integrin expression. The α 6-integrin and β 1-integrin expression was not affected (data not shown). To further characterize the α 5-integrin expression, we assessed the effects of the expression of CBD^{wt}, shRhoC and shRhoA on α 5-integrin mRNA levels. We found that only the CBD^{wt} or shRhoC expression profoundly reduced the mRNA levels of α 5-integrin (Figure 3g). Notably, the expression of CBD^{wt} had no negative impact on RhoC expression. The reduced expression of α 5-integrin in cells expressing CBD^{wt} or shRhoC is consistent with their inability to metastasize.

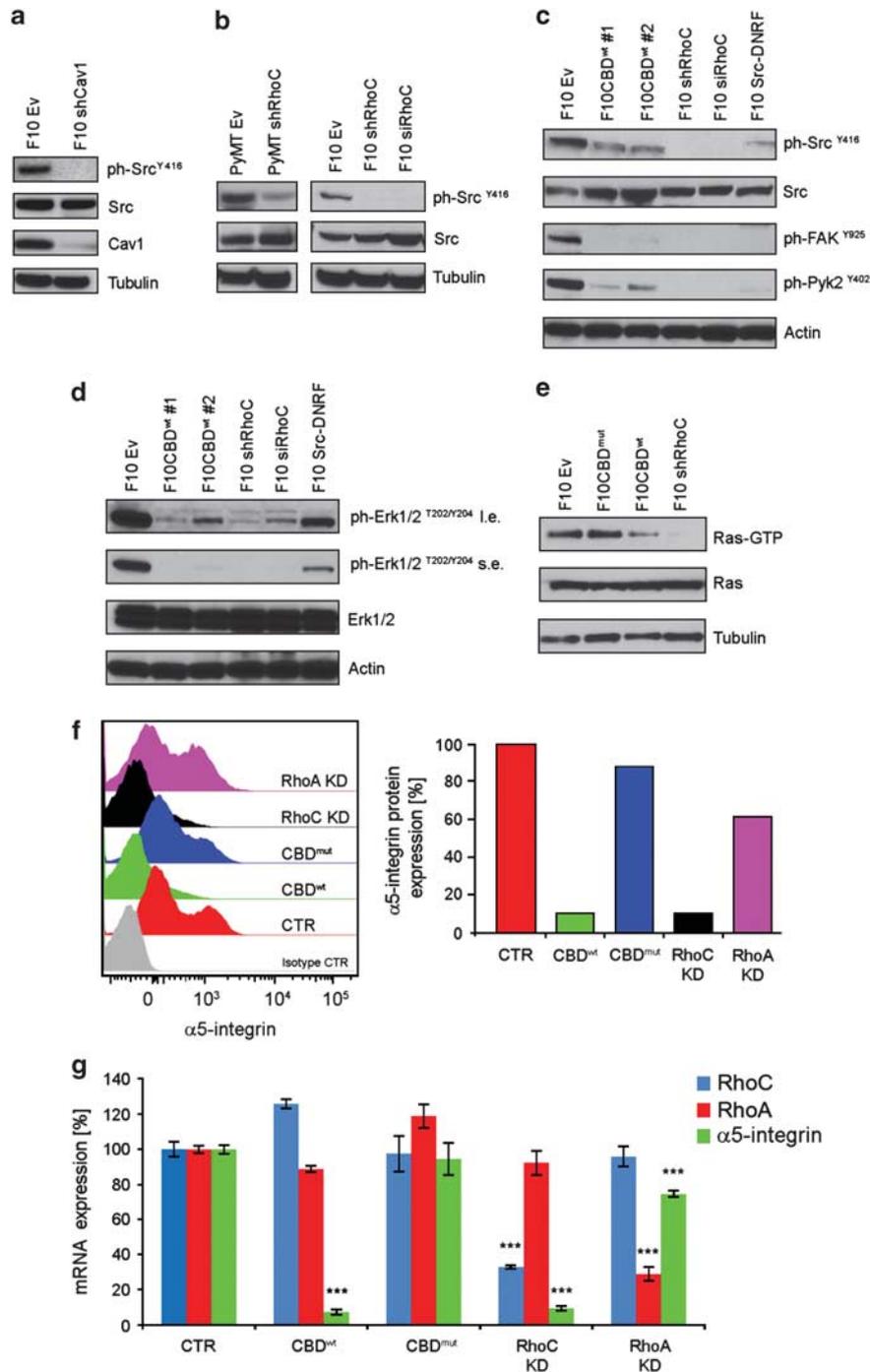
Taken together, our results suggest that Cav1-Rho-GTPase interaction is important not only for Src, Ras and Erk1/2 activation but also for α 5-integrin expression required for cancer cell migration/invasion. As the reduction of RhoC has a major impact on these molecules, we believe that RhoC is the main GTPase contributing to the migratory and invasive properties of melanoma cells.

