# The C terminus of talin links integrins to cell cycle progression

Pengbo Wang, Christoph Ballestrem, and Charles H. Streuli

Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, England, UK

Integrins are cell adhesion receptors that sense the extracellular matrix (ECM) environment. One of their functions is to regulate cell fate decisions, although the question of how integrins initiate intracellular signaling is not fully resolved. In this