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ORIGINAL ARTICLE

PIPKIγ and talin couple phosphoinositide and adhesion signaling to control the epithelial to mesenchymal transition

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Epithelial cells acquire migratory/invasive and stemness traits upon conversion to the mesenchymal phenotype. The expression of E-cadherin is a key to this transition; yet precise understanding of the pathways involved in integrating E-cadherin loss to the gain of mesenchymal traits remains poorly understood. Here, we show that phosphoinositide-generating enzyme, PIPKIγ, expression is upregulated upon epithelial–mesenchymal transition (EMT) and together with the cytoskeletal protein talin assemble into a signaling complex upon E-cadherin loss. PIPKIγ and talin together control the adhesion and phosphoinositide signaling that regulates conversion to the mesenchymal phenotypes. PIPKIγ and talin regulate the stability of E-cadherin transcriptional repressors, snail and slug, induced by transforming growth factor-β1 or extracellular matrix protein. Loss of PIPKIγ or talin or their interaction impaired EMT and the acquisition of cell motility and stemness. This demonstrates a mechanism where a phosphoinositide-generating enzyme PIPKIγ couples with a cytoskeletal protein talin to control the acquisition of mesenchymal phenotypes.

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INTRODUCTION

A prevailing theme in cancer specifies that the loss of E-cadherin-mediated adherens junctions and acquisition of migratory/invasive traits in conjunction with self-replicating stemness property determine the success of tumor metastasis.[1–4] Epithelial–mesenchymal transition (EMT) is a normal embryonic development program often hijacked by metastasizing tumor cells, whereby tumor cells acquire different traits required for metastasis.[3,4] However, the precise understanding of signaling molecules that couple E-cadherin loss to gain of migratory/invasive and stemness traits remains poorly understood.[1] Uncovering the role of molecules and signaling pathways that are involved is key to the development of effective therapeutic approaches in cancer treatment as the majority of carcinomas originate from epithelial cells.[3,5] Arguably, the signaling pathways commonly deregulated in cancer are responsible for orchestrating these processes, thus provoking us to interrogate the role of molecules in phosphoinositide signaling.

Phosphatidylinositol-4,5-bisphosphate (PIP_2) is a lipid messenger and a substrate for the generation of other messengers (PIP_3, DAG and IP_3), all of which regulate cell polarity and motility.[6,7] PIP_2 is synthesized by type I phosphatidylinositol 4-phosphate kinase (PIPKI) enzymes encoded by three genes in mammalian cells, PIPKIα, PIPKIβ and PIPKIγ.[8,9] In epithelial cells, different splice variants of PIPKIγ colocalize and associate with E-cadherin at adherens junctions, and they also regulate E-cadherin trafficking and epithelial morphogenesis.[10–12] PIPKIγ is also found over-expressed in triple-negative breast cancer,[13] as it regulates cell migration/anchorage-independent growth of tumor cells[14–17] and functions as a proximal regulator of PI3K/Akt signaling.[18] PIPKIγi2, a focal adhesion targeting variant of PIPKIγ, interacts with talin and regulates adhesion signaling by generating PIP_2 that modulates the assembly of adhesion complexes.[19,20] Talin, an FERM-domain containing cytoskeletal protein, is the structural and functional unit of integrin-mediated adhesion complexes that mediate 'inside-out' and 'outside-in' signaling at cell–matrix interaction sites.[21] Although EMT is accompanied by a profound increase in adhesive and migratory activity of the transitioning cells, roles for talin and PIPKIγ in EMT are not defined.

Here, we show that upon E-cadherin loss, PIPKIγ couples with talin to form a signaling complex that regulates the adhesion-stimulated PI3K/Akt signaling required for epithelial cells undergoing EMT. PIPKIγ/PIPKIγi2 expression and PI3K/Akt signaling were increased in mesenchymal cells induced by transforming growth factor-β1 (TGFβ1) treatment. The integrity of PIPKIγ and talin complex was required for the stability of E-cadherin transcriptional repressors and the gain of mesenchymal traits, highlighting the integrative role of adhesion and PI3K/Akt signaling in EMT. The assembly of PIPKIγ/PIPKIγi2 with talin and their collaborative functions provide the signaling platform for the regulation of PI3K/Akt signaling downstream of extracellular matrix (ECM) proteins and growth factors. These are required for the stability of EMT-regulating transcription factors and the maintenance of mesenchymal phenotypes, including cell motility and stemness properties. This demonstrates that E-cadherin loss in EMT is coupled with the assembly of PIPKIγ and talin for regulation of adhesion and PI3K/Akt lipid signaling required for gain of mesenchymal phenotypes.

RESULTS

Mesenchymal cells displays increased PI3K/Akt signaling

Epithelial cells acquire properties essential for cancer progression upon transition into the mesenchymal state.[3] We used the EMT model of murine mammary epithelial cells, NMuMG, that can be progressively transformed into mesenchymal state by TGFβ1 treatment or by culturing on ECM protein or E-cadherin knockdown as illustrated in this study. EMT was assessed by loss of epithelial markers and increased expression of mesenchymal marker proteins (Figure 1a) and change in cell morphology (e.g.

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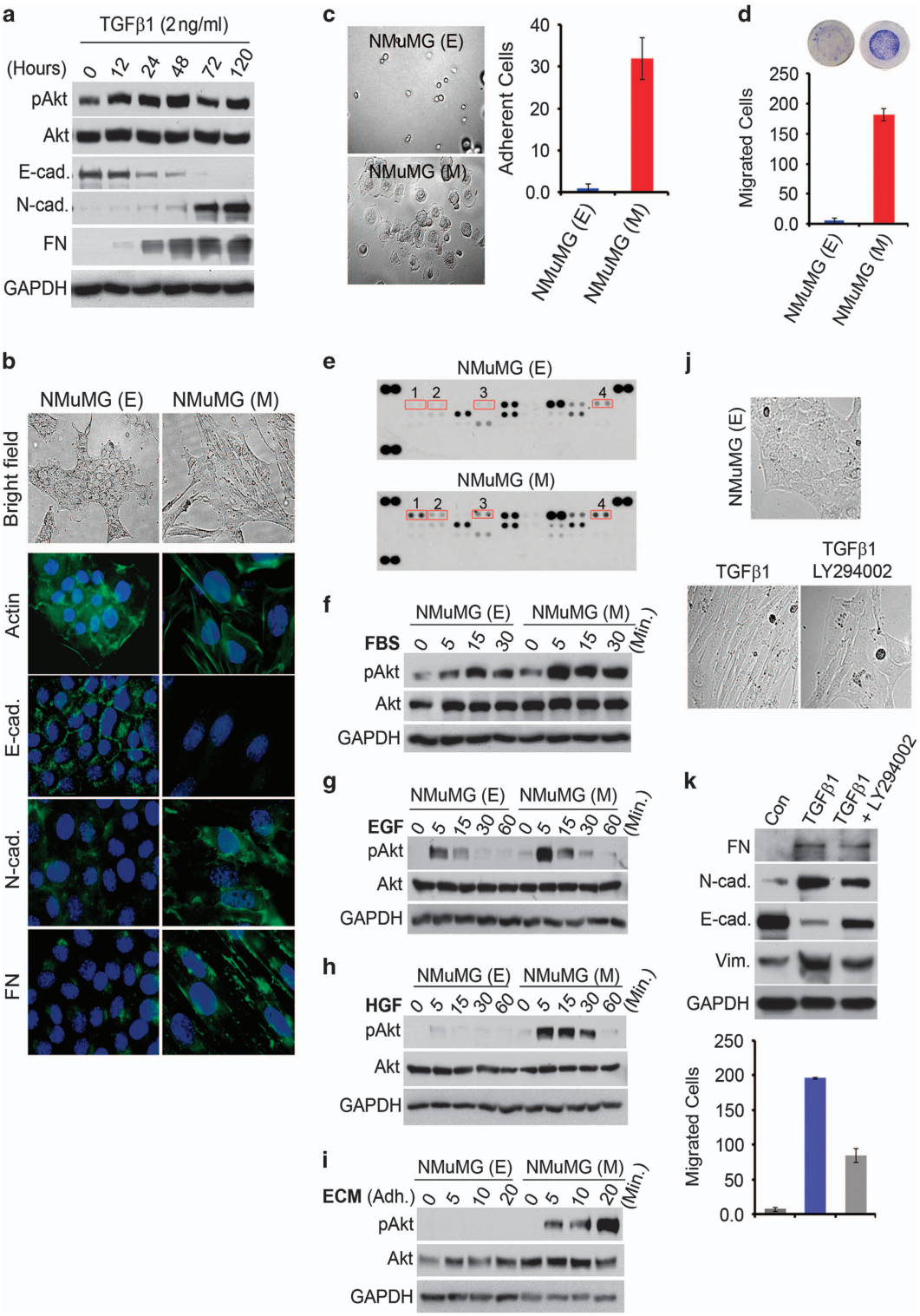
loss of organized compact cell islands and gain of front–back polarity) (Figure 1b). The progressive changes in the morphology of NMuMG cells undergoing EMT upon TGFβ1 treatment is demonstrated in Supplementary Figure S1. Consistent with previous studies[3,5] epithelial cells converted into mesenchymal state showed dramatically increased adhesive and migratory activity (Figures 1c and d).

