

Galectin-3– and phospho-caveolin-1–dependent outside-in integrin signaling mediates the EGF motogenic response in mammary cancer cells

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ABSTRACT In murine mammary epithelial cancer cells, galectin-3 binding to β 1,6-acetylglucosaminyltransferase V (Mgat5)–modified N-glycans restricts epidermal growth factor (EGF) receptor mobility in the plasma membrane and acts synergistically with phospho-caveolin-1 to promote integrin-dependent matrix remodeling and cell migration. We show that EGF signaling to RhoA is galectin-3 and phospho-caveolin-1 dependent and promotes the formation of transient, actin-rich, circular dorsal ruffles (CDRs), cell migration, and fibronectin fibrillogenesis via Src- and integrin-linked kinase (ILK)–dependent signaling. ILK, Src, and galectin-3 also mediate EGF stimulation of caveolin-1 phosphorylation. Direct activation of integrin with Mn^{2+} induces galectin-3, ILK, and Src-dependent RhoA activation and caveolin-1 phosphorylation. This suggests that in response to EGF, galectin-3 enables outside-in integrin signaling stimulating phospho-caveolin-1–dependent RhoA activation, actin reorganization in CDRs, cell migration, and fibronectin remodeling. Similarly, caveolin-1/galectin-3–dependent EGF signaling induces motility, peripheral actin ruffling, and RhoA activation in MDA-MB-231 human breast carcinoma cells, but not HeLa cells. These studies define a galectin-3/phospho-caveolin-1/RhoA signaling module that mediates integrin signaling downstream of growth factor activation, leading to actin and matrix remodeling and tumor cell migration in metastatic cancer cells.

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INTRODUCTION

Stimulation of cell motility in response to motogenic factors is associated with remodeling of the actin cytoskeleton and enhanced turnover of substrate adhesions, reflecting cross-talk between receptor tyrosine kinase (RTK) and integrin signaling pathways. Epidermal growth factor receptor (EGFR) and integrins cooperate and synergize to promote cell migration (Moro *et al.*, 1998; Li *et al.*, 1999; Cabodi

et al., 2004). EGFR is up-regulated in many cancers, including breast cancer, and is associated with a poor prognosis (Masuda *et al.*, 2012). EGF stimulates cancer cell migration through transcriptional regulation, actin reorganization, and integrin-dependent adhesion (Rabinovitz *et al.*, 1999; Katz *et al.*, 2007; Sidani *et al.*, 2007; Morello *et al.*, 2011). For example, EGF promotes cell motility through Fyn-dependent β 4-integrin phosphorylation, which decreases cell adhesion (Mariotti *et al.*, 2001). The α 2-integrin intracellular domain also participates in EGF-induced cell migration by promoting EGFR and integrin trafficking and recycling (Klekotka *et al.*, 2001; Ning *et al.*, 2007). EGF pretreatment decreases α 5 β 1-integrin–dependent cell spreading in a filamin- and ROCK-dependent manner (Vial and McKeown-Longo, 2012). In vivo EGF signaling to Akt and ERK1/2 leads to β 1-integrin–dependent tumor growth, similar to EGF-induced cell migration (Morello *et al.*, 2011). All of these results support the idea that EGF promotes cell migration by regulating integrin signaling and trafficking. However, the mechanisms underlying growth factor–mediated integrin signaling are not well understood.

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Abbreviations used: Cav1, caveolin-1; CDR, circular dorsal ruffle; EGF, epidermal growth factor; FN, fibronectin; Gal3, galectin-3; Mgat5, β 1,6-acetylglucosaminyltransferase V; pCav1, Y14 phosphorylated caveolin-1; PR, peripheral ruffle.

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Plasma membrane subdomains, including lipid rafts, caveolin-dependent domains, and galectin-glycan lattices, organize the spatial distribution and regulate the activity of cytokine receptors, adhesion molecules, and proximal effectors of signaling (Lajoie *et al.*, 2009). Galectins are galactose-binding proteins that bind and cross-link branched N-glycans on glycoproteins (Rabinovich and Toscano, 2009; Boscher *et al.*, 2011). Of the 15 mammalian galectins, galectin-3 (Gal3) is unique in that it can oligomerize to form complexes, up to pentameric, forming a lattice (Nieminen *et al.*, 2007). Recruitment to the lattice promotes RTK signaling as a function of both N-glycan number (NXS/T sites) and N-glycan branching, the latter generated principally by activity of the Golgi enzymes (β 1,6-acetylglucosaminyltransferase V [Mgat5]; Lau *et al.*, 2007). Lipid-based signaling domains, such as cholesterol-rich lipid rafts, and associated caveolin-1 are also important regulators of cell signaling. In cancer cells, caveolin-1 (Cav1) interacts via its scaffolding domain with a number of receptor tyrosine kinases, including EGFR, to inhibit their activity (Couet *et al.*, 1997; Park *et al.*, 2000). The Mgat5-dependent Gal3 lattice promotes EGF signaling by reducing receptor down-regulation and protecting EGFR from negative regulatory Cav1 scaffold domains (Partridge *et al.*, 2004; Lajoie *et al.*, 2007).

The galectin lattice also promotes integrin activation and endocytosis (Furtak *et al.*, 2001; Lagana *et al.*, 2006). Src kinase-dependent Cav1 tyrosine phosphorylation increases focal adhesion turnover and tumor cell migration in a Gal3-dependent manner (Goetz *et al.*, 2008). Overexpression of a nonphosphorylatable Cav1 mutant (Cav1Y14F) or disruption of the lattice inhibits cell motility. In other words, phosphorylated Cav1 (pCav1) and Gal3 are synergistic with respect to activation of focal adhesions. pCav1 activates RhoA and mediates RhoA/ROCK/Src-dependent cell migration and invasion (Grande-Garcia *et al.*, 2007; Joshi *et al.*, 2008). Coordinated expression of Cav1 and Gal3 induces focal adhesion turnover, RhoA activation, and cell migration in thyroid cancer cells and represents a highly selective marker to distinguish differentiated thyroid cancers from benign thyroid nodules (Shankar *et al.*, 2012).

As conditional modifiers of EGFR availability to ligand and of adhesion dynamics, galectin lattice and Cav1 domains will therefore influence actin cytoskeleton and adhesion dynamics and thereby cell migration and invasion (Lagana *et al.*, 2006; Goetz *et al.*, 2008; Lajoie *et al.*, 2009; Boscher *et al.*, 2011). The signaling mechanisms underlying the synergistic action of Gal3 and Cav1, however, remain to be determined. EGF is a motogenic factor that induces cell migration and is frequently associated with membrane ruffling activity (Harms *et al.*, 2005). In epithelial and fibroblastic cells, EGF can induce the formation of particular actin structures defined as circular dorsal ruffles (CDRs; Orth *et al.*, 2006; Abella *et al.*, 2010; Azimifar *et al.*, 2012). These dynamin-cortactin-Arp2/3-dependent actin structures contribute to actin remodeling and internalization processes promoting the migratory response (Krueger *et al.*, 2003; Hoon *et al.*, 2012). Here we show that EGF stimulation of CDR, RhoA activation, tumor cell migration, and matrix remodeling in breast cancer cells is mediated by Gal3- and pCav1-dependent integrin signaling.

RESULTS

Integrin and Src dependence of EGF stimulation of circular dorsal ruffles, cell migration, and RhoA activation

Addition of EGF to PyMT murine mammary epithelial cancer cells induces the formation of circular actin rings at the cell surface stained for cortactin, corresponding to growth factor-induced CDRs (Figure 1, A and B). These actin ruffles appear 2 min after EGF addition, forming a large actin ring that becomes a tight annulus at 12.5 min

before collapsing. EGF-induced CDRs are inhibited by the dynamin inhibitor Dynasore and cholesterol extraction by methyl- β -cyclodextrin but not by clathrin small interfering RNA (siRNA), which prevents transferrin internalization (Figure 1C and Supplemental Figure S1). The raft marker ganglioside GM1, bound by the B subunit of cholera toxin, accumulates at EGF-induced CDR (Figure 1D). EGF-induced CDR formation is therefore dynamin and raft dependent and clathrin independent.

As previously described (Gu *et al.*, 2011; Azimifar *et al.*, 2012), focal adhesion-associated proteins, including integrin-linked kinase (ILK) and β 1-integrin (Figure 1E), and paxillin (not shown) are recruited to EGF-induced CDRs. As described previously (Orth *et al.*, 2006), EGFR is also recruited to CDRs (Figure 1F). ILK is an atypical serine/threonine kinase that interacts with the intracellular domain of β -integrins and actin and is involved in the regulation of cell-matrix adhesion (McDonald *et al.*, 2008). We observed that siRNA against ILK significantly reduces the number of cells forming CDRs in response to EGF (Figure 1G) and also decreases EGF-induced cell migration through fibronectin (FN)-coated Boyden chambers (Figure 1F). CDR formation has been proposed to be required for the ECM-dependent chemotaxis response but not for ECM-independent oriented migration during wound healing (Suetsugu *et al.*, 2003). EGF-induced CDR formation and cell migration therefore require signaling through ILK.