Disruption of Cav1-Rho-GTPase interaction reduces metastasis in mice

Erk1/2 have roles in cell motility but these molecules are also crucial for cancer cell proliferation and survival (Dent *et al.*, 1999; Shaul and Seger 2007; Mebratu and

Tesfaigzi 2009). As CBD^{wt} expression reduced Erk1/2 activation (Figure 3d), we assessed whether CBD^{wt} expression could alter the metastatic potential of cancer cells *in vivo*. Luciferase-expressing F10Ev, F10 CBD^{wt} and F10 CBD^{mut} cell lines were tail-vein injected into C57/B6 mice, and monitored for metastasis and survival. Non-transfected F10 cells and F10Ev cells had metastasized by 2 weeks post-injection (Figure 4a, top). Similarly, mice injected with F10 CBD^{mut} cells also showed extensive metastasis (Figure 4a, middle). How-

ever, mice injected with F10 CBD^{wt} cells did not develop lung tumors until 3 weeks post-injection, and no significant spread of cancer cells was observed until 4 weeks post-injection (Figure 4a, bottom). As a consequence, mice injected with F10 CBD^{wt} cells had a lifespan that was double that of mice injected with either F10Ev or F10 CBD^{mut} cells (Figure 4b). Comparable results were obtained when these studies were repeated using female mice (Supplementary Figure S3). In line with our earlier results (Figures 3c and d), SrcDNRF expression also



greatly diminished the aggressiveness of the injected F10 cells and prolonged mouse lifespan (Figure 4c). Similar results were obtained when luciferase-expressing PyMT-CBD^{wt} or PyMT-Ev cells were injected into the tail veins of SCID mice. Whereas PyMT-Ev cells readily

established tumors in the lungs, PyMT-CBD^{wt} cells failed to do so (Figure 5a). As a consequence, mice that received PyMT-CBD^{wt} cells lived much longer than animals injected with PyMT-Ev cells (Figure 5b). PyMT cells expressing SrcDNRF were also less metastatic

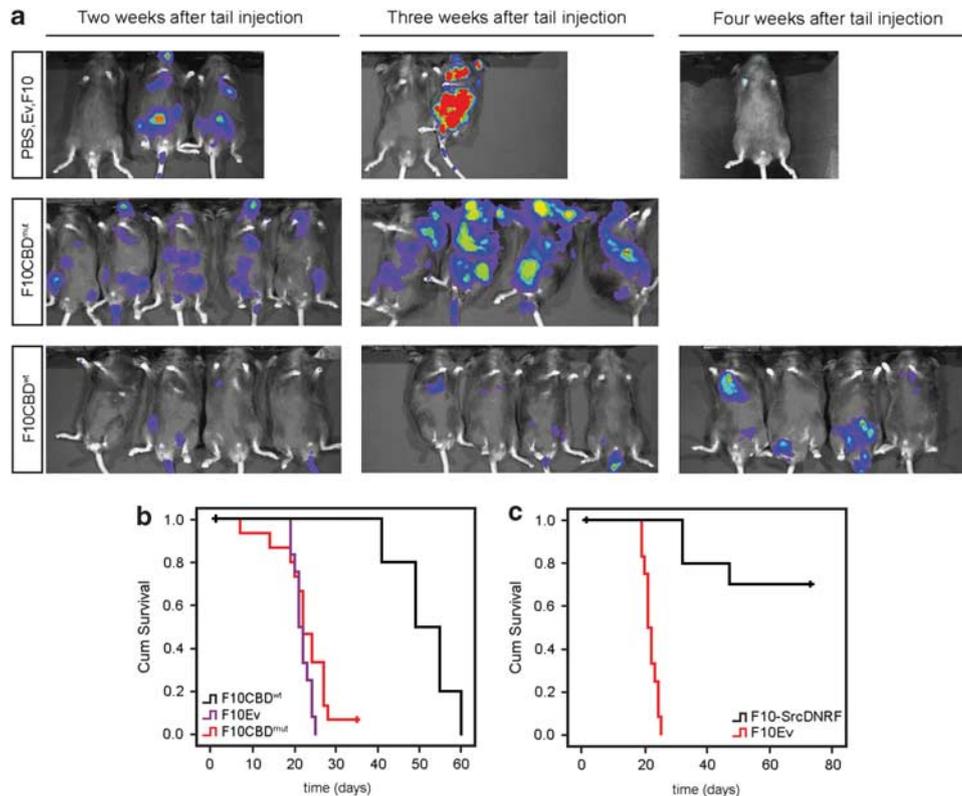


Figure 4 Disruption of the Cav1-Rho-GTPase interaction in F10 cells: *in vivo* effects. (a) CBD^{wt} expression reduces metastasis. Top panel, controls. Middle and bottom panels, male mice were tail-vein injected with F10CBD^{mut} or F10CBD^{wt} cells, respectively. Mice were imaged at the indicated time points post-injection using a Xenogen apparatus (Caliper Life Sciences (Corporate Headquarters), Hopkinton, MA, USA). (b) Extended lifespan of mice tail-vein injected with F10CBD^{wt} cells. The survival of the indicated mice from (a) was examined using Kaplan Meier plots. Median overall survival: F10Ev, 21.75 days ($n = 6$ mice); F10CBD^{mut}, 22.6 days ($n = 10$ mice); F10CBD^{wt}, 51.4 days ($n = 10$ mice). Log-rank test: F10Ev vs F10CBD^{wt}, $P < 0.001$; F10CBD^{mut} vs F10CBD^{wt}, $P < 0.001$; F10Ev vs F10CBD^{mut}, $P = 0.106$. (c) Kaplan-Meier survival plots for C57/B6 mice tail-vein injected with F10Ev or F10SrcDNRF cells. Median overall survival: F10Ev, 21.75 days ($n = 10$ mice); F10SrcDNRF, 62.2 days ($n = 10$ mice). Log-rank test, $P < 0.001$.