Next, the activation level of signaling molecules in epithelial vs mesenchymal cells were interrogated by an array kit, which detects the phosphorylation levels of 26 different kinases. As shown in Figure 1e, the phosphorylation level of molecules in the phosphoinositide signaling pathway (e.g. Akt1, total Akt and GSK3β) was significantly higher in mesenchymal cells over epithelial cells. We also demonstrated the upregulated activation level of Akt during TGFβ1-induced EMT (Figure 1a). This was further confirmed by demonstrating the higher activation level of Akt in mesenchymal cells in response to fetal bovine serum (FBS) or growth factor (e.g. epidermal growth factor (EGF), hepatocyte growth factor (HGF))



stimulation of the cells (Figures 1f–h), and upon adhesion to ECM protein (Figure 1i). This indicates that EMT is accompanied by increased PI3K/Akt lipid signaling. Consistently, the inhibition of PI3K/Akt signaling (e.g. by PI3K inhibitor) impaired the transition of epithelial cells into mesenchymal cells and acquisition of cell motility (Figures 1j and k), indicating that PI3K/Akt signaling is an integral part of the EMT process.[22,23]

PIPKIγ expression is increased in mesenchymal cells

After demonstrating the increased PI3K/Akt lipid signaling in mesenchymal cells and its role in EMT, the expression levels of type I PIPKI and PI3K enzymes during EMT were examined. As shown in Figure 2a, the expression level of PIPKIγ and its focal adhesion targeting variant, PIPKIγi2, showed a progressive increase, whereas PIPKIα progressively decreased during TGFβ1-induced EMT. However, no obvious changes were observed in the expression level of PIPKIβ. Although the expression levels of PIPKIα and PIPKIγ appeared inversely related, their precise role in the regulation of EMT remains undefined. The expression level of PI3K or PTEN also remained largely unchanged. PIPKIγ knockdown affected the phosphorylation level of the different molecules in the phosphoinositide signaling pathway (e.g. Akt1, total Akt and GSK3β) as indicated by the array kit (Figure 2b). This was further substantiated by demonstrating impaired activation level of Akt in PIPKIγ knocked down cells (Figure 2c), and is consistent with a previous study.[18] Furthermore, the expression level of ectopically expressed PIPKIγi2 and PIPKIγi4 splice variants was upregulated in NMuMG cells upon induction of EMT by TGFβ1 treatment (Supplementary Figure S2A), indicating post-transcriptional control of PIPKIγ expression during EMT.

In addition to TGFβ1, ECM proteins and growth factors (e.g. EGF and HGF) are common regulators of phosphoinositide signaling and EMT.[5,24,25] NMuMG cells cultured into type I collagen (Col. I)-coated culture plates in the presence or absence of EGF or HGF demonstrated a conversion into the mesenchymal state, which was also accompanied by strongly induced PIPKIγ expression (Figures 2d and e). Upregulated expression of PIPKIγ was also observed in MDA-MB-231 and SUM159 breast cancer cells upon culturing into ECM protein-coated culture plates or treatment with TGFβ1 (Supplementary Figure S2B). As PIPKIγ and the PIP_2 lipid messenger are integral parts of multiple cellular functions, PIPKIγ appeared important for EMT as loss of PIPKIγ diminished EMT (Figures 2f and g), consistent with a loss of Akt activation (Figure 2c).

PIPKIγ displays increased association with talin and other pro-migratory molecules during EMT

During EMT, epithelial cells lose E-cadherin-mediated cell–cell adherens junction and simultaneously gain increased interactions with ECM proteins.[3,5] Different variants of PIPKIγ, including PIPKIγi2, associate with E-cadherin[10] and are localized at cell–cell contact sites of adherens junctions in epithelial cells (Supplementary Figure S3). PIPKIγi2 also associate with talin, and is localized at integrin-mediated cell–matrix interaction sites, focal adhesions.[19] Upon transition of these cells into mesenchymal phenotype by TGFβ1 treatment, PIPKIγi2, but not PIPKIγi1, becomes extensively localized at the interface of cell–matrix interaction sites along with talin (Figure 3a). Furthermore, with a decreased expression level of E-cadherin and loss of PIPKIγi2 interaction with E-cadherin during EMT, PIPKIγi2 showed a progressive increase in its association with various pro-migratory and pro-invasive molecules, including talin, N-cadherin, Src and IQGAP1 (Figure 3b).

Among the PIPKIγvariants, PIPKIγi2 showed the strong association with talin, N-cadherin, moesin and Src upon EMT (Figure 3c). This was further demonstrated in NMuMG cells transitioning into mesenchymal cells by culturing into the ECM proteins (Figure 3d). E-cadherin, N-cadherin, talin, IQGAP1 and Src are previously identified interacting partners of PIPKIγ/PIPKIγi2.[10,14,17] Moesin, an FERM-domain containing protein is involved in cell polarity.[26] Moesin was identified by mass spectrometry in the immunocomplex of PIPKIγi2 isolated from NMuMG cells induced to mesenchymal state by TGFβ1 treatment and associates with PIPKIγi2 via talin (Supplementary Figures S4A and B). However, unlike pan-PIPKIγ, the knockdown of PIPKIγi2 alone was not sufficient to block EMT induced by TGFβ1 treatment, indicating the collective role of PIPKIγ variants in the EMT process (Figure 3e). Alternatively, a less efficient knockdown of PIPKIγi2 might have also contributed to the lack of effect on TGFβ1-induced EMT.

Remarkably, the knockdown of talin, a key cytoskeletal protein at integrin-mediated adhesion complex, diminished EMT induced by TGFβ1 (Figure 3e) and more significantly by ECM proteins (see below). In NMuMG cells fully converted into mesenchymal state by TGFβ1 treatment, the knockdown of PIPKIγ or PIPKIγi2 or its associating partners (such as talin and Src) significantly impaired cell migration, one of the key phenotypes of mesenchymal cells (Figures 3f and g). Consistently, all of these molecules are also known to play essential role in cell migration of different tumor cells.[14–16] These results indicate that upon the loss of PIPKIγ interaction with E-cadherin, PIPKIγ shows increased association with talin and other pro-migratory molecules to regulate EMT and gain of cell motility.

E-cadherin loss promotes PIPKIγ association with talin and PI3K/Akt signaling

E-cadherin and talin, cornerstones of cell–cell adherens junctions and integrin-mediated adhesion complexes, respectively, are the direct interacting partners of PIPKIγ/PIPKIγi2, which appear to shuttle between these two molecules in epithelial and mesenchymal cells as indicated in Figure 3a. Consistently, talin knockdown promoted PIPKIγi2 association with E-cadherin (Figure 4a). Conversely, E-cadherin knockdown promoted PIPKIγi2 association

Figure 1. EMT is associated with increased PI3K/Akt signaling. (**a**, **b**) NMuMG cells cultured into complete growth medium were treated with TGFβ1 (2 ng/ml) before harvesting the cells at different time points. For culturing the cells for more than 3–4 days, cells were subcultured into new culture plates and the TGFβ1 concentration reduced to half (1 ng/ml). Transition to mesenchymal state was examined by downregulation of E-cadherin expression and gain of mesenchymal marker proteins by immunoblotting (**a**) and immunofluorescence study/change in cell morphology (**b**) (images taken using ×10 and ×40 objective lenses). (**c**, **d**) NMuMG cells in epithelial state, NMuMG (**e**) or after conversion into mesenchymal state by TGFβ1 treatment for 3–4 days, NMuMG (M) were cultured in serum- and insulin-free medium overnight before adhesion and migration assays as described in 'Materials and methods'. For cell adhesion, attached cells to Col. I-coated culture plates after 15- min of incubation were counted. For cell migration, migrated cells towards underside of membrane after 5–6 h of incubation were stained and counted (results are represented as mean ± s.d. from three independent experiments and error bars represents s.d.). (**e**) The activation of cellular signaling in NMuMG (E) or NMuMG (M) were analyzed by the MAPK array kit following the protocol provided by the manufacturer (1, Akt1; 2, Akt2; 3, total Akt; 4, GSK3β). (**f–i**) Examination of Akt activation in response to FBS or growth factors (e.g. EGF, HGF) stimulation of the cells, and upon adhesion to ECM protein. NMuMG (E) or NMuMG (M) cells were cultured in serum- and insulin-free medium overnight before cell stimulation with indicated factors. Cells were harvested at different time points for examining the activation level of Akt by immunoblotting. (**j**, **k**) NMuMG cells were treated with PI3K inhibitor (LY294002) along with TGFβ1 (2 ng/ml) and cultured for 2-days. The effect of PI3K/Akt inhibition on EMT was examined by gain of mesenchymal marker proteins and cell motility. Results are represented as mean ± s.d. from three independent experiments and error bars represents s.d.