Inhibition of Src kinase (PP2), ROCK (γ 27632), and PI3K (LY-294002) prevented EGF-induced CDRs and partially inhibited EGF-induced cell migration, whereas inhibition of MEK1/2 activity by U0126 did not significantly affect EGF-induced CDR formation or EGF-induced cell migration (Figure 2A). Using a rhotekin pull-down assay, we observed that EGF treatment induced the temporal activation of RhoA at 5 min, followed by down-regulation after 15 min (Figure 2B). No activation of Rac1 was observed in response to EGF in these cells. EGF-induced RhoA activation is inhibited by the Src inhibitor PP2 and by ILK siRNA (Figure 2C and Supplemental Table S1). As for EGF-induced CDR, RhoA activation was also dependent on raft integrity (Figure 2C and Supplemental Table S1). EGF signaling to RhoA in these mammary carcinoma cells is therefore Src, ILK, and raft mediated.

Gal3 and pCav1 promote EGF-induced CDR, signaling to RhoA, and cell migration

Gal3 and pCav1 act synergistically to promote focal adhesion turnover, cell migration, and RhoA activation (Goetz *et al.*, 2008; Shankar *et al.*, 2012). We therefore tested whether Gal3 and pCav1 are required for EGF-induced RhoA activation, CDR formation, and cell migration. Competitive disruption of Gal3 binding to cell surface glycoproteins with lactose, but not control sucrose, inhibited EGF-induced RhoA activation and reduced EGF-stimulated CDR formation and cell migration (Figure 3A). Furthermore, siRNA knock-down of Gal3 or Cav1 inhibited EGF-induced RhoA activation, CDR formation, and migration (Figure 3B). To specifically test the role of pCav1, we expressed wild-type Cav1-mRFP, a nonphosphorylatable Cav1Y14F mutant, or a phosphomimetic Cav1Y14D mutant (Joshi *et al.*, 2008, 2012). Overexpression of Cav1Y14D induced RhoA activation in the absence of EGF but not dorsal ruffle formation, whereas Cav1Y14F prevented EGF-induced RhoA activation and CDR formation (Figure 3C). We then tested the effect of transient overexpression of Cav1Y14F and Cav1Y14D on cell migration. Although transfection efficiency was limited to ~25% of the cell population, overexpression of Cav1Y14F significantly decreased (~15%), whereas Cav1Y14D significantly increased both basal cell migration and EGF-induced cell migration (~20%; Figure 3C). Our results show

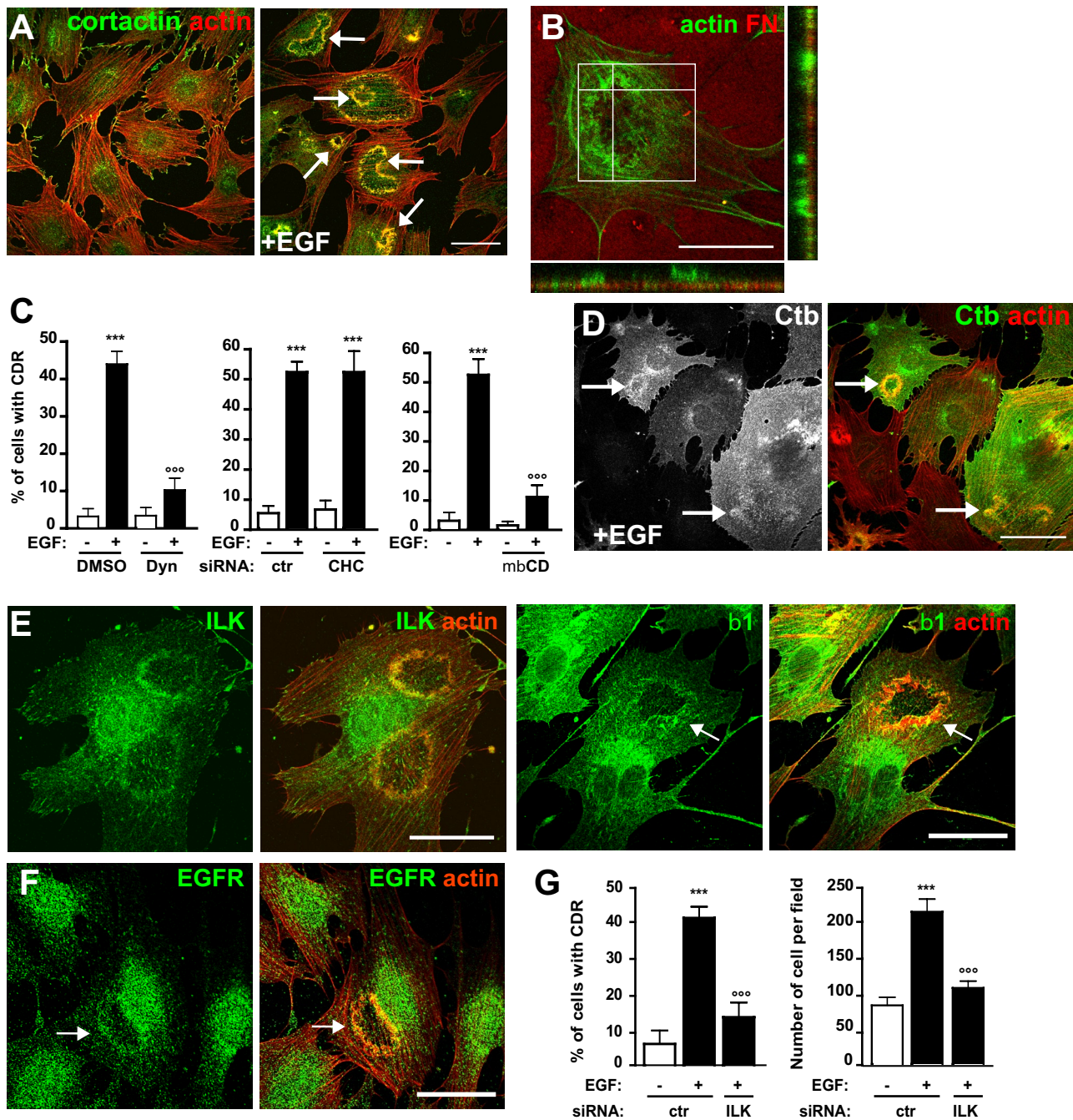


FIGURE 1: EGF induces raft-, dynamin-, and ILK-dependent actin reorganization in CDR. (A) *Mgat5*^{+/+} cells were plated on FN for 24 h and treated with 100 ng/ml EGF for 8 min before fixation and labeling for cortactin (green) and F-actin (red). Actin reorganization in CDR is indicated by arrows. Bar, 80 μ m. (B) *Mgat5*^{+/+} cells were plated on FN (red) and treated with EGF before fixation and staining. Actin is shown in green. Y, Z (right) and X, Z (bottom) sections from a 3D confocal stack are shown. Bar, 30 μ m. (C) *Mgat5*^{+/+} cells were plated on FN and treated with 75 μ M Dynasore (Dyn) for 45 min or with an equivalent amount of dimethyl sulfoxide (DMSO) before addition of 100 ng/ml EGF for 8 min. In parallel, *Mgat5*^{+/+} cells were transfected with clathrin siRNA (clathrin-heavy chain [CHC]) or treated with methyl- β -cyclodextrin (m β CD) 45 min before EGF treatment. After fixation and F-actin labeling, the percentage of cells with ruffles was counted in 10 fields; the mean is presented +SE. (D) *Mgat5*^{+/+} cells were incubated with the B subunit of cholera toxin (Ctb) before EGF treatment, fixation, and F-actin staining. Actin reorganization in ruffles is indicated by arrows. Bar, 50 μ m. (E) *Mgat5*^{+/+} cells plated on FN substrate were treated with EGF and labeled for F-actin (red) and ILK or β 1-integrin (green) as indicated. Bar, 30 μ m. (F) EGF-treated *Mgat5*^{+/+} cells were stained for EGFR (green) and F-actin (red). Bar, 40 μ m. (G) *Mgat5*^{+/+} cells were plated on FN-coated substrate and transfected with control (ctr) or ILK siRNA (siILK) 48 h before EGF treatment. The mean number of cells forming CDRs in response to EGF is represented. In parallel, control (ctr) or ILK siRNA (siILK) transfected cells were plated in Transwell migration chambers, and after 5 h of EGF treatment, the mean number of migrating cells per field was calculated from 10 independent fields for each condition. *** $p < 0.001$, comparing with or without EGF condition; ^{ooo} $p < 0.001$, comparing control or treated cells both in EGF condition.