Figure 3 Cav1-Rho-GTPase interaction or RhoC regulate Ras/Erk1/2 and FAK/Pyk2 activation through Src. (a) Cav1 controls Src activity. Src activation (Src phosphorylated at Y416) is severely impaired upon loss of Cav1 expression while the total level of Src is not affected. (b) RhoC is involved in Src activation. Src activation was assessed by IB in extracts of F10 and PyMT cells stably expressing siRhoC or shRhoC. (c) Cav1-Rho-GTPase interaction or RhoC control Src, FAK and Pyk2 activation. Phosphorylated tyrosines of Src, FAK and Pyk2 were detected by IB in extracts of two independent F10 clones stably expressing the CBD^{wt} and clones of F10 cells stably expressing shRhoC, siRhoC or Src-DNRF. The expression of the CBD^{wt}, the RhoC silencing or the expression of a Src dominant-negative form have all the effect of reducing the activation of Src, FAK and Pyk2. (d) Cav1-Rho-GTPase interaction or RhoC control Erk1/2 activation. Activated Erk1/2 were detected by IB in extracts of two independent clones of F10 cells stably expressing the CBD^{wt} and clones of F10 cells stably expressing shRhoC, siRhoC or Src-DNRF. The expression of the CBD^{wt}, RhoC silencing or the expression of Src-DNRF reduce the activation of Erk1/2. (e) Cav1-Rho-GTPase interaction or RhoC control Ras activation. Active Ras (Ras-GTP) was pulled down with agarose beads GST-RBD-conjugated (RBD: Raf 1-binding domain) from extracts of F10 cells stably expressing the CBD^{wt}, the CBD^{mut} or shRhoC. Ras was detected by IB. Either the expression of the CBD^{wt} or RhoC silencing impairs Ras activation when compared with the expression of the CBD^{mut}. (f) Fluorescence-activated cell sorting analysis of $\alpha 5$ -integrin expression. The graphs are representative of three independent experiments. The expression of $\alpha 5$ -integrin of F10 cells expressing the CBD^{wt} or shRhoC is drastically reduced in comparison with cells expressing the CBD^{mut}. The silencing of RhoA has a minimal effect on $\alpha 5$ -integrin expression. (g) Cav1-Rho-GTPase interaction or RhoC control $\alpha 5$ -integrin expression. The mRNA expression levels of RhoA, RhoC and $\alpha 5$ -integrin were assessed by quantitative reverse transcriptase-PCR. The expression of the CBD^{wt} decreases $\alpha 5$ -integrin mRNA level without affecting RhoC expression. RhoC silencing also decreases $\alpha 5$ -integrin mRNA level. RhoA silencing has a minimal effect on $\alpha 5$ -integrin mRNA level, *** = statistically significant (two-tailed *t*-test with s.e.m.), $P < 0.001$.