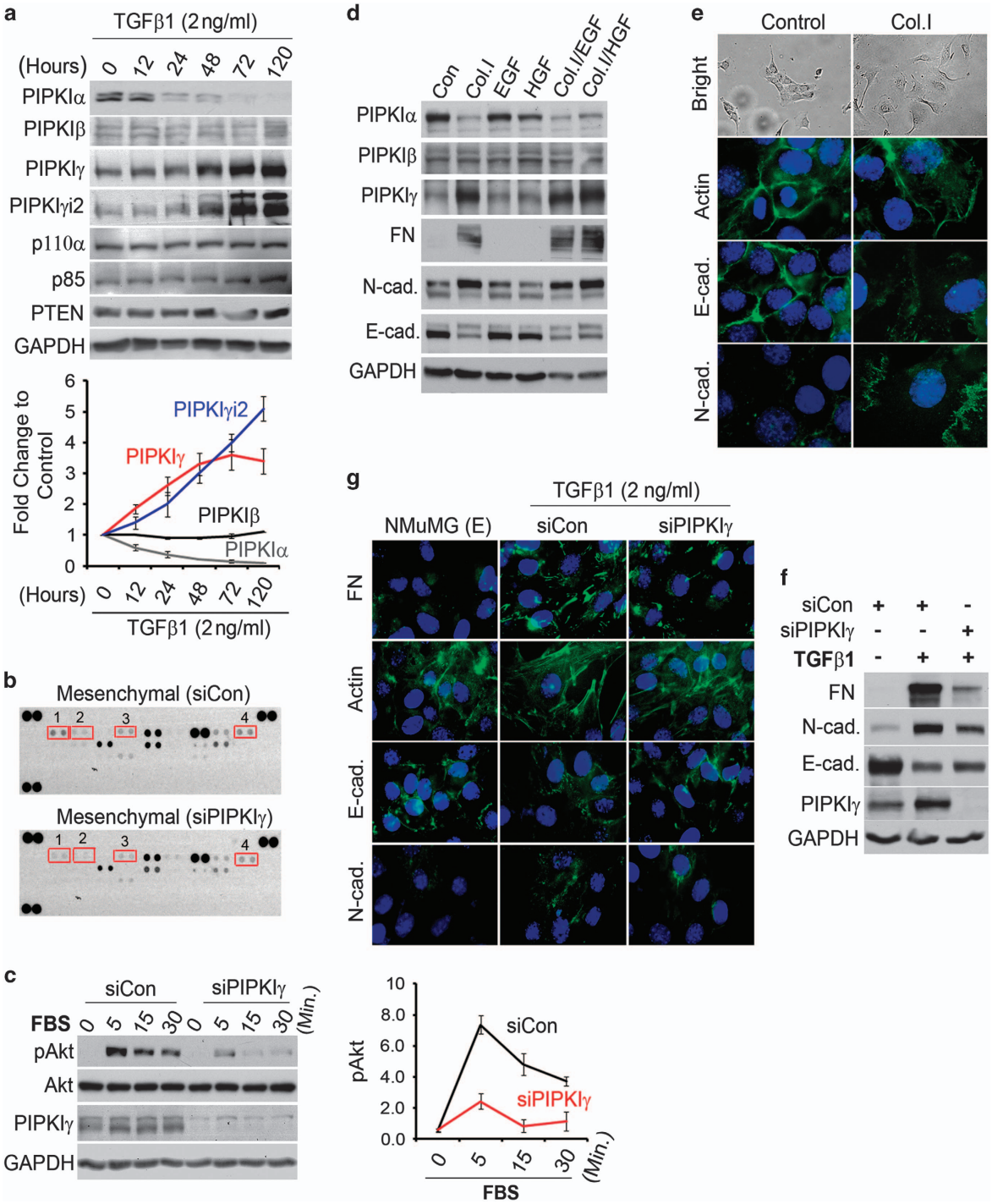


Figure 2. PIPKIγ expression is increased during EMT. (**a**) The expression levels of type I PIPKI (PIPKIα, PIPKIβ and PIPKIγ) and PI3K enzymes in NMuMG cells during TGFβ1-induced EMT were examined. Cells were harvested at different time points following TGFβ1 treatment and immunoblotted with specific antibodies. (**b**) siRNA was used to knockdown PIPKIγ from the NMuMG cells transformed into mesenchymal state by TGFβ1 treatment. Then, the effect of PIPKIγ knockdown on cellular signaling was examined by the MAPK array kit as described above (1, Akt1; 2, Akt2; 3, total Akt; 4, GSK3β). (**c**) NMuMG cells converted into mesenchymal cells by TGFβ1 treatment were transfected with siRNA for PIPKIγ knockdown. At 48–72 h after transfection, cells were cultured in serum- and insulin-free medium overnight before stimulating with 10%FBS. The cells were harvested at different time points and the activation level of Akt examined by immunoblotting using phosphor-Akt antibody. (**d**, **e**) The examination of PIPKIγ expression in NMuMG cells converted into mesenchymal cells by ECM protein. Cells were cultured into type I collagen (Col. I)-coated culture plates in the presence or absence of EGF or HGF for 2–3 days before harvesting for the expression of mesenchymal marker proteins by immunoblotting or examination by immunofluorescence study (image taken at ×40; bright field at ×10). (**f**, **g**), the effect of PIPKIγ knockdown on TGFβ1-induced EMT and gain of cell motility. At 24–36 h post-transfection with siRNA for the knockdown of PIPKIγ, NMuMG cells were treated with TGFβ1. Induction of EMT was assessed 36–48 h later either by immunoblotting for the expression of mesenchymal marker proteins or by immunofluorescence study (image taken at ×40).