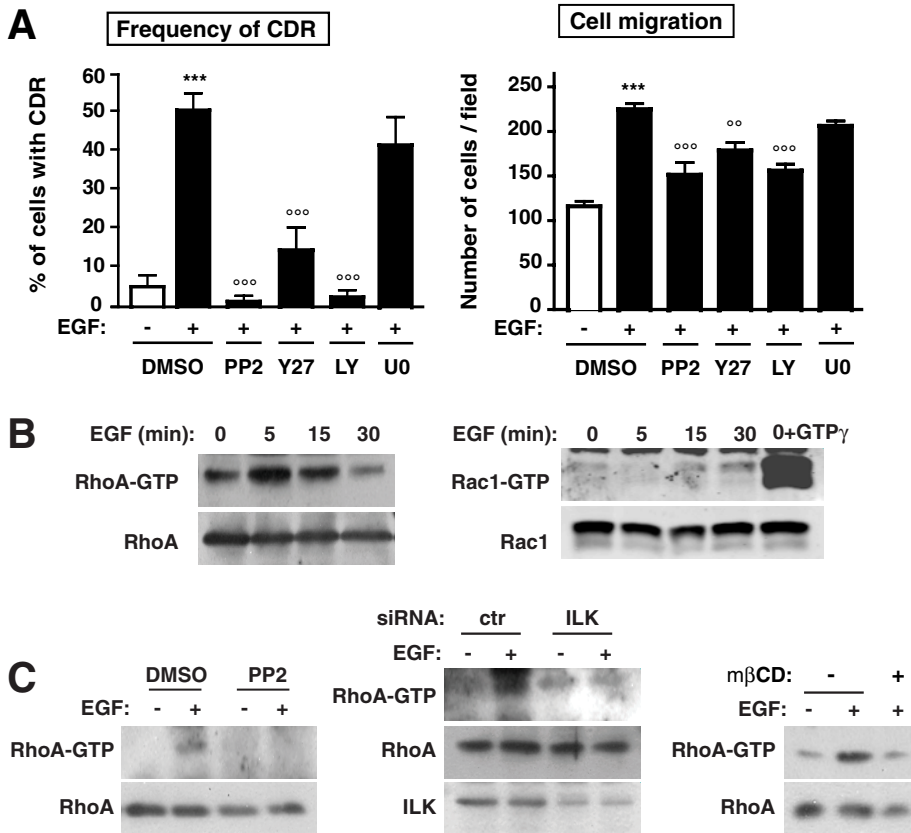


FIGURE 2: EGF induces ROCK-dependent CDR and cell migration and ILK-dependent RhoA activation. (A) *Mgat5*^{+/+} cells were treated with 10 μ M Y27632 (Y27), 10 μ M PP2, 20 μ M LY29003 (LY), 10 μ M U0126 (U0), or with an equivalent amount of DMSO for 30 min before addition of 100 ng/ml EGF for 8 min. The percentage of cells forming CDRs is presented. In parallel, *Mgat5*^{+/+} cells were plated on FN-coated Transwell chambers, treated for 30 min with 10 μ M Y27632 (Y27), 10 μ M PP2, 20 μ M LY29003 (LY), 10 μ M U0126 (U0), or with an equivalent amount of DMSO, and treated with 100 ng/ml EGF for 5 h. The mean number of migrating cells per field is presented (***) $p < 0.001$, comparing with or without EGF condition; (°°) $p < 0.001$, (°°°) $p < 0.005$ relative to EGF-treated cells. (B) *Mgat5*^{+/+} cells were treated with EGF and submitted to lysis. Cell extracts were subjected to Rhotekin-RBD or PAK1-PBD pull down. The amount of activated RhoA (RhoA-GTP) or Rac1 (Rac1-GTP) relative to the total amount of RhoA (RhoA) or Rac1 (Rac1) was found by Western blot. (C) Cells were treated for 30 min with DMSO or 10 μ M PP2 (left), transfected with control (ctr) or ILK-targeting siRNA (middle), or treated with methyl- β -cyclodextrin (m β CD, right) before EGF treatment and Rhotekin-RBD pull down. Level of activated RhoA (RhoA-GTP) relative to total amount of RhoA (RhoA) is shown.

that the Gal3 lattice and pCav1 synergize to promote EGF-induced RhoA activation, CDR formation, and cell migration.

Cav1Y14D overexpression does not increase the number of cells with CDRs in the absence of EGF, but it significantly prolongs CDR expression in response to EGF; the number of Cav1Y14D-overexpressing cells with CDRs after 15 min EGF is significantly higher than nontransfected or Cav1-overexpressing cells with CDRs (Figure 4A). Cav1Y14F overexpression reduces the lifetime of EGF-induced CDRs. As previously described (Azimifar *et al.*, 2012), we observed that EGF-induced CDR formation is dependent on FN coating (Figure 4A). In the absence of a FN coating, overexpression of Cav1 and Cav1Y14D but not Cav1Y14F stimulates CDR formation in response to EGF, defining a stimulatory role for pCav1 in CDR formation. We also observed that Cav1Y14D accumulates in EGF-induced CDRs, whereas Cav1 and Cav1Y14F are not detected (Figure 4B). Incubation with Cy3-Gal3 after fixation revealed strong accumulation of the exogenous protein in CDRs (Figure 4C). This suggests that *Mgat5*-modified branched N-glycans are recruited to CDRs.

After addition of Cy3-Gal3 at 37°C at different times before or after EGF addition, we observed recruitment of Gal3 at cell-cell junctions as previously described (Boscher *et al.*, 2012) but did not detect Gal3 accumulation in CDRs (Figure 4D). This suggests that, whereas N-glycans are present in CDRs, Gal3 does not stably accumulate in CDRs. Taken together, the results indicate that Gal3 and pCav1 promote EGF signaling to CDRs and cell migration.

Gal3 and pCav1 mediate EGFR activation of RhoA through integrin signaling

To specifically determine the role of Gal3 and pCav1 in integrin signaling independently of EGF, we directly activated integrins with Mn^{2+} , known to result in dynamic actin reorganization (Chen *et al.*, 1999; Mould, 2002; Cluzel *et al.*, 2005). The Mn^{2+} -treated *Mgat5*^{+/+} cells display actin reorganization in small β 1-integrin- and cortactin-labeled annuli (Figure 5A). Mn^{2+} treatment induces RhoA activation, which is inhibited by pretreatment with the RGD peptide (Figure 5B), suggesting that Mn^{2+} treatment is integrin specific. Inhibition of Src with PP2 and of Gal3 binding with lactose also prevents Mn^{2+} activation of RhoA (Figure 5B), as does siRNA targeting Gal3, Cav1, and ILK (Figure 6C). Integrin signaling to RhoA therefore requires Gal3, Src/Cav1 signaling, and ILK.

Both EGF and Mn^{2+} induce Cav1Y14 phosphorylation (Figure 5D). Inhibition of ROCK or Src by Y27632 and PP2, respectively, prevents EGF or Mn^{2+} induction of Cav1 phosphorylation (Figure 5D). EGF and Mn^{2+} induction of Cav1 phosphorylation is therefore mediated by Src and Rho/ROCK signaling (Joshi *et al.*, 2008). Gal3 induces Cav1 phosphorylation (Goetz *et al.*, 2008), and treatment with lactose or Gal3 siRNA prevents EGF- and Mn^{2+} -induced Cav1 phosphorylation (Figure 5D). ILK siRNA also prevents EGF and Mn^{2+} induction of Cav1 phosphorylation, demonstrating that it is integrin mediated. Taken together, our results suggest that Gal3 promotes integrin-dependent phosphorylation of pCav1 and downstream activation of RhoA.