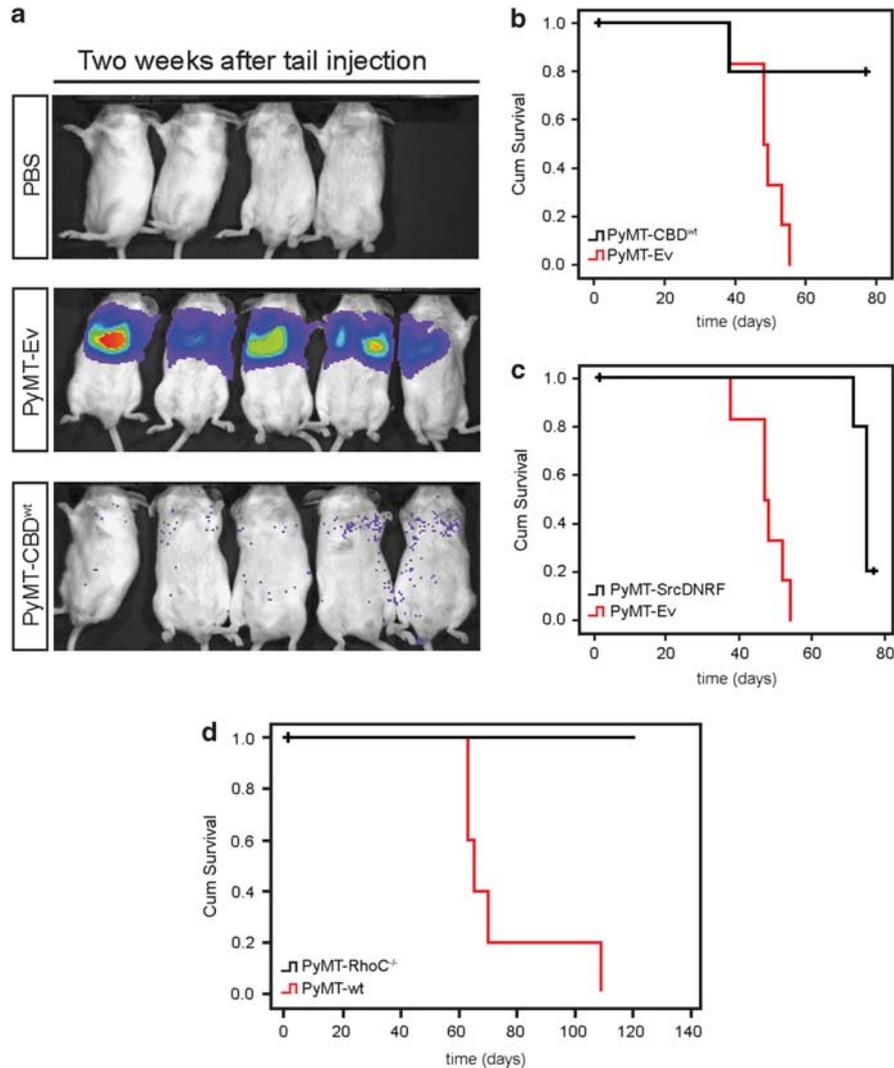


Figure 5 Disruption of the Cav1-Rho-GTPase interaction in PyMT tumor cells: *in vivo* effects. **(a)** Less metastasis by CBD^{wt} expression. SCID mice were tail-vein injected with PBS alone (top panel), PyMT-Ev cells (middle panel) or PyMT-CBD^{wt} cells (bottom panel). Mice were imaged at 2 weeks post-injection using Xenogen. **(b, c)** Increased survival of mice bearing tumor cells expressing CBD^{wt} or SrcDNRF. Kaplan–Meier survival plots for SCID mice tail-vein injected with PyMT-Ev, PyMT-CBD^{wt} or PyMT-SrcDNRF cells. Median overall survival: PyMT-Ev, 47 days ($n=6$ mice); PyMT-CBD^{wt}, not reached at 84 days ($n=6$ mice). Log-rank test, $P=0.021$. PyMT-SrcDNRF, 76 days ($n=6$ mice). Log-rank test, $P<0.001$. **(d)** Kaplan–Meier survival plots for 129P2 mice tail-injected with PyMT-wt or PyMT-RhoC^{-/-} cells. Median overall survival: PyMT-wt, 65 days ($n=5$ mice), PyMT-RhoC^{-/-} not reached.

than controls, as judged by mouse survival (Figure 5c). Remarkably, the injection of RhoC null PyMT cells (PyMT-RhoC^{-/-} cells; Hakem *et al.*, 2005) did not show detectable metastasis or caused the death of any experimental animal (Figure 5d and data not shown). These *in vivo* results suggest that disruption of Cav1-Rho-GTPase interaction, and/or the inhibition of Src, Ras and Erk1/2 activation that stems from it, can reduce the metastatic potential of cancer cells.

Disruption of Cav-Rho-GTPase interaction impairs cancer cell extravasation and survival *in vivo*

The reduced metastasis of CBD^{wt}-expressing cells could be due to either a decreased capacity of these cells to extravasate or a compromised ability to engraft and

survive in a secondary site. To distinguish between these possibilities *in vivo*, we examined the behavior of F10 cells injected into chicken embryos. We generated F10CBD^{wt} cells expressing GFP (F10CBD^{wt}GFP cells), and control F10 cells expressing Ev and the fluorescent protein tomato (F10Ev-tomato cells). The engraftment capacity of these cells was then assessed by injecting them into a vein in the chorioallantoic membrane of the chicken embryo. Quantitative *alu* PCR was used to determine the number of mouse tumor cells migrating to the lungs and liver (Zijlstra *et al.*, 2002, 2008). At 5 days post-injection, the metastatic load in the lung (Figures 6a and b) and liver (Figures 6c and d) attributable to F10CBD^{wt}GFP cells was about fourfold lower than the metastatic load associated with F10Ev-tomato cells. Thus, cells lacking Cav1-Rho-GTPase interaction have