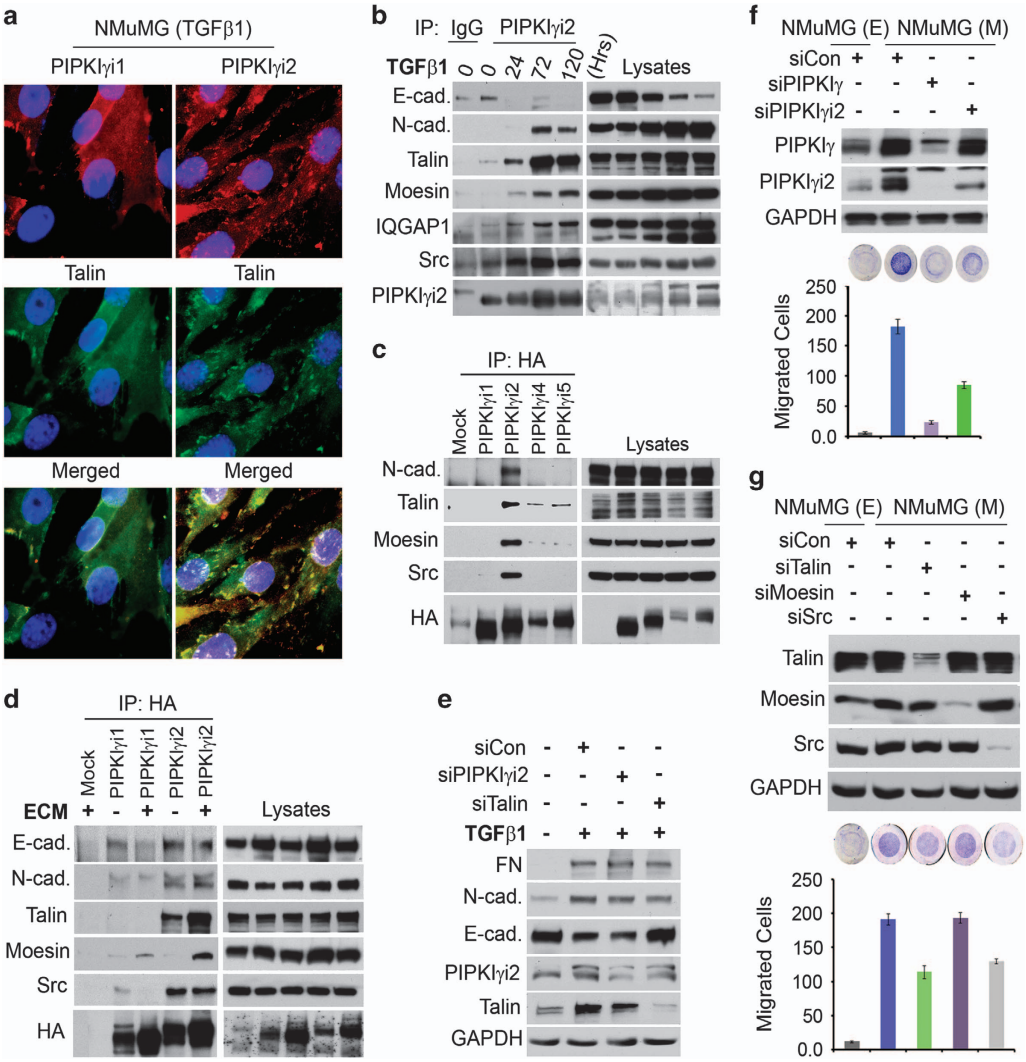


Figure 3. PIPKIγ displays increased association with talin and other pro-migratory molecules during EMT. (**a**) Localization of HA-tagged and ectopically expressed PIPKIγi1 and PIPKIγi2 in epithelial and mesenchymal cells were examined by immunofluorescence study. Upon induction of EMT, PIPKIγi2 showed extensive localization at cell–matrix interaction sites along with talin. (**b**) Endogenous PIPKIγi2 was immunoprecipitated from NMuMG cells at different time points following TGFβ1 treatment and associated molecules (e.g. talin, N-cadherin, moesin, IQGAP1 and Src) examined by immunoblotting. (**c**) NMuMG cells expressing different variants of HA-tagged PIPKIγ were transformed into mesenchymal state by TGFβ1 treatment. PIPKIγ was immunoprecipitated using anti-HA antibody and associated molecules were examined by immunoblotting. (**d**) NMuMG cells expressing different variants of HA-tagged PIPKIγ were transformed into mesenchymal cells by culturing into Col. I-coated culture plates. PIPKIγ was immunoprecipitated using anti-HA antibody and associated molecules were examined by immunoblotting. The increased association of PIPKIγi2 with talin, N-cadherin, moesin and Src was observed. (**e**), NMuMG cells were treated with TGFβ1 for 36–48 h after siRNA transfection for knockdown of PIPKIγi2 or talin. Cells were harvested 48 h after TGFβ1 treatment. The conversion of NMuMG cells into mesenchymal state was assessed by examining the expression level of mesenchymal marker proteins. The knockdown of PIKIγi2 unlike talin was not sufficient to impair EMT induced by TGFβ1 treatment. (**f**, **g**), PIPKIγ or PIPKIγi2 or their associating partners (e.g. talin and Src) were knocked down using siRNA in NMuMG cells fully converted into mesenchymal state by TGFβ1 treatment. Cells were cultured in serum- and insulin-free medium overnight before cell migration assay. Results are represented as mean ± s.d. from three-independent experiments and error bars represent s.d.

with talin (Figure 4b), and PIPKIγi2 was extensively reorganized to the leading edge adhesion complexes along with talin in E-cadherin knocked down cells (Figure 4c). These results indicate that E-cadherin loss promotes the assembly and reorganization of PIPKIγ/PIPKIγi2 with talin in transitioning epithelial cells.

The loss of E-cadherin is a hallmark of EMT[5,27] and the knockdown of E-cadherin was sufficient to convert NMuMG cells from epithelial into mesenchymal state, which was further substantiated by culturing into Col. I-coated culture plates (Figure 4d). However, knockdown of talin and PIPKIγ/PIPKIγi2 abrogated the cell motility gained by E-cadherin knockdown cells (Figure 4e). Furthermore, E-cadherin knockdown cells acquired increased PI3K/Akt signaling, which was severely impaired upon PIPKIγ and talin knockdown (Supplementary Figures S5A–D), suggesting that the PIPKIγ and talin assembly provides a platform for the regulation of PI3K/Akt signaling in mesenchymal cells. As expected, the pharmacological inhibition of PI3K blocked cell motility and the expression of mesenchymal marker proteins in the E-cadherin knockdown cells (Figures 4f and g). Together, these results indicate that mesenchymal traits acquired by epithelial cells upon E-cadherin loss depend upon the integrity of the talin and PIPKIγ/PIPKIγi2 complex assembled in the vicinity of the cell–matrix interface, and their signaling.

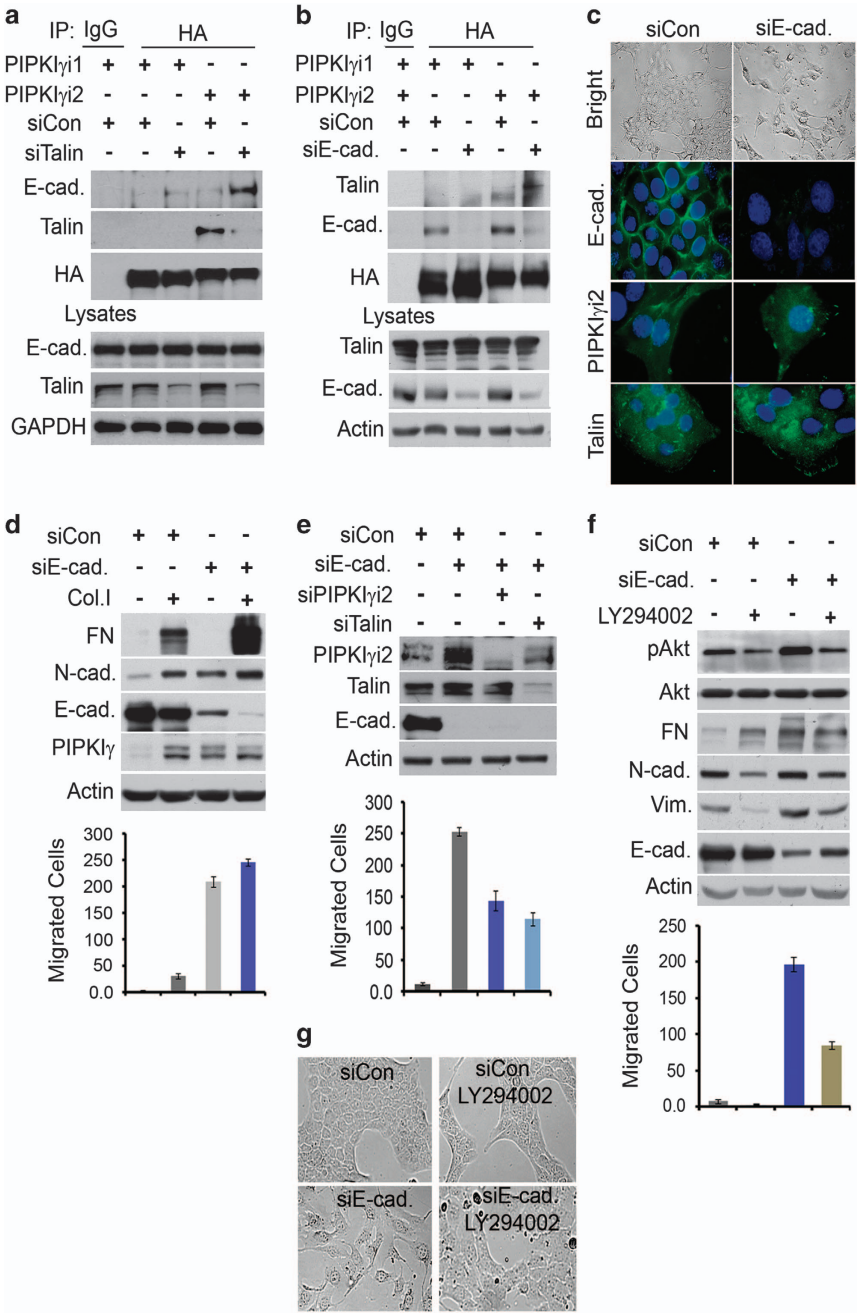


Figure 4. E-cadherin loss promotes PIPKIγ association with talin and PI3K/Akt signaling. (**a**) PIPKIγi2 was immunoprecipitated from control or talin knockdown cells, and co-immunoprecipitation of E-cadherin with PIPKIγi2 was examined by immunoblotting. (**b**, **c**) PIPKIγi2 was immunoprecipitated from NMuMG cells after E-cadherin knockdown and co-immunoprecipitation of talin with PIPKIγi2 was examined. The localization of PIPKIγi2 and talin in E-cadherin knockdown cells was examined by immunofluorescence study. E-cadherin knockdown induced extensive reorganization of PIPKIγi2 to leading edge adhesion complexes along with talin. (**d**) siRNA was used to knockdown E-cadherin expression in NMuMG cells. Cells were harvested 48–72 h post-transfection to examine the expression level of mesenchymal marker proteins (fibronectin and N-cadherin). The cell motility was examined as described above. The knockdown of E-cadherin was sufficient to promote EMT of NMuMG cells and gain of cell motility. The culture into Col.I-coated culture plates further substantiated the EMT process. (**e**) NMuMG cells were co-transfected with siRNA for knockdown of E-cadherin along with talin or PIPKIγ or PIPKIγi2. At 48–72 h after transfection, cell motility was assessed as described above. However, cell motility gained by E-cadherin knockdown was abrogated upon talin or PIPKIγ/PIPKIγi2 knockdown. (**f**, **g**) NMuMG cells were transfected with siRNA for the knockdown of E-cadherin. Cells were treated with DMSO or LY294002 compound 36–48 h post-transfection, and allowed to grow for another 24–36 h. The activation level of Akt and expression level of mesenchymal marker proteins were examined by immunoblotting. Cell motility was examined as described above. Mesenchymal phenotype and cell motility gained by E-cadherin knockdown cells were significantly compromised upon inhibition of PI3K/Akt signaling by LY294002 compound. Results are represented as mean ± s.d. from three-independent experiments and error bars represent s.d.