EGF-induced migration, actin ruffling, and RhoA activation in human mammary cancer cells is Cav1 and Gal3 dependent

We previously showed that pCav1 and RhoA positively regulate each other in human mammary cancer MDA-MB-231 cells (Joshi *et al.*, 2008). As previously reported, MDA-MB-231 cells migrate in response to EGF (Price *et al.*, 1999), and we observed that this response requires Cav1 and Gal3 expression (Figure 6A). However, only Cav1 was required for EGF-induced migration of human epithelial carcinoma HeLa cells. MDA-MB-231 cells show strong ruffling activity in response to EGF (Figure 6B), predominantly peripheral ruffles (PRs) but also CDRs (Gu *et al.*, 2011; Chen *et al.*, 2012). The

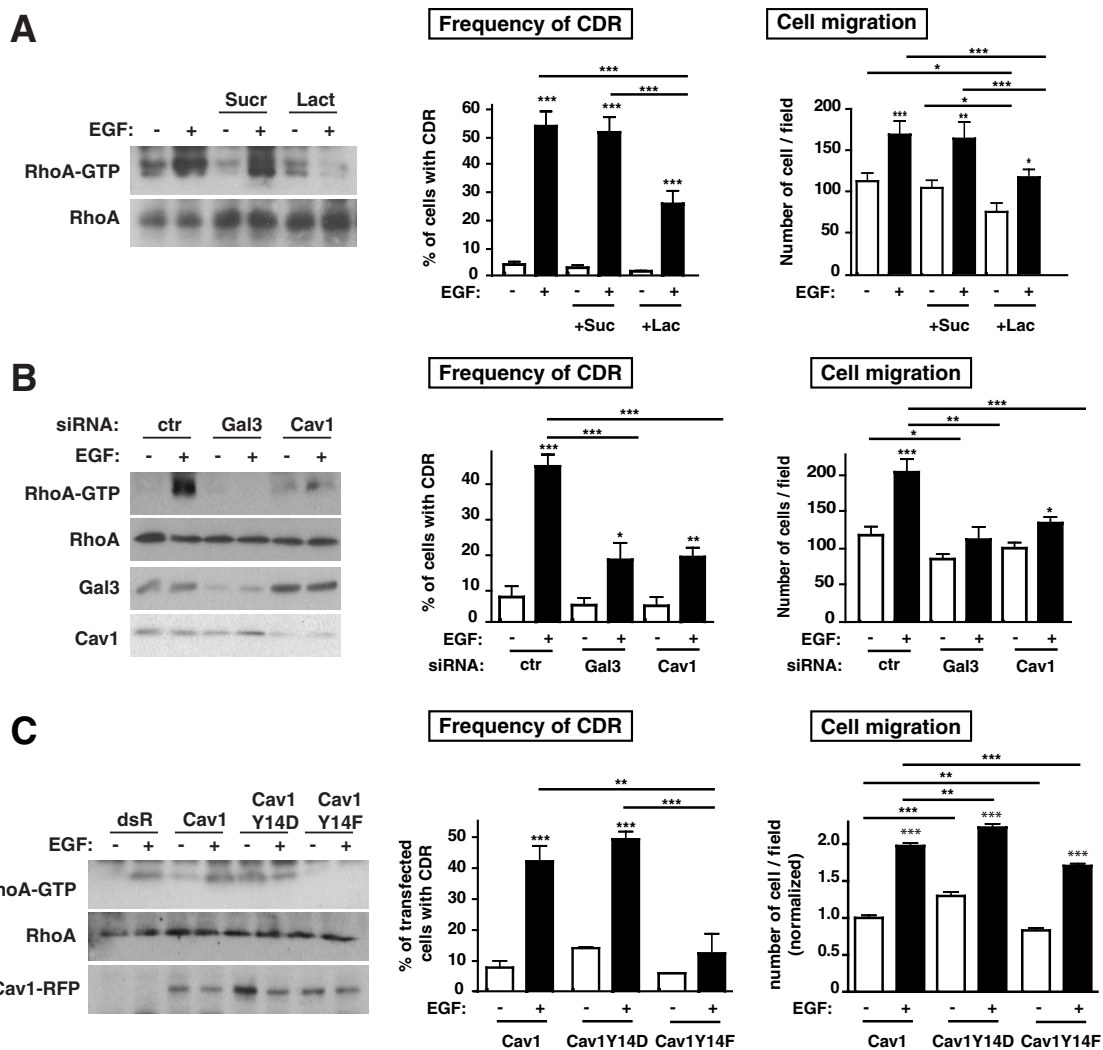


FIGURE 3: EGF-induced RhoA activation, CDR formation and cell migration are regulated by the Gal3 lattice and pCav1. After the indicated treatments, cell lysates were subjected to Rhotekin-RBD pull down to assess levels of activated RhoA (left), percentage of cells presenting CDRs (center), or cell migration in a Transwell assay (right). (A) Mgat5^{+/+} cells were treated with 25 mM sucrose (Suc) or lactose (Lac) continuously for 48 h before treatment with 100 ng/ml EGF. (B) Mgat5^{+/+} cells were transfected with control (ctr) or Gal3-targeted (Gal3) or Cav1-targeted (Cav1) siRNA 48 h before EGF treatment. Cav1 and Gal3 knockdown was assessed by Western blot. (C) Mgat5^{+/+} cells were transfected with dsRed, Cav1, Cav1Y14F, or Cav1Y14D plasmids 48 h before EGF treatment. Cav1-RFP indicates the expression level of the Cav1 constructs. For all experiments, ****p* < 0.001, ***p* < 0.005, **p* < 0.05.

percentage of cells forming EGF-induced ruffles was strongly decreased by Cav1 and Gal3 depletion with siRNA (Figure 6C). Similarly, EGF stimulation of active RhoA levels in MDA-MB-231 cells is Cav1 and Gal3 dependent (Figure 6D). Gal3 siRNA knockdown also prevented EGF stimulation of Cav1 phosphorylation (Figure 6D). Cav1 and Gal3 depletion also decreased cell migration, ruffling, and basal levels of RhoA activation in the absence of EGF (Figure 6, A, C, and D). Taken together, our results show that EGF triggers Cav1- and Gal3-dependent migration, ruffling, and RhoA activation in human MDA-MB-231 breast cancer cells.

EGF-induced FN fibril formation is Gal3, pCav1, ILK, and ROCK dependent

In addition to their potential role in cell migration, CDRs have been proposed to participate to cell invasion and matrix remodeling (Krueger *et al.*, 2003; Hoon *et al.*, 2012). Indeed the metalloprotease MMP2 has been located in CDRs, and the formation of CDRs

and consequent cell migration have been shown to require the presence of extracellular matrix (Suetsugu *et al.*, 2003). We previously showed that the Mgat5-dependent Gal3 lattice activates integrins, leading to FN remodeling (Lagana *et al.*, 2006), and we therefore investigated whether Gal3/pCav1-dependent integrin signaling regulates EGF-dependent matrix remodeling. At 24 h after EGF addition to Mgat5^{+/+} cells, we observed a significantly increased number of FN fibrils (Figure 7A). Similar to EGF-induced CDR, RhoA activation, and cell migration, EGF-induced FN remodeling is reduced by ILK siRNA or PP2 treatment and prevented by ROCK inhibition but not MEK1/2 or phosphatidylinositol 3-kinase (PI3K) inhibition (Figure 7, B and C). Moreover, siRNAs targeting Cav1 or Gal3, as well as Cav1Y14F overexpression and lactose treatment, prevent EGF-induced FN remodeling (Figure 7, D–F). Of interest, independent of EGF, Cav1Y14D overexpression stimulates FN remodeling, which is inhibited by lactose treatment, providing further evidence for Gal3-pCav1 as regulators of integrin signaling. We propose that