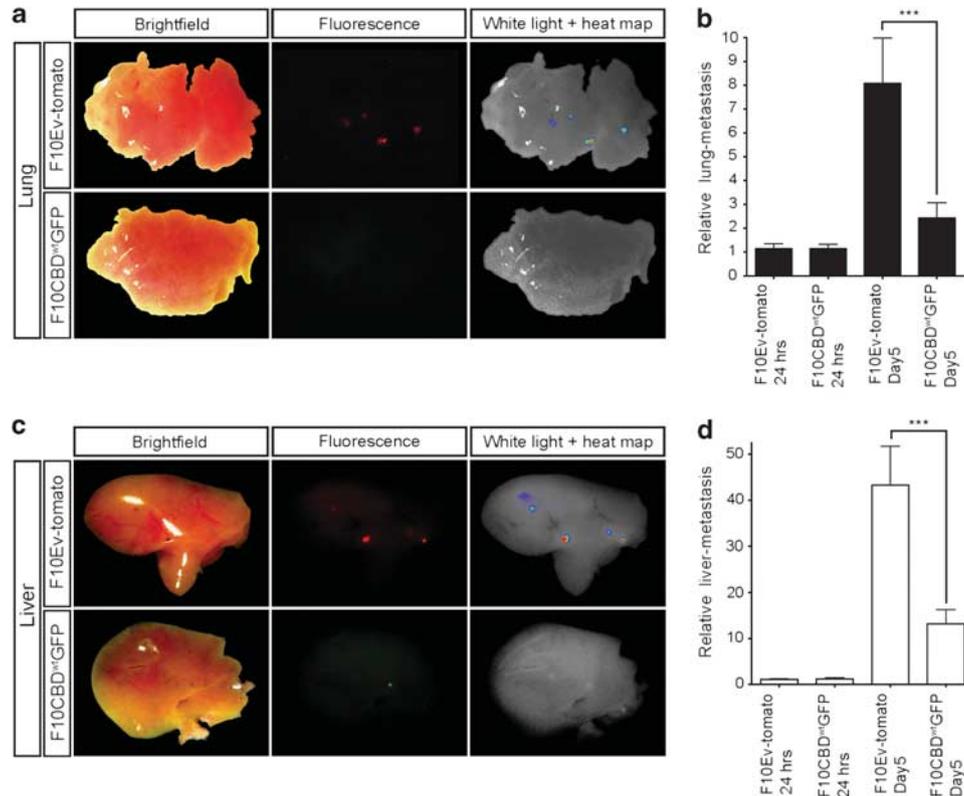


Figure 6 Disruption of the Cav1–Rho-GTPase interaction reduces metastasis in chicken embryos. F10Ev-tomato and F10CBD^{wt}GFP cells were injected intravenously into the chorioallantoic membrane of chicken embryos and the metastatic spread of these cells to the lungs (a, b) and liver (c, d) was assessed at 24 h and 5 days post-injection. (a, c) Imaging of dissected lungs and liver of chicken embryos injected with F10Ev-tomato (top) or F10CBD^{wt}GFP cells (bottom), showing brightfield, fluorescence and overlay images at 5 days post-injection. (b, d) Quantitation of metastases in lungs (b) and liver (d) at 24 h and 5 days post-injection was performed using quantitative alu PCR, *** = statistically significant (two-tailed *t*-test with s.e.m.), $P < 0.001$.

a diminished ability to engraft not only in mice but also in chicken embryos.

Next, we used intravital imaging to compare the extravasation of F10CBD^{wt}GFP and F10Ev-tomato cells. This technique allows the visualization of tumor cells within blood vessels (Lewis *et al.*, 2006), as they are extravasating, and after they have successfully extravasated into the surrounding stroma. Although the extravasation rate of F10CBD^{wt}GFP cells was similar to that of F10Ev-tomato cells (Figure 7a), the attrition rate of F10CBD^{wt}GFP cells during extravasation was double that of F10Ev-tomato cells (Figure 7b). Consequently, fewer F10CBD^{wt}GFP cells than F10Ev-tomato cells successfully completed extravasation (Supplementary Movies 1, 2). This decrease in the viability of F10CBD^{wt}GFP cells coincided with morphological alterations not seen in F10Ev-tomato cells, either during or after extravasation. Specifically, F10CBD^{wt}GFP-expressing cells showed a greater number of branched protrusions (Supplementary Figure S4) that may have affected the ability of these cells to extravasate. These data imply that the reduced metastasis that follows CBD^{wt} expression may be due in part to detrimental effects on tumor cell survival during extravasation and in the secondary site.

Discussion

Cav1 and Rho-GTPases have important roles in cell movement, growth, survival, cancer progression and metastasis. Cav1 orchestrates various signaling molecules among Rho-GTPases, which bear a CBD and interact directly with Cav1. Cav1 regulates cell polarization and directional migration through a process involving Src and Rho-GTPases (Grande-Garcia *et al.*, 2007). RhoC contains a putative CBD and interacts directly with Cav1 (Lin *et al.*, 2005). Therefore, the Cav1–Rho-GTPase interaction should be of important biological significance.

We have provided evidence that the expression of the CBD^{wt} does not affect RhoC expression but disrupts the Cav1–Rho-GTPase interaction and compromises cell migration/invasion and survival by altering the expression of $\alpha 5$ -integrin, the activity of Src and downstream activation of p130^{Cas}/Rac1, FAK/Pyk2 and Ras/Erk1/2. The expression of $\alpha 5$ -integrin is virtually absent in CBD^{wt}-expressing cells or in cells lacking RhoC but it is only marginally affected by RhoA silencing. This argues that the absence of $\alpha 5$ -integrin is due to the disrupted interaction of Cav1 and RhoC. At the biochemical level, the expression of the CBD^{wt} or the