PIPKIγ and talin interaction is required for EMT

During EMT, epithelial cells lose E-cadherin-mediated cell–cell adherens junctions and simultaneously gain the increased adhesive interaction with ECM proteins through the integrin-mediated adhesion complex.[2,5] Talin, a cytoskeletal protein in the cytosolic compartment of the integrin-mediated adhesion complex, provides the platform for bidirectional signaling ('outside-in' and 'inside-out').[21,28] The interaction of cells with ECM proteins and derived cellular signals appears pivotal for acquisition of mesenchymal traits as knockdown of talin abrogated the transition of epithelial cells into mesenchymal state induced by ECM proteins (Figures 5a and b) or E-cadherin knockdown (Figures 5c and d). Talin knockdown cells appeared similar to epithelial NMuMG control cells and the expression level of E-cadhein and N-cadherin was similar to that of epithelial control cells, although the expression level of FN was higher. Similarly, EMT induced by E-cadherin knockdown was also effectively blocked by the knockdown of talin as indicated by lack of induced N-cadherin expression and impaired cell motility (Figure 4e), although, the expression level of FN was subtly affected. In contrast, moesin knockdown promoted EMT, which was accompanied by increased talin association with integrin (Figure 5e), suggesting that moesin controls EMT by regulating talin assembly with integrins at adhesion complexes, which is in agreement with reported study.[29] Furthermore, the knockdown of talin, but not moesin, promoted reversion of NMuMG cells from mesenchymal (after TGFβ1 treatment) into the epithelial state (Figures 5f and g). These results indicate that talin, a cytoskeletal protein at integrin-mediated adhesion complex, plays crucial role in the initiation of EMT as well as in the maintenance of mesenchymal phenotypes.

Next, we tried to define the functional role of talin interaction with PIPKγ in EMT and the acquisition of mesenchymal traits as talin association with PIPKIγi2 is significantly increased during EMT. Furthermore, the lipid messenger PIP_2 regulates the recruitment and activation of talin at the adhesion complex required for bidirectional signaling.[30] The PIP_2 is putatively provided by PIPKIγi2 associated with talin[16,19,21] (Figure 5h). To explore this, GFP-fusion proteins of the F2 or F3 subdomains of talin FERM domain were constructed and expressed into NMuMG cells by lentiviral infection. The F3 subdomain harbors the binding sites for PIP_2, PIPKIγi2 and integrins.[20,21] As expected, the expression of talin F3 subdomain abrogated the co-immunoprecipitation between talin and PIPKIγi2 (Supplementary Figures S6A and B). Furthermore, when NMuMG cells expressing F2 or F3 subdomains of talin FERM domain were induced to undergo EMT by E-cadherin knockdown, the expression of talin F3 subdomain completely blocked talin association with PIPKIγi2, this also impaired adhesion (as indicated by phosphorylation level of FAK) and PI3K/Akt lipid signaling (Figure 5i). These cells showed defects in acquiring mesenchymal traits as indicated by impaired N-cadherin expression and cell motility (Figure 5j). The expression of talin F3-subdomain also impaired adhesion and PI3K/Akt lipid signaling as well as cell motility of highly aggressive MDA-MB-231 tumor cells (Supplementary Figures S6C and D). These results indicate that talin interaction with PIPKIγ is required for gain of mesenchymal properties in transitioning epithelial cells.

PIPKIγ and talin regulate the stability of snail and slug

Different agonists inducing EMT, such as TGFβ1, ECM proteins, growth factors, WNT and NOTCH, converge to inducing E-cadherin transcriptional repressors (e.g. snail, slug and twist) to orchestrate EMT.[5,31,32] EMT induced by ECM or TGFβ1 in NMuMG cells is associated with increased expression levels of snail and slug (Figure 6a). Consistently, knockdown of snail and/or slug severely impaired the induction of EMT by TGFβ1 treatment (not shown). These transcriptional repressors are short-lived proteins requiring cellular signaling emanating from plasma membrane for their stability[31] and PI3K/Akt signaling regulates the stability of these transcriptional repressors by inhibiting the GSK3β, which promotes ubiquitination and proteasomal degradation of these transcriptional repressors.[31,33] This suggests that PIPKIγ and talin might regulate the stability of these E-cadherin transcriptional repressors during EMT. In alignment with this notion, the expression level and half-life of snail and slug, but not β-catenin, were dramatically reduced upon PIPKIγ or talin knockdown in NMuMG cells transformed into mesenchymal state by TGFβ1 treatment (Figure 6b). However, the treatment of cells with proteosomal inhibitor but not lysosomal inhibitors (not shown) completely replenished the expression level of snail and slug in talin or PIPKIγ knockdown cells (Figures 6c–e), indicating that PIPKIγ and talin are required for the stability of snail and slug by preventing proteasomal degradation of these transcription factors. This fully reconciled with increased ubiquitinylated snail upon knockdown of PIPKIγ or talin in NMuMG cells overexpressing snail and also in cancer cells (Figure 6f). Consistently, the expression level of mesenchymal markers and mesenchymal attributes (e.g. migratory) acquired by ectopic expression of snail in NMuMG cells was significantly abrogated upon knockdown of talin or PIPKIγ (Figures 6g–i). A decreased expression level of ectopically expressed snail in talin or PIPKIγ knockdown cells concurs with increased E-cadherin expression and decreased mesenchymal phenotypes. All of these results indicate that PIPKIγ and talin provide a critical signaling nexus for the stability of snail and slug during EMT.

PIPKIγ regulates the stemness property of mesenchymal and tumor cells

Stemness trait is one of the key properties acquired by epithelial cells upon conversion into mesenchymal cells.[34,35] The ability to form mammospheres/tumorspheres in suspension culture is the commonly used assay to measure stemness property.[34] Induction of EMT by TGFβ1 treatment or E-cadherin knockdown or snail overexpression (not shown) substantially increased the ability of NMuMG cells to form mammospheres/tumorspheres, which was significantly compromised upon talin or PIPKIγ knockdown (Figures 7a and b). The overexpression of PIPKIγ further substantiated the ability of E-cadherin knockdown cells to form mammospheres/tumorspheres (Figures 7c and d). However, kinase dead PIPKIγi2-expressing cells or knockdown of talin in PIPKIγi2-overexpressing cells all formed significantly fewer mammospheres/tumorspheres upon E-cadherin knockdown (Supplementary Figure S7A). Together, these results indicate the key role of PIPKIγ and talin complex in acquisition of self-replicating stemness property upon EMT.

To further explore PIPKIγ functions, mammospheres/tumorspheres formed by different tumor cells were isolated. The expression level of PIPKIγ/PIPKIγi2 and level of activated Akt were higher in mammospheres/tumorspheres formed by different tumor cells (Figure 7e). The expression of pluoripotent stem cell marker genes was highly upregulated in isolated mammospheres/tumorspheres as indicated by the pluoripotent stem cell array kit (Supplementary Figure S7B). In a complementary assay, anoikis-resistant tumor cells isolated at different time points after culturing in non-adherent condition also showed the increased expression of PIPKIγ that maintained a higher activation level of Akt and mammospheres/tumorspheres forming capacity (Supplementary Figures S7C and D). These results indicate that PIPKIγ and PI3K/Akt signaling is increased in the subpopulation of tumor cells enriched in stemness property. Corroborating this, the loss of PIPKIγ or PIPKIγi2 or talin (not shown) expression in different tumor cells (e.g. Cal51, A431 and T47D) significantly compromised their ability to form mammospheres/tumorspheres (Figure 7f). With these results, we conclude that PIPKIγ/PIPKIγi2 and the cytoskeletal protein talin provide an important signaling nexus for EMT and acquisition of mesenchymal phenotypes as summarized in the schematic diagram (Figure 8).