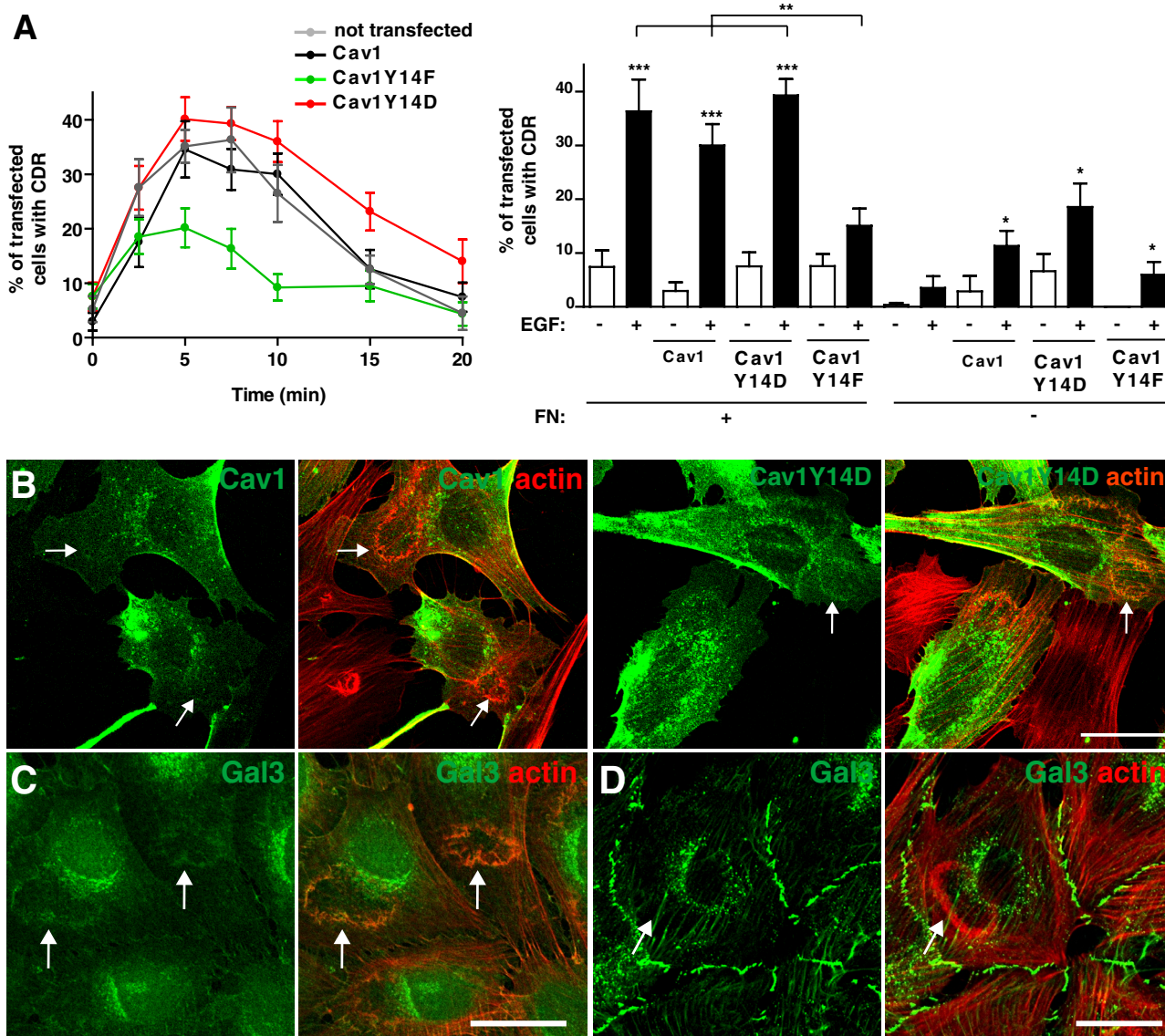


FIGURE 4: The Cav1Y14D mutant promotes EGF-induced CDR formation and accumulates in CDRs. (A) *Mgat5*^{+/+} cells were plated on FN-coated or uncoated coverslips, transfected with Cav1, Cav1Y14F, or Cav1Y14D mutants, treated with EGF, fixed at different times, and stained for F-actin. Left, percentage of transfected cells displaying CDRs on FN-coated coverslips over time. Right, percentage of cells with CDR on either FN-coated or uncoated coverslips before or after 7.5 min of EGF. ****p* < 0.001, ***p* < 0.005, **p* < 0.05. Three to eight transfected cells were counted per field for 10 different fields; *n* = 3. The number of untransfected cells forming CDRs was quantified as control. (B) Cav1- or Cav1Y14D-overexpressing cells (green) are shown after EGF treatment. CDRs are identified with F-actin (red, arrows), and recruitment of Cav1Y14D but not Cav1 to CDRs is observed. Bar, 40 μ m. (C) To reveal the presence of Gal3 ligands in EGF-induced CDRs, *Mgat5*^{+/+} cells were treated with 100 ng/ml EGF, fixed, and incubated with Cy3-coupled Gal3 (green) for 30 min, fixed again, and stained for actin (red). Bar, 40 μ m. (D) *Mgat5*^{+/+} cells were treated with Cy3-coupled Gal-3 (green) and 100 ng/ml EGF together, fixed, and stained for actin (red). Gal3 was not detected in EGF-induced CDR. Bar, 40 μ m.

EGF triggers Gal3- and ILK-dependent integrin signaling, leading to Src/pCav1/RhoA/ROCK-dependent CDR formation, tumor cell migration, and FN remodeling (Figure 8).

DISCUSSION

A Gal3-integrin-pCav1 signaling module mediates the EGF motogenic response

Cell motility induced by growth factors involves complex signaling networks that are incompletely understood. Gal3 and pCav1 were shown to act in concert to promote focal adhesion turnover and cell

migration (Goetz *et al.*, 2008; Shankar *et al.*, 2012). Furthermore, coordinate Cav1 and Gal3 expression is associated with human differentiated thyroid cancer (Shankar *et al.*, 2012). We show here that the Gal3 lattice and pCav1 coordinately promote EGFR signaling to integrins, leading to ILK- and Src-dependent RhoA activation, CDR formation, cell migration, and FN remodeling (Figure 8). Consistently, ILK was shown to mediate EGF-induced CDR formation (Azimifar *et al.*, 2012) and was proposed to promote RTK/integrin cross-talk, leading to cell migration through Nck1/2, PINCH, and ELMO2 (Tu *et al.*, 1999; McDonald *et al.*, 2008; Ho and Dagnino,

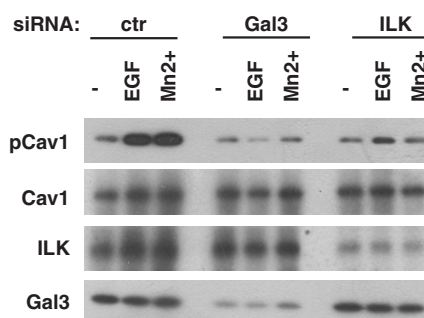
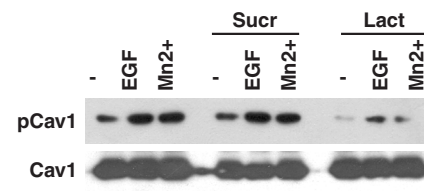
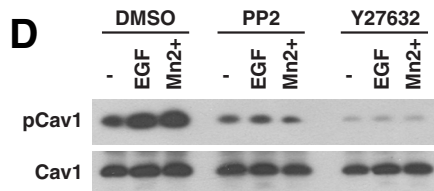
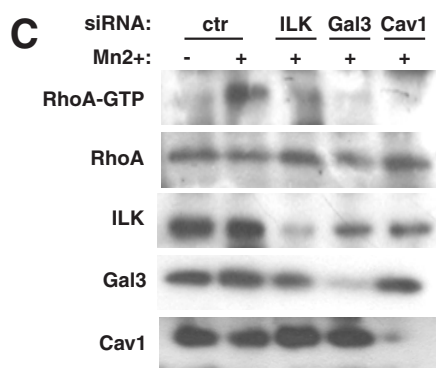
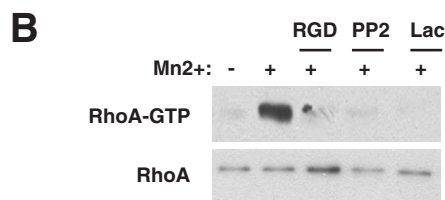
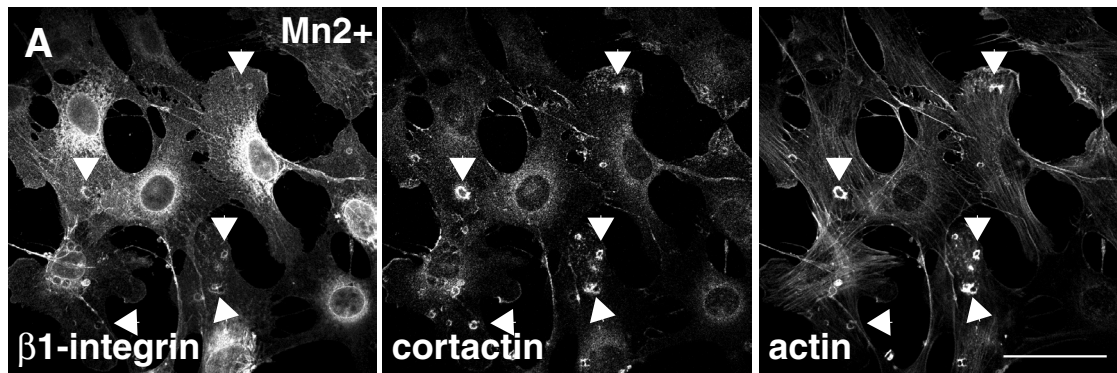


FIGURE 5: EGF and Mn^{2+} induce Gal3- and ILK-dependent RhoA activation and Cav1 phosphorylation. (A) $Mgat5^{+/+}$ cells were treated with $0.5 \mu M Mn^{2+}$ for 10 min before fixation and staining for cortactin, $\beta 1$ -integrin, and F-actin. Mn^{2+} treatment induced small, circular structures (arrowheads). Bar, 100 μm . (B) $Mgat5^{+/+}$ cells were treated for 48 h with 100 $\mu g/ml$ RGD peptide or 25 mM lactose, or for 30 min with 10 μM PP2, before addition of $0.5 \mu M Mn^{2+}$ for 5 min and analysis of cell lysates for RhoA activation by Rhotekin-RBD pull down. (C) $Mgat5^{+/+}$ cells were transfected with control (ctr) or ILK, Gal3 or Cav1-targeting siRNA 48 h before EGF treatment. RhoA activation was determined by Rhotekin-RBD pull down and levels of Gal-3, Cav1, ILK, and EGFR assessed by Western blot. (D) $Mgat5^{+/+}$ cells were treated with 10 μM PP2 or Y27632 for 30 min (top) or 25 mM lactose or sucrose for 48 h (middle) or transfected with control (ctr), Gal-3, or ILK siRNA before addition of EGF or Mn^{2+} . Cell extracts were Western blotted for Y14-phosphorylated Cav1 (pCav1), total Cav1, and (bottom) ILK and Gal-3.