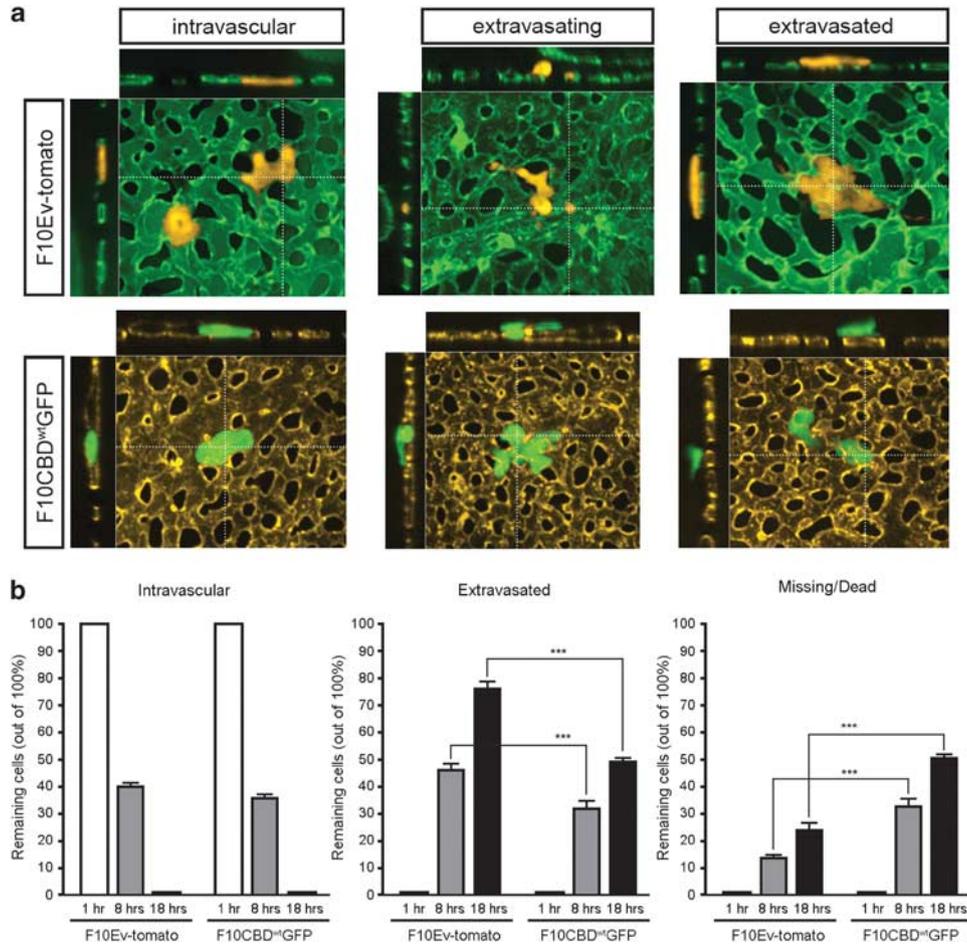


Figure 7 CBD^{wt} expression interferes with extravasation and cell survival *in vivo*. F10Ev-tomato and F10CBD^{wt}GFP cells were injected intravenously into the chorioallantoic membrane of a chicken embryo and monitored using 3D intravital fluorescence imaging. **(a)** Imaging. The status of each tumor cell relative to the vasculature was visualized, whether intravascular (left), in the process of extravasating (middle), or extravasated (right). Both F10Ev-tomato and F10CBD^{wt}GFP cells were able to extravasate in the CAM. **(b)** CBD^{wt} expression decreases extravasation of tumor cells and profoundly reduces tumor cell survival. The extravasation of individual tumor cells (F10Ev-tomato, $n = 1375$ cells; F10CBD^{wt}GFP, $n = 1365$ cells) was monitored over 18 h. After 1 h, all cells were intravascular. After 8 h, 44.9% more F10Ev-tomato cells had extravasated than F10CBD^{wt}GFP cells ($P = 0.016$). After 18 h, 54.2% more F10Ev-tomato cells had extravasated than F10CBD^{wt}GFP cells ($P = 0.0007$). *** = statistically significant (two-tailed *t*-test with s.e.m.), $P < 0.001$. This difference in extravasation rate coincides with higher cell death (missing/dead) of F10CBD^{wt}GFP cells.

silencing of RhoC has similar effects. Both CBD^{wt}-expressing cells and cells lacking RhoC exhibit a drastic reduction of Src activity. The reduction of Src activation has several effects. First, Src-dependent p130^{Cas} phosphorylation is reduced causing the consequent drastic decrement of Rac1 activation. Rac1 has been shown to be a crucial mediator of Src-dependent actin cytoskeleton organization and cell migration (Timpson *et al.*, 2001; Bosco *et al.*, 2009). The observed changes on p130^{Cas}/Rac1 activation and altered actin cytoskeleton by CBD^{wt}-expressing cells do explain the migration/invasion defects observed *in vitro*. Also, Src-dependent activation of FAK and Pyk2 is affected by CBD^{wt} expression or lack of RhoC. In addition to their known involvement in cell adhesion (Schaller 2010), FAK and Pyk2 have been shown to support cell proliferation in a p53-dependent manner (Lim *et al.*, 2008, 2010). Therefore, CBD^{wt}-expressing and RhoC

knockdown cells lack survival signals provided by either FAK or Pyk2.