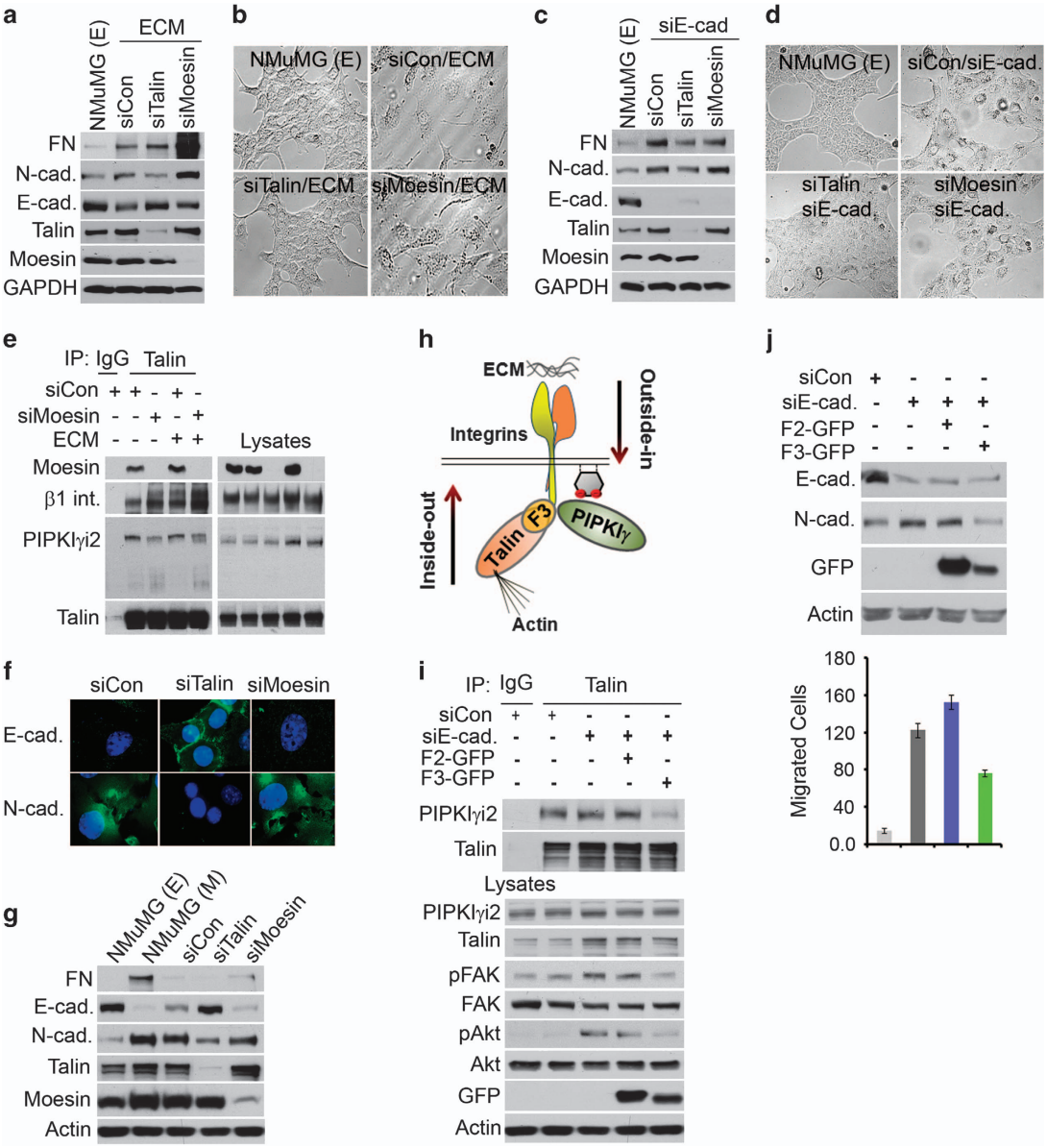


Figure 5. The PIPKIγ–talin interaction is required for EMT. (**a**, **b**) NMuMG cells seeded into a Col. I-coated culture plate and induced to undergo EMT were transfected with siRNA for the knockdown of talin or moesin. Cell morphology was examined 48 h post-transfection (image taken using ×10 objective lens), and the expression level of E-cadherin and mesenchymal marker proteins were examined by immunoblotting after harvesting the cells. (**c**, **d**) NMuMG cells were co-transfected with siRNA for the knockdown of E-cadherin along with talin or moesin. Cell morphology was examined 48 h post transfection (image taken using ×10 objective lens). Gain of mesenchymal marker protein expression was examined by immunoblotting after harvesting the cells. (**e**) Endogenous talin was immunoprecipitated from NMuMG cells cultured into Col. I-coated culture plates and/or after the knockdown of moesin. Co-immunoprecipitation of β1 integrin with talin was examined by immunoblotting. Moesin knockdown promoted EMT and the association of talin with integrin. (**f**, **g**) NMuMG cells fully converted into mesenchymal state by TGFβ1 treatment was transfected with siRNA for knockdown of talin or moesin. At 48–72 h post-transfection, the expression level of E-cadherin and N-cadherin was examined by immunofluorescence study and immunoblotting. (**h**) Schematic diagram depicting talin and PIPKIγ as a platform for bidirectional signaling at cell–matrix interaction sites for epithelial cells transitioning into mesenchymal state. Increased association/expression of talin and PIPKIγ is essential for transitioning epithelial cells which is accompanied by increased adhesion and PI3K/Akt signaling. (**i**, **j**) NMuMG cells infected with lentiviral vector for expression of GFP-fusion protein of F2 or F3 subdomains of talin FERM domain were induced to undergo EMT by E-cadherin knockdown/culture into Col. I-coated culture plates. Cells were harvested 48–72 h post-transfection. Talin was immunoprecipitated and co-immunoprecipitation of PIPKIγi2 was examined to determine the inhibition of talin interaction with PIPKIγi2 by F3 subdomain. The effect on focal adhesion and PI3K/Akt signaling by F3 subdomain were determined by examining the phosphorylated FAK and Akt. The effect of F3 subdomain on acquisition of mesenchymal phenotypes were assessed by N-cadherin expression and cell motility. Results are represented as mean ± s.d. from three-independent experiments and error bars represent s.d.

DISCUSSION

The present study indicates that coupling of adhesion and PI3K/Akt signaling by talin and PIPKIγ provides the integral signaling platform for acquisition of mesenchymal traits by transitioning epithelial cells. The conversion of epithelial into mesenchymal phenotype is accompanied by profound changes in adhesion and PI3K/Akt signaling that are initiated and sustained by ECM proteins and growth factors known to constitute key components