2012). ILK is shown here to be required not only for EGF-induced CDR formation and cell migration, but also for EGF-stimulated Cav1 phosphorylation, RhoA activation, and FN remodeling.

Gal3 induces integrin activation and Cav1 phosphorylation (Lagana *et al.*, 2006; Goetz *et al.*, 2008). The demonstration here that Gal3 is required for direct Mn^{2+} -mediated, ILK-dependent integrin signaling leading to Cav1 phosphorylation and RhoA activation defines a synergistic role for extracellular Gal3 and intracellular pCav1 in outside-in integrin signaling. EGF and Mn^{2+} responses are both Gal3 and ILK dependent and prevented by Src and ROCK inhibition. Downstream Gal3-, ILK-, and pCav1-dependent integrin signaling therefore mediates EGF signaling to RhoA, CDR formation, fibronectin

remodeling, and cell migration via a similar Src/ROCK-dependent pathway (Figure 8). Consistently, expression of phosphomimetic Cav1Y14D induces RhoA activation, cell migration, and FN remodeling independent of EGF, whereas a nonphosphorylatable Cav1Y14F prevents these cellular responses to EGF. Use of these Cav1 Y14 mutants further defined a role for Cav1 tyrosine phosphorylation in EGF stimulation of RhoA, CDR formation, cell migration, and FN remodeling. Based on the established role of pCav1 as an activator of RhoA signaling (Grande-Garcia *et al.*, 2007; Joshi *et al.*, 2008), this suggests that EGF promotes Gal3-dependent activation of integrin signaling, leading to pCav1-dependent RhoA activation, CDR formation, cell migration, and FN remodeling (Figure 8).

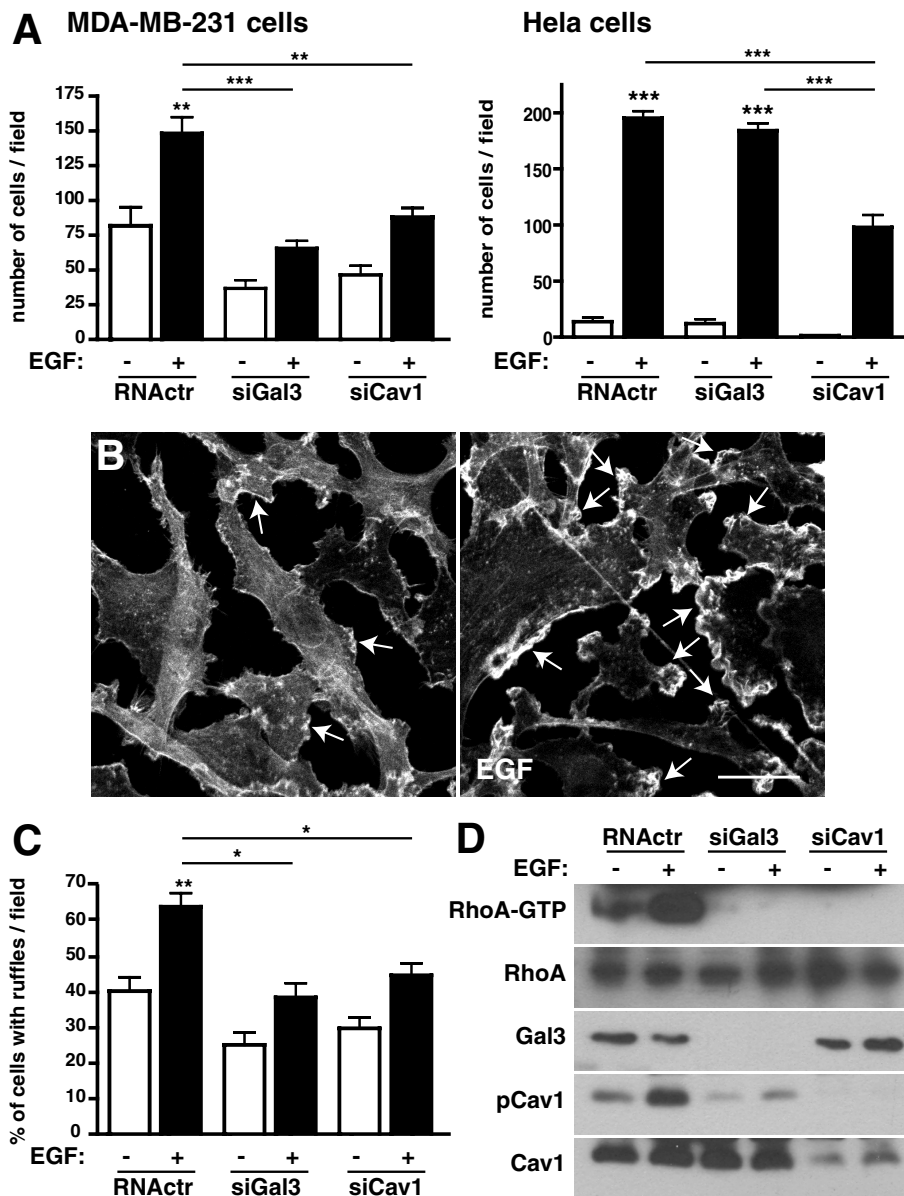


FIGURE 6: EGF induces Cav1- and Gal3-dependent cell migration, actin remodeling, and RhoA activation in MDA-MB-231 cells. For all experiments, MDA-MB-231 or HeLa cells were transfected with control (ctr) or Gal3-targeted (Gal3) or Cav1-targeted (Cav1) siRNA 48 h before 10 ng/ml EGF treatment. *** $p < 0.001$, ** $p < 0.005$, or * $p < 0.001$. (A) MDA-MB-231 or HeLa cells were plated on FN-coated Transwell chambers and treated with EGF for 5 h. The mean number of migrating cells per field is presented. (B) MDA-MB-231 cells were treated with EGF for 4 min before fixation and actin staining, revealing actin reorganization in ruffles. Cells presenting ruffles are indicated by arrows. Bar, 30 μ m. (C) The percentage of MDA-MB-231 cells forming ruffles. (D) MDA-MB-231 cells were treated with EGF for 5 min and cell lysates subjected to Rhotekin-RBD pull down. Western blot analysis revealed the amount of activated RhoA (RhoA-GTP) relative to the total amount of RhoA (RhoA), as well as Cav1, Gal3, and pCav1 expression levels.

Gal3/pCav1 and EGF induction of actin remodeling and membrane ruffling

Ruffling activity in response to growth factors has been described in many cell types and linked to cell motility. Growth factor-induced ruffles have been characterized as either PRs or CDRs based on morphology, associated signaling, or actin-binding proteins and proposed functions (Suetsugu *et al.*, 2003; Hoon *et al.*, 2012). CDR formation might correspond to the reorganization of actin from

stress fibers to lamellipodial type ruffles, thereby promoting the transition from a static to a motile state (Krueger *et al.*, 2003; Sero *et al.*, 2011; Zeng *et al.*, 2011). CDRs are also endocytic structures; CDR collapse leads to the formation of macropinosomes that internalize EGFR and integrins and might contribute to integrin recycling to the leading edge (Orth *et al.*, 2006; Gu *et al.*, 2011). The absence of Gal3 in CDR may be due to its rapid endocytosis in these cellular structures: Gal3 has been reported to internalize from the apical surface and to promote raft-dependent integrin endocytosis and endosomal trafficking (Furtak *et al.*, 2001; Delacour *et al.*, 2007; Gao *et al.*, 2012).