The activation of Ras and Erk1/2 is also impaired in both CBD^{wt}-expressing cells and cells lacking RhoC. This is consistent with the notion that the Src kinase family can affect cell proliferation via the Ras/ERK/mitogen-activated protein kinase pathway (Minden *et al.*, 1995; Kim *et al.*, 2009). Moreover, Ras and Erk1/2 have been shown to be involved in cell migration, proliferation and survival (Giehl 2005; Torii *et al.*, 2006). We show for the first time that Ras and Erk1/2 activation as well as $\alpha 5$ -integrin expression might be entirely dependent on RhoC signaling in B16 melanoma cells. Mechanistically, RhoC signaling might affect $\alpha 5$ -integrin expression first and that this event, in turn, activates the Src/p130^{Cas}/Rac1 signaling cascade. Rho-GTPases are important for integrin clustering/function (Keely *et al.*, 1998), because the prevention

of plasma membrane localization of Rho-GTPases decreases $\alpha 5$ -integrin mRNA and protein expression (Kidera *et al.*, 2010). On the other hand, Ras can activate integrin signaling although integrin expression is not controlled directly by Ras (Kinbara *et al.*, 2003). The importance of $\alpha 5$ -integrin in cell survival/apoptosis becomes evident when considering that high $\alpha 5$ -integrin expression is detrimental for breast cancer patients (Nam *et al.*, 2010). Moreover, various other cancer cell types depend on $\alpha 5$ -integrin expression/function for their survival (O'Brien *et al.*, 1996; Qian *et al.*, 2005; Haenssen *et al.*, 2010). Besides, block of $\alpha 5$ -integrin expression/function has been shown to decrease the invasiveness and adhesiveness of cancer cells (Mierke *et al.*, 2011).

We demonstrate that mice or chicken embryos injected with CBD^{wt}-expressing melanoma cells show great reduction of metastatic burden. The effects of the CBD^{wt}-expression were not limited to melanoma cells because PyMT mammary gland tumor cells were similarly affected *in vivo*. Intravital microscopy revealed that CBD^{wt}-expressing cells encounter problems during extravasation and also display markedly reduced ability to survive post extravasation. We provide the first evidence that the Cav1–Rho-GTPase interaction is important for melanoma cells to extravasate and survive *in vivo*.

In conclusion, the disruption of the Cav1–Rho-GTPase interaction and the absence of RhoC have the same biochemical/phenotypic effects, therefore we speculate that RhoC is responsible for the metastatic properties of B16 melanoma cells. We suggest that Cav1–RhoC interaction and downstream signaling controls $\alpha 5$ -integrin expression. Subsequently, $\alpha 5$ -integrin signaling activates the Src/FAK/p130^{Cas} pathway (Wu *et al.*, 2008) that leads to Rac1 and Ras/Erk1/2 activation, which altogether is crucial for the migration/invasion and survival of metastatic melanoma cells.

Materials and methods

Additional details of material and methods can be found in Supplementary Information.

Plasmids

Plasmids containing the wild-type and mutant forms of CBD were created by ligating synthetic duplex oligonucleotides into the *Bam*HI/*Eco*RI sites of pcDNA3.1. The G418- or hygromycin-resistant versions of pcDNA3.1 were used for all cloning. SrcDNRF construct (Millipore, Billerica, MA, USA; Cat#21–154).

Cancer cell lines and transfections

B16 F0, F1 and F10 melanoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Iscove's modified Dulbecco's media containing 10% fetal bovine serum. B16 F10 cells expressing luciferase were purchased from Caliper LifeSciences (Corporate Headquarters), Hopkinton, MA, USA). PyMT primary mammary tumor cells were derived in-house from MMTV–PyMT mice. Mammary tumor cells were cultured in

Dulbecco's modified Eagle's medium/HAM-F12 (1:1) media containing 10% fetal bovine serum, 0.5 μ g/ml hydrocortisone (Sigma-Aldrich, St Louis, MO, USA), 5 μ g/ml insulin (US Biological, Marblehead, MA, USA) and 5 ng/ml epidermal growth factor (US Biological) under standard conditions (37 °C, 5% CO₂) until spontaneous immortalization occurred. Tumor cells were transfected with plasmids using a standard lipofectamine transfection procedure (Invitrogen Canada, Burlington, ON, Canada) and the desired clones were isolated by selection in the appropriate agents/antibiotics.

RNA interference-mediated gene silencing

For transient silencing, cells were transfected with 200 pmoles siRNA (either control scrambled siRNA or siRNA against RhoC from Ambion, Applied Biosystems, Carlsbad, CA, USA) using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. siRNA sequences are listed in Supplementary Information. Transfected cells were analyzed by immunoblotting at 72 h post-transfection as described below. For stable knockdowns, control siRNA and short hairpin RNA against RhoC, Cav1, RhoA were either cloned into the pSilencer vector (Ambion) or were purchased ready for lentiviral expression (Open Biosystems Products, Huntsville, AL, USA; Sigma-Aldrich). Lentiviral particles were produced in 293FT cells and virus-containing culture supernatants were used to infect cells in the presence of 8 μ g/ml polybrene (Sigma).

Immunoblotting

Extracts of transfected or transduced cells were prepared in RIPA buffer. Equal amounts of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to immunoblotting using the following antibodies: rabbit polyclonal anti-Cav1 (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit polyclonal anti-p130^{Cas} (Sigma); rabbit polyclonal antibodies specific for the Y165 or Y410 phosphorylated forms of p130^{Cas}, or phosphorylated Src-Y416 (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal antibodies specific for FAK-Y925, Pyk2-Y402, total Erk1/2; or phosphorylated forms of Erk1/2-T202/Y204 (Cell Signaling). To detect Cav1 bound to Rho-GTPases in the presence of the CBD^{wt} and CBD^{mut}, immunoblotting of immunoprecipitates obtained using the Rho activation kit (Millipore) was performed using anti-Cav1 antibody as above. Anti-Rho (-A, -B, -C) antibody, clone 55 (Millipore), anti-Rac1 mouse monoclonal antibody (Cytoskeleton Inc., Denver, CO, USA) and anti-Ras monoclonal antibody clone RAS10 (Millipore).