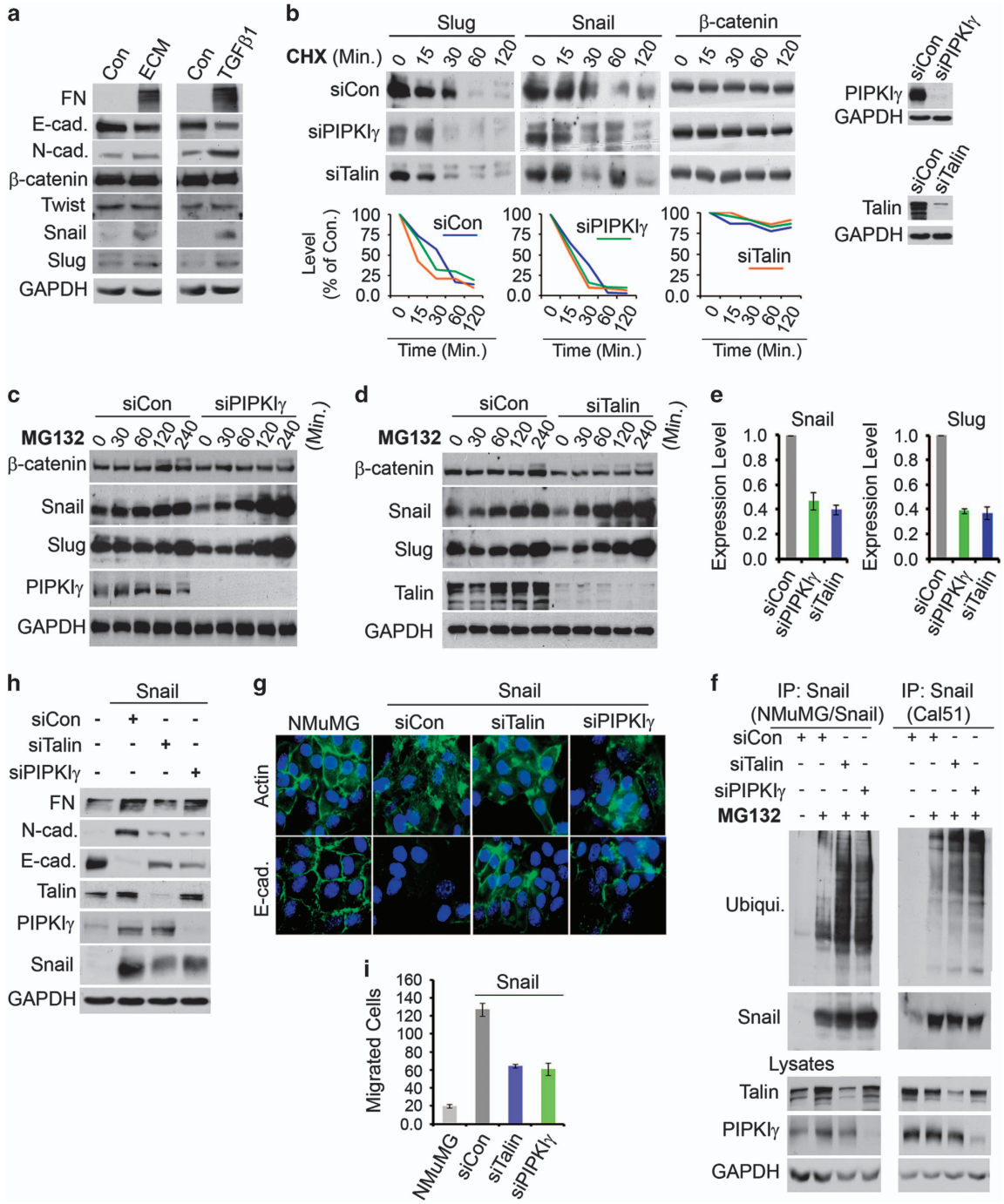


Figure 6. PIPKIγ and talin are required for the stability of EMT-regulating transcription factors. (**a**) Induction of E-cadherin transcriptional repressors (e.g. snail, slug and twist) during transition of NMuMG cells into mesenchymal state by TGFβ1 treatment or culture into ECM protein was examined by immunoblotting. (**b**) NMuMG cells transformed into mesenchymal cells by TGFβ1 treatment were transfected with siRNA for knockdown of talin or PIPKIγ expression. At 48 h post-transfection, cells were treated with cyclohexamide (CHX, 50 μg/ml) to block protein synthesis. Cells were harvested at different time points following cyclohexamide treatment to examine the expression level of EMT-regulating transcription factors by immunoblotting. The protein level of snail, slug and β-catenin at different time points is shown as % of control (before cyclohexamide treatment). The knockdown of PIPKIγ and talin is shown by immunobloting. (**c–e**) PIPKIγ or talin knockdown cells were treated with proteasomal inhibitor, MG132 (50 μg/ml), before harvesting the cells at different time points. The expression level of snail, slug and β-catenin was examined by immunoblotting. (**e**) The expression level of snail and slug in PIPKIγ or talin knockdown cells before MG132 treatment was quantified by measuring the intensity of immunoblot using ImageJ. The data are presented as mean ± s.d. from three-independent experiments. (**f**) siRNA was used to knockdown PIPKIγ/PIPKIγi2 or talin expression in NMuMG cells overexpressing snail or from Cal51 cells. At 48–72 h post-transfection, cells were treated with MG132 for 3 h before immunoprecipitating the snail. Immunoprecipiates were resolved by sodium dodecyl sulfateS–polyacrylamide gel electrophoresis followed by immunoblotting with HRP-conjugated ubiquitin antibody. (**g–i**) siRNA was used to knockdown talin or PIPKIγ in NMuMG cells overexpressing snail. At 72- h post-transfection, cell morphology and E-cadherin expression were examined. The effect of talin or PIPKIγ knockdown on cell motility of snail-overexpressing cells was examined as described above. Results are represented as mean ± s.d. from three independent experiments and error bars represent s.d.

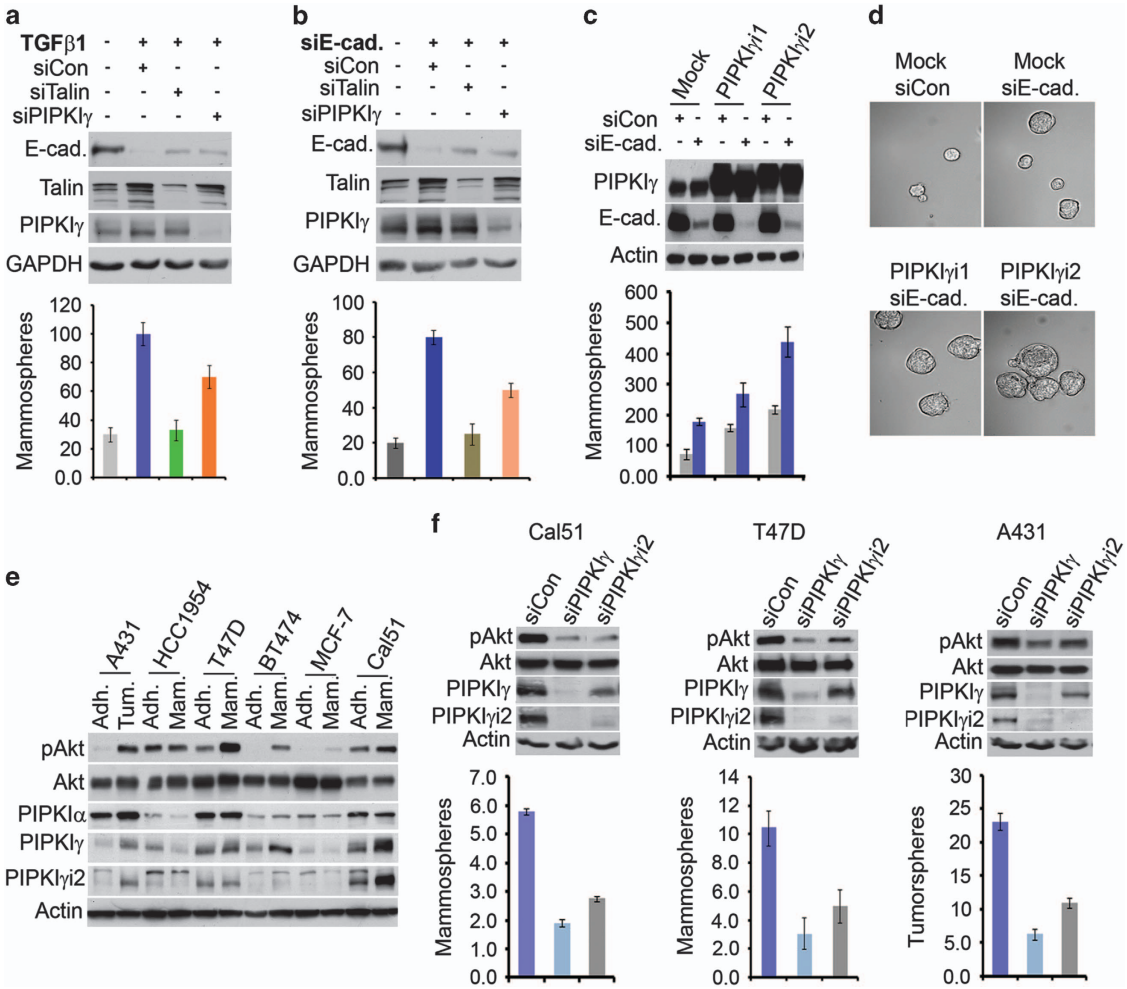


Figure 7. PIPKIγ and talin control mammosphere/tumorspher formation of mesenchymal and tumor cells. (**a**) NMuMG cells were transfected with siRNA to knock down talin or PIPKIγ. At 36–48 h after siRNA transfection, cells were treated with TGFβ1 (2 ng/ml) to induce EMT. Cells were allowed to grow in non-adherent suspension condition 48 h after the TGFβ1 treatment. Mammospheres/tumorspheres formed were counted 3–5 days after incubation. (**b**) NMuMG cells were co-transfected with siRNA for the knockdown of E-cadherin and talin or PIPKIγ. 48–72 h after siRNA transfection, cells were allowed to grow in non-adherent condition. Mammospheres/tumorspheres formed were counted 3–5 days after incubation. (**c**, **d**) NMuMG cells ectopically overexpressing PIPKIγi1 or PIPKIγi2 were transfected with siRNA for knockdown of E-cadherin. At 48–72 h post-transfection, cells were resuspended and cultured in non-adherent condition to examine their ability to form mammospheres/tumorspheres. The representative images of mammospheres/tumorspheres formed are shown (images taken using ×10 objective lens). (**e**) Different tumor cells were cultured in non-adherent condition and mammospheres/tumorspheres formed by these tumor cells were isolated and harvested to examine the expression level of PIPKIγ/PIPKIγi2 and phosphorylated Akt by immunoblotting. (**f**) siRNA was used to knockdown PIPKIγ or PIPKIγi2 in different tumor cells (e.g. Cal51, A431 and T47D). 48–72 h post-transfection, cells were harvested and cultured in non-adherent condition to examine their ability to form mammospheres/tumorspheres. Results are represented as mean ± s.d. from three independent experiments and error bars represent s.d.

of tumor microenvironment.[2,4,5] Furthermore, concomitant with E-cadherin loss, talin and PIPKIγ gained increased expression/interaction and reorganization from cell–cell contact sites to adhesion complexes. This provides a critical signaling nexus for adhesion and PI3K/Akt signaling in transitioning epithelial cells.

The loss of talin or PIPKIγ expression or abrogation of their molecular interaction by overexpression of talin F3 fragment profoundly affected PI3K/Akt and adhesion signaling in transitioning epithelial cells, and these cells retained epithelial-like morphology even after E-cadherin knockdown or culturing on the ECM protein. These cells also failed to acquire full-fledged mesenchymal attributes, including cell motility and stemness properties. This shows that E-cadherin loss in EMT is coupled with a gain of increased PI3K/Akt and adhesion signaling regulated by PIPKIγ and, the cytoskeletal protein, talin that are required for mesenchymal attributes. This is fully consistent with the prevailing notion that spatial generation of PIP_2 lipid messenger by PIPKIγ regulates the development of integrin-mediated adhesion complex at cell–matrix interface, where talin assumes key roles in executing inside-out and outside-in bidirectional signaling. However, the precise mechanism and dynamics of molecular interaction among talin, PIPKIγi2 and integrins at the adhesion complex and their regulation by PIP_2 lipid messenger still remain poorly understood.[21] In alignment with increased adhesive/invasive property of transitioning epithelial cells, the predominant changes observed in EMT are the profound increase in the synthesis of ECM proteins and integrin expressions, which probably occurs irrespective of agonists used for inducing EMT (e.g. TGFβ1, oncogenes or overexpression of EMT-inducing transcription factors).[27,36–38] Again, these events also result in initiation and sustenance of more profound PI3K/Akt lipid signaling downstream of integrins/growth factors in