The role of Cav1 in CDR formation and cell migration may be specifically associated with its phosphorylation. Indeed, Gal3 stimulates Cav1 phosphorylation (Goetz *et al.*, 2008). However, whereas Cav1Y14D induces RhoA activation and prolonged CDR lifetime, it is not sufficient to induce CDRs. This might be a temporal issue: CDRs form soon after growth factor stimulation, a synchronous event that would not be observed after transfection. Furthermore, direct integrin activation with Mn^{2+} induces Cav1 phosphorylation and RhoA activation in a lattice-dependent manner but does not induce CDRs, suggesting that Cav1 phosphorylation requires an additional signal to trigger CDR formation. In human breast cancer MDA-MB-231 cells, we observed strong EGF-induced ruffling activity, corresponding mostly to PRs but also to CDRs (Gu *et al.*, 2011; Chen *et al.*, 2012). CDRs have been proposed to result from the formation of PR in non-gradient-induced cell migration (Suetsugu *et al.*, 2003). Whereas ruffling is Rac1 dependent, overexpression of active Rac1 is not enough to induce these structures, suggesting the presence of another, unknown mechanism (Suetsugu *et al.*, 2003). In many carcinoma cells, as observed for Mgat5^{+/+} and MDA-MB-231 cells, the Rho pathway is involved in ruffling activity (Nishiyama *et al.*, 1994; O'Connor *et al.*, 2000), and EGF-induced ruffles are dependent on Rho signaling (Chen *et al.*, 2012). We observed that EGF-induced PRs and CDRs are both associated with migration and dependent on Cav1 and Gal3 expres-

sion. We also show a critical role of RhoA and pCav1/Src/integrin signaling downstream of EGF in ruffle formation. EGF has been reported to activate both Rac1 and RhoA (Tamas *et al.*, 2003; Han *et al.*, 2010; Balanis *et al.*, 2011). Cav1 and Gal3 expression are strongly correlated in differentiated thyroid cancers, where they regulate focal adhesion signaling, RhoA activation, and cell migration (Shankar *et al.*, 2012). In HeLa cells, EGF-induced migration appears to be Cav1 dependent but Gal3 independent, suggesting

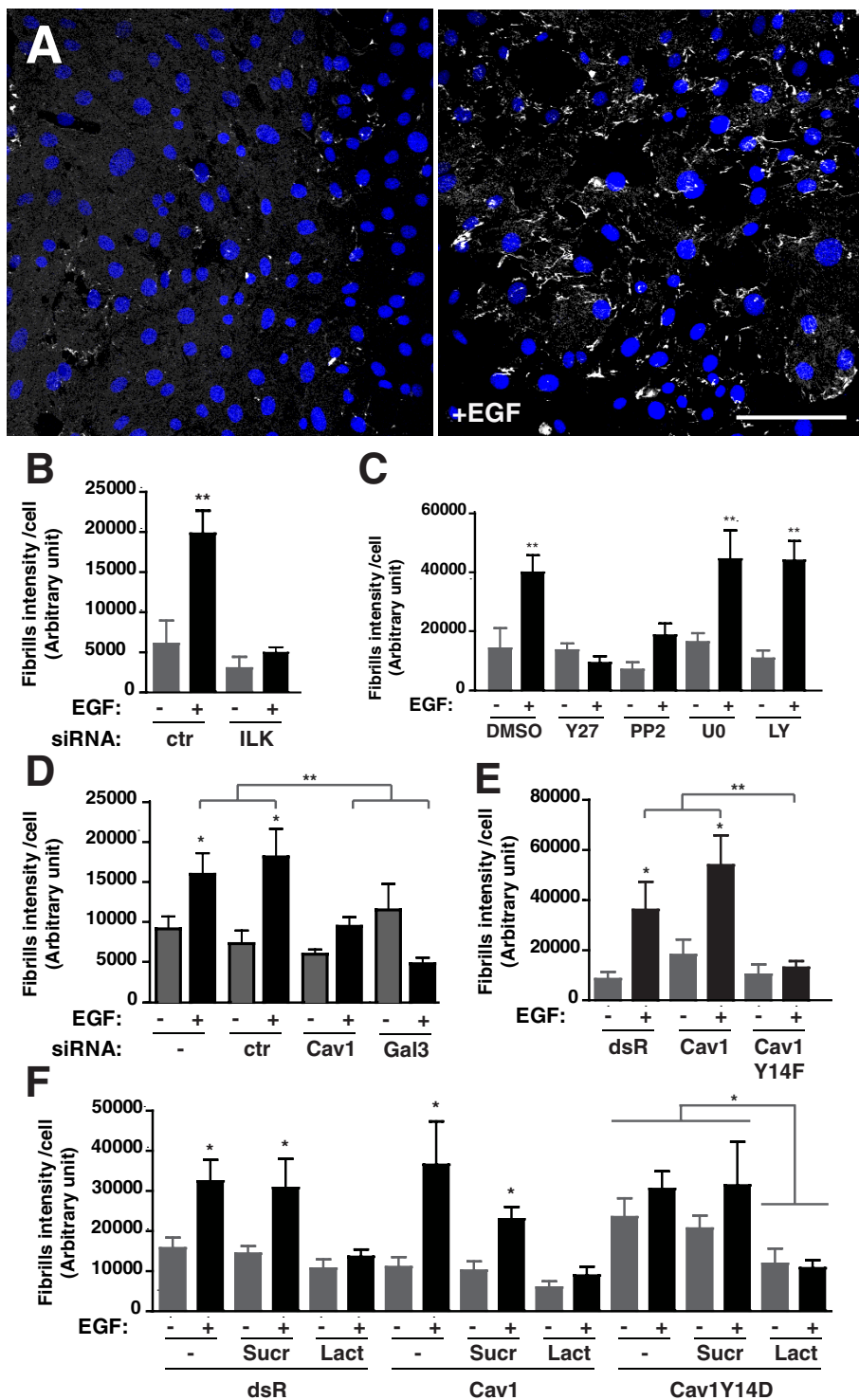


FIGURE 7: EGF-induced FN remodeling requires pCav1/ROCK/Src, Gal3 lattice, and ILK. (A) *Mgat5*^{+/+} cells were plated on FN-coated coverslips and treated with 100 ng/ml EGF for 24 h before fixation and staining. Untreated or EGF-treated (+EGF) cells, as indicated, were labeled for FN (white) and nuclei (blue), showing EGF stimulation of FN fibril formation. Bar, 200 μ m. (B) *Mgat5*^{+/+} cells were transfected with control (ctr) or ILK-targeting siRNA 24 h before EGF treatment. (C) Cells were pretreated for DMSO, 10 μ M Y27632 (Y27), 10 μ M PP2, 10 μ M U0126 (U0), or 20 μ M LY29300 (LY) before EGF treatment. (D) *Mgat5*^{+/+} cells were transfected with control (ctr) or Cav1- or Gal3-targeting siRNA 24 h before EGF treatment. (E) *Mgat5*^{+/+} cells were transfected for dsRed, Cav1-RFP, or Cav1Y14F-RFP at 24 h before addition of EGF. (F) *Mgat5*^{+/+} cells were transfected with dsRed, Cav1, or Cav1Y14D as indicated and treated or not with sucrose (Sucr; 25 mM) or lactose (Lact; 25 mM) for 48 h. For B–F, cells were labeled with anti-FN and Hoechst and the density of FN fibrils/cell quantified. ** $p < 0.01$, * $p < 0.05$.

that other factors, such as *Mgat5* expression, may regulate functionality of this signaling module. We propose that in tumor cells, the *Mgat5*-dependent Gal3 lattice enables EGF signal transduction to ILK-dependent integrin signaling that activates a pCav1/Src/RhoA positive feedback loop that drives downstream actin remodeling, cell migration, and FN remodeling (Figure 8). Coordinate Gal3 and pCav1 regulation is therefore critical for integrin-dependent transduction of the EGFR motogenic response.

EGFR, ILK, and integrin interact with Cav1 (Wary *et al.*, 1996; Couet *et al.*, 1997; Meyer *et al.*, 2005), and recruitment to the lattice sequesters EGFR away from negative inhibitory Cav1 scaffolds (Lajoie *et al.*, 2007). As observed for PDGF-induced ruffles (Berrou and Bryckaert, 2009), raft integrity is required for EGF-induced CDR and RhoA activation, and GM1 accumulates in CDRs (Figures 1 and 2). Local Gal3 and pCav1 organization of raft membrane domains may therefore contribute to EGF induction of CDRs. Indeed the presence of signaling proteins, including Src, in rafts is affected by Cav1 expression and integrity of the galectin lattice (Zheng *et al.*, 2011; Boscher *et al.*, 2012). Moreover, Cav1 regulates lipid order in focal adhesions (Gaus *et al.*, 2006), and Gal3 regulates the dynamics of both N-cadherin and GM1 ganglioside in adhesion junctions (Boscher *et al.*, 2012).