Transwell migration/invasion and wound-healing assays

For Transwell migration/invasion, cells (1×10^5) that had been cultured overnight in media containing 0.2%. Fetal bovine serum was plated into the upper chamber of a Transwell insert (8 μ m pore size) that had been coated with collagen-IV for migration assays, or with Matrigel for invasion assays (BD - Canada, Mississauga, ON, Canada). The upper and lower chambers were then filled with medium containing 5% fetal bovine serum and the cells were cultured for 24 h under standard conditions. For visualization, cells were fixed and stained with 1% methylene blue in 50% methanol. For the wound-healing assay, cell cultures at near-confluence were wounded by creating a 'scratch' in the cell monolayer. At 48 h post-wounding, cell migration was examined after cell cultures were fixed by staining with 1% methylene blue in 50% methanol.

Rho, Rac1 and Ras activation assays

Cells (5×10^6) were serum-starved overnight before stimulation for 15 min with 50 ng/ml insulin-like growth factor (IGF; US Biologicals) for Rho-GTPase and Ras activation, or for 5 min with 10 ng/ml insulin-like growth factor for Rac1 activation. Specific activation of GTP-loaded Rho-GTPases, Rac1 or Ras was detected using the Rho activation kit (Millipore), the Rac1 activation kit (Cytoskeleton Inc.) or the Ras activation kit (Millipore), respectively, according to each manufacturer's protocol.

Actin visualization

Cells (2×10^4) were plated on uncoated 12 mm glass coverslips and allowed to attach overnight before fixation in 2% formaldehyde/phosphate-buffered saline (PBS) and permeabilization in 0.5% Triton-X-100/PBS. Permeabilized cells were washed in PBS and actin was detected by rhodamine-phalloidin (PHDR1; Cytoskeleton Inc.). Fluorescent images were obtained using an Olympus-Fluoview-FV1000 confocal microscope (Olympus Canada Inc., Markham, ON, Canada).

Integrin assays and quantitative reverse transcriptase-PCR

The expression of $\alpha 5$ -integrin was assessed by fluorescence-activated cell sorting analysis on a BD FACSCanto II flow cytometer. Cells (10^6) were stained with anti-CD49e ($\alpha 5$ -integrin) antibody BD clone 5H10-27.

The mRNA level was assessed by quantitative reverse transcriptase-PCR. TRIzol reagent (Invitrogen) was used for RNA extraction and the iScript reverse transcription-kit (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON, Canada) to produce complementary DNA. The quantitative reverse transcriptase-PCR reaction was performed using SYBR Green (Applied Biosystems, Carlsbad, CA, USA) and the Applied Biosystems Real-time PCR-7900HT. The *gapdh* gene amplification was used as internal reference.

In vivo analyses of metastasis in mice

Tumor cells (5×10^5 in 100 μ l PBS) were injected into the tail-vein of C57/B6 or SCID mice. *In situ* imaging of luciferase-expressing metastatic cells was performed using a Xenogen apparatus (IVIS Imaging Systems, Caliper LifeSciences). Mouse survival over 60–80 days was evaluated using Kaplan–Meier methodology. All animal experiments were approved by the Animal Care and Use Committee of the University Health Network (Toronto, Canada).

Quantitation of in vivo metastasis in chicken embryos

Fertilized chicken eggs were incubated in a rotary incubator at 37 °C with 70% humidity for 4 days before being removed

from the shell and placed in covered dishes. On day 12 of development, 2×10^5 tumor cells in 50 μ l PBS were injected into a vein within the chorioallantoic membrane. Chicken embryonic liver and lung were harvested at 24 h and 5 days post-injection and mouse tumor cells in these organs were measured using quantitative PCR to detect the mouse *alu* sequence. The cT values obtained from the mouse *alu* amplification were normalized to the chicken GAPDH as previously described (Zijlstra *et al.*, 2002, 2008).

Intravital imaging of tumor cell migration and extravasation

Real-time imaging of the intravascular migration and extravasation of mouse tumor cells in chicken embryos was performed using a chicken embryo imaging unit as previously described (Zijlstra *et al.*, 2008). Briefly, the embryonic chicken vasculature was labeled with fluorescent Lens Cularis Agglutinin (LCA, Vector Laboratories, Burlingame, CA, USA), F10CBD⁺GFP or F10Ev-tomato cells (2×10^5) were injected into a vein within the chorioallantoic membrane and the embryos were visualized using a spinning-disk confocal microscope (Quorum Technologies, Waterloo, ON, Canada).

Statistics

Kaplan–Meier survival plots and intergroup comparison statistics were determined by using GraphPad PRISM-5 software (GraphPad Software, Inc., La Jolla, CA, USA). The log-rank test was used to evaluate differences in animal survival among experimental groups (*P*-value). The two-tailed *t*-test with s.e.m. was used to evaluate differences in tumor cell extravasation and survival in chicken embryos.

Conflict of interest

The authors declare no conflict of interest.

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