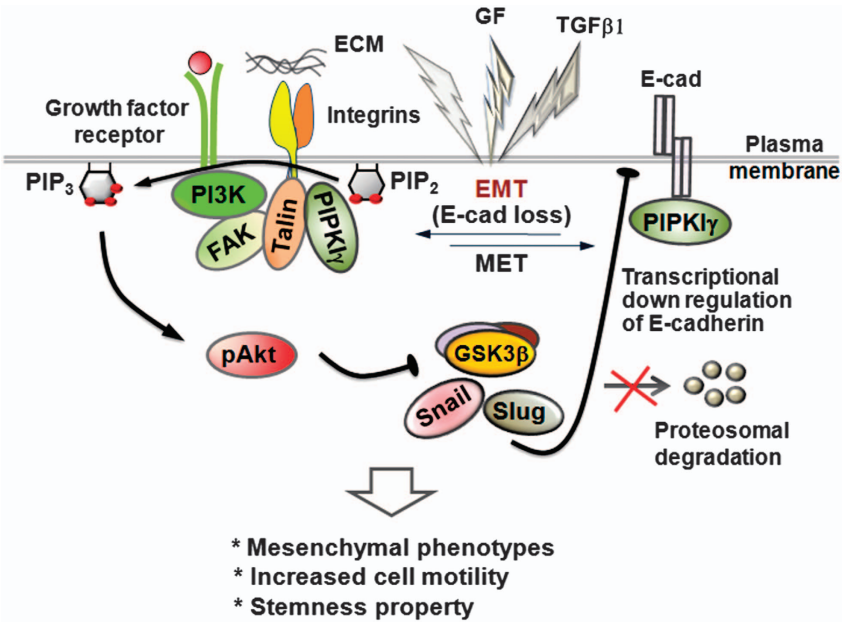


Figure 8. Schematic diagram depicting PIPKIγ and talin control of EMT and mesenchymal phenotypes. During EMT, loss of E-cadherin expression promotes the assembly of PIPKIγ with talin at adhesion complex, which is required for the increased adhesion and PI3K/Akt signaling in the transitioning epithelial cells. PIPKIγ and talin control the stability of E-cadherin transcriptional repressors, snail and slug by preventing their ubiquitination and proteosomal degradation. Acquisition of mesenchymal phenotypes (e.g. cell motility and stemness property) further depends upon the integrity of PIPKIγ and talin (ECM, extracellular matrix; GF, growth factor; E-cad., E-cadherin; MET, mesenchymal epithelial transition).

transitioning epithelial cells indicating that all of these are interconnected events of the EMT program, and the integrity of PIPKIγ and talin is essential for acquisition as well as sustenance of mesenchymal attributes in transitioning epithelial cells.

In mesenchymal cells, talin, PIPKIγ and their interaction appear to have pleiotropic roles that range from the initiation and sustenance of adhesion and PI3K/Akt signaling to regulation of cell motility and the stability of EMT-regulating transcription factors. In different physio-pathological conditions, upregulated expression of the transcription factors snail and slug block E-cadherin expression promoting EMT. Different agonists that induce EMT, such as TGFβ1, ECM protein, growth factors, WNT and NOTCH, all converge into inducing these E-cadherin transcriptional repressors.[5,31] In the absence of talin or PIPKIγ, the expression of EMT-regulating transcription factors snail and slug was compromised. The integrity of talin and PIPKIγ, and their cellular signaling, appears to play a pivotal role in the stability of snail and slug by preventing their proteosomal degradation, possibly by inhibiting the GSK3β phosphorylation and ubiquitination, as number of studies indicate crucial roles of cellular signals, such as PI3K/Akt and Erk1/2, in the stability of these EMT-regulating transcription factors by inhibiting GSK3β.[33,39]

Along with increased motility, another key facet of EMT and many cancer cells is increased stemness property, which dictates the ability of cancer cells to metastasize and colonize to distant places.[34,35,40] PIPKIγ expression and PI3K/Akt signaling are selectively enriched in anoikis-resistant cell populations with stemness traits. The loss of PIPKIγ or disruption of the PIPKIγ and talin interaction and their signaling nexus affected the gain of stemness traits by transitioning epithelial cells, and also impacted the stemness property of many tumor cells. In summary, we illustrate the comprehensive mechanism how coupling of phosphoinositide kinase PIPKIγ and cytoskeletal protein talin provides a platform for the acquisition of mesenchymal traits by transitioning epithelial cells.

MATERIALS AND METHODS

Cell culture

Mouse mammary epithelial cells, NMuMG were cultured in Dulbecco's modified Eagle's medium-containing 10% FBS supplemented with 10 μg/ml insulin (Sigma, St Louis, MO, USA). MDA-MB-231, Cal51, A431, MCF-7, HCC1954 and HEK293FT cells were cultured into Dulbecco's modified Eagle's medium-containing 10% FBS. T47D cells were cultured in RMPI-1640 containing 10% FBS. SUM159 cells were culture in Ham's F12 supplemented with 5% FCS. All cells were grown at 37 °C in a 5% CO_2 incubator.

For the induction of EMT, NMuMG cells were treated with TGFβ1 (2 ng/ml), and harvested at different time points. The mesenchymal cells indicated in this study refer to NMuMG cells treated with TGFβ1 for 3–5 days. Similarly, for studying the effect of PI3K inhibitor, NMuMG cells were treated with LY294002 (2 μg/ml) during induction of EMT by TGFβ1 treatment. For induction of EMT by ECM protein, cells were seeded into Col. I-coated culture plate for 2–3 days in the presence or absence of EGF or HGF.

For examination of activated phosphoinositide signaling (PI3K/Akt) by FBS or different growth factors, cells were serum- and insulin-starved overnight before stimulating with FBS or indicated growth factor and then harvested at different time points. Similarly, cells, after resuspension in serum-free medium, were seeded into Col. I-coated culture plate and incubated for 15–20 min. Non-attached cells were removed by phosphate-buffered saline washing before cell harvesting.

For examining the stability of EMT-regulating transcription factors, NMuMG cells fully converted into mesenchymal state by TGFβ1 treatment were treated with cyclohexamide (50 μg/ml) before harvesting them at different time periods. Similarly, for inhibition of proteosomal degradation of EMT-regulating transcription factors, cells were treated with MG132 (50 μg/ml) before harvesting them at different time points.

Transfection or lentiviral infection

For transfection of siRNA, LipofectamineRNAiMAX (Invitrogen, Carlsbad, CA, USA) was used following the protocol provided by the manufacturer, and cells were assayed 48–72 h post-transfection. For transient transfection into HEK293 cells, Lipofectamine-2000 (Invitrogen) was used. Cells were harvested 24 h post-transfection. For the expression of PIPKIγ variants, snail or talin fragments into NMuMG or other cells, the lentiviral system was used as described previously.[16] Cells were harvested 48–72 h post-

infection and 70–80% of infection efficiency was achieved for the experiments.

Immunoprecipitation and immunoblotting

Cells were lysed using lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton-X100, 1 mM EDTA, 10 mM NaF, 5 mM Na_3VO_4 and protease inhibitors). Clear supernatants were incubated with indicated antibodies for 3–4 h to overnight at 4 °C followed by isolation of immunocomplexes using protein G Sepharose 4B beads (Amersham, Uppsala, Sweden). Beads were washed three times with lysis buffer before eluting the immunocomplexes with 2× sample buffer, run through sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and subjected to immunoblotting using specific antibodies.

Mammospheres/tumorspheres formation

For mammospheres/tumorspheres formation, different cell types at the density of 5×10^3 cells/ml were suspended into the complete mammoCults medium (StemCell Tech, Vancouver, BC, Canada) containing methylcellulose were seeded into 24-well ultra-low attachment plates (Corning, Kennebun, ME, USA). The mammospheres/tumorspheres formed were isolated or counted after 3–5 days of incubation. For examination of protein expression by the pluoripotent stem cell array kit, the clear cell lysates (200 μg) prepared from isolated mammospheres/tumorspheres were used. For isolation of anoikis-resistant tumor cell subpopulation, single cells suspended at the density of 1×10^5 cells/ml in Dulbecco's modified Eagle's medium medium-containing 0.5% FBS were seeded into 10 cm dish culture plates precoated with 0.5% agar for preventing the cell attachment. Cells surviving under this condition were isolated at different time points and harvested.

Cell adhesion and migration

Cell adhesion and migration assays were carried out as described previously.[7,16] In brief, cells were serum- and insulin-starved overnight before resuspension in Dulbecco's modified Eagle's medium medium containing 0.2% bovine serum albumin. For cell adhesion, cells were seeded into Col. I-coated culture plate and incubated for 15–30 min before removing the non-adherent cells by phosphate-buffered saline washing. Adherent cells were fixed with paraformaldehyde and cells counted from different areas of the plate. For cell migration, cells were allowed to migrate towards 0.25% FBS as chemoattractant. Time allowed for cell migration was 5–6 h. The cell migrated to the underside of membrane were fixed, stained and counted.

Immunofluorescence microscopy

For immunofluorescence study, colonies developed in the soft agar were fixed with 3.7% paraformaldehyde followed by cell permeabilization with 0.1% Triton-X and blocked with 3% bovine serum albumin in phosphate-buffered saline. Cells were incubated with a primary antibody overnight at 4 °C followed by incubation with Alexa555- and/or Alexa488-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Slides were mounted using Vectashield and visualized with a Nikon TE2000-U microscope, Japan. Images were acquired using MetaMorph and processed using Adobe Photoshop.

Statistical analysis

The data are presented mean ± s.d. from at least three-independent experiments. Unpaired *t*-test was conducted to determine the *P*-value and the statistical significance between two groups (*P*-value less than 0.05 were considered significant).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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