Gal3/pCav1 and FN remodeling

The signaling pathway leading to EGF-induced FN remodeling is Gal3, pCav1, ROCK, Src, and ILK dependent. This is in agreement with our previous work showing that Gal3 lattice promotes tumor cell migration, focal adhesion turnover, and FN remodeling (Lagana *et al.*, 2006; Goetz *et al.*, 2008) and with previous reports showing that FN assembly involves Rho-associated α 5 β 1-integrin centripetal translocation (Zhang *et al.*, 1997; Zhong *et al.*, 1998; Clark *et al.*, 2005). The signaling pathway observed for FN remodeling is similar to that observed for CDR formation and cell migration, suggesting that cell migration might be downstream of CDRs and lead to FN remodeling. Indeed, recruitment of MMP-2 in CDRs and the formation of CDRs in three-dimensional (3D) cultures in response to EGF suggest that these actin structures might participate in 3D motility and matrix invasion and remodeling (Suetsugu *et al.*, 2003; Orth *et al.*, 2006). The PI3K requirement for EGF-induced CDR formation and cell migration but not for FN remodeling, however, suggests that the mechanisms

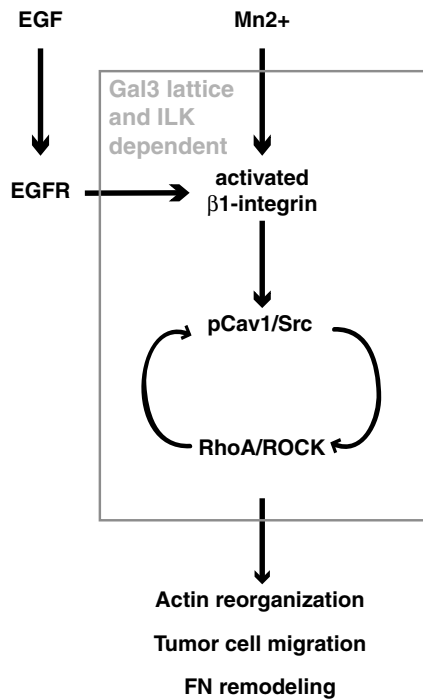


FIGURE 8: EGF induces Gal3- and ILK-dependent integrin signaling, activating the pCav1/RhoA positive feedback loop that drives CDR formation, cell migration, and FN remodeling. We propose that EGF triggers Gal3-dependent integrin activation, which induces Src-dependent phosphorylation of Cav1 and RhoA/ROCK signaling, which feeds back to promote Cav1 phosphorylation. This Gal3-integrin-pCav1 signaling module transduces EGF signaling to promote downstream CDR formation, cell migration, and FN remodeling.

driving EGF-induced CDR and cell migration are similar but not identical to those involved in EGF-induced FN remodeling.

In conclusion, we describe here a novel signaling mechanism in which the motogenic EGF signal to RhoA, CDR, cell migration, and FN remodeling is transduced by Gal3- and pCav1-dependent integrin signaling. A required role for both extracellular Gal3 and intracellular pCav1 defines coordinate outside-inside control of this integrin signaling response, which plays a critical role in mediating growth factor-stimulated tumor cell migration.

MATERIALS AND METHODS

Reagents and antibodies

Plasmids encoding Cav1-mRFP Y14 constructs were as previously described (Goetz *et al.*, 2008; Joshi *et al.*, 2008). ON-TARGETplus SMARTpool siRNA targeting Cav1, Gal3, and clathrin-heavy chain, as well as nontargeting siRNA, were purchased from Thermo Scientific Dharmacon (Lafayette, CO). siRNA targeting ILK was kindly provided by Shoukat Dedhar (University of British Columbia, Vancouver, Canada). Bovine plasma FN, sucrose, lactose, MnCl₂, RGD peptide, fluorescein isothiocyanate-coupled cholera toxin B subunit, and methyl-β-cyclodextrin were purchased from Sigma-Aldrich (St. Louis, MO). Dynasore, U0126, PP2, LY29300, and Y27632 were purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase (HRP)-conjugated mouse and rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). EGF, phalloidin, and secondary antibodies conjugated to Alexa 488, 568, or 647 were purchased from Invitrogen (Carlsbad, CA). Mouse anti-RhoA (26C4), rabbit anti-Cav1 (N20), and mouse anti-Gal-3 (B2C10) antibodies were purchased from Santa Cruz Biotechnology (Santa

Cruz, CA). Mouse anti-pY14Cav1 and rabbit anti-cortactin (H222) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-ILK (6G6.3), rat anti-mouse β1-integrin (MAB1997), and mouse anti-Rac1 (23A8) antibodies were purchased from Millipore (Billerica, MA). Mouse anti-FN was purchased from BD Biosciences (San Diego, CA).

Cell culture, transfection, and cell treatments

Mgat5^{+/+}, Mgat5^{-/-}, and Mgat5^{-/-Res} (rescued: Mgat5^{-/-} cells rescued with retroviral expression of Mgat5) cells were maintained in 10% fetal bovine serum (FBS) containing DMEM (Invitrogen) as previously described (Lagana *et al.*, 2006). MDA-MB-231 and HeLa cells were cultured in RPMI or DMEM, respectively, containing 10% FBS. For transfection, cells were spread 24 h before on FN-coated plates or plastic plates. Lipofectamine 2000 (Invitrogen) was mixed in OptiMEM (Invitrogen) with siRNA or DNA at a 1:1 ratio and added to the cells for 4 h. Experiments were performed after 48 h, and transfection efficiency was verified by Western blot or immunofluorescence. Cells were treated with 25 mM sucrose, 25 mM lactose, or 100 μg/ml RGD peptide continuously for 48 h before treatment or treated with 75 μM Dynasore, 10 μM U0126, 10 μM PP2, 10 μM LY29300, or 10 μM Y27632 for 30 min before EGF or Mn²⁺ treatment.

CDR and FN fibril formation assay, immunofluorescence, and image acquisition

Cells were spread on coverslips coated with a solution of 10 μg/ml FN as previously described (Lagana *et al.*, 2006). After 4 h in complete medium, cells were transfected and switched to serum-free medium. For CDR formation, cells were treated after 48 h with EGF for 8 min before fixation in 3% paraformaldehyde (PFA). For FN fibril formation, cells were treated with EGF for 18 h before fixation. After washing, cells were permeabilized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and preparations blocked with PBS containing 1% BSA. Primary and secondary antibodies and phalloidin were incubated for 1 h in the same solution before mounting in Gelvatol. For quantification, 10 independent fields were acquired for each condition using equivalent settings with a 60x PlanApo objective of an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan) or an A1R-A1 confocal Nikon microscope (Nikon, Melville, NY). The percentage of cells with CDR was calculated, and statistical analyses were performed on Prism 4 (GraphPad, San Diego, CA) using an unpaired *t* test (two tailed with a confidence interval of 95%). For FN fibril quantifications, the same threshold was applied to each image, and the FN intensity was determined relative to the number of cells in each image. The 3D reconstruction was made from confocal stacks (*z* = 0.1 μm step) using ImageJ (National Institutes of Health, Bethesda, MD).

Cell migration assay

We plated 50,000 Mgat5^{+/+} cells on FN-precoated well chamber and treated them after 2 h with chemical inhibitors for 30 min before adding 100 ng/ml EGF in the bottom chamber. After 5 h, cells in the top part of the chamber were gently removed with a swab and cells on the bottom fixed in PFA and stained with 0.1% crystal violet solution. Ten independent fields were manually counted using a 20x objective for statistical analysis.

RhoA pull-down assay and Western blot

We treated 100-mm plates containing cells at 90% confluency with EGF or Mn²⁺ and extracted them in Mg²⁺ Lysis/Wash Buffer

(Millipore). Cell extracts were submitted to pull down with glutathione–agarose beads coupled with Rhotekin-RBD or PAK1-PBD, according to the manufacturer's protocol (Millipore). The total pull down and 2% of the total extract were analyzed by Western blot for RhoA or Rac1. For Western blot of tyrosine-phosphorylated Cav1, cells were extracted in 50 mM Tris, pH 7.5, buffer containing 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 150 mM NaCl, 1% SDS, 1% Triton-X100, 1 mM NaVO₃, 50 mM NaF, 2 mM NaPPi, and a cocktail of protease inhibitors (Roche, Indianapolis, IN), and cell extracts were Western blotted with antibody to pCav1. Briefly, cell extracts were loaded on 13% acrylamide gels and proteins were transferred on polyvinylidene fluoride membrane, blocked in PBST (1% Tween) containing 5% milk, and incubated with primary antibodies in PBST overnight. Membranes were probed with HRP-coupled secondary antibodies and revealed with enhanced chemiluminescence (Millipore).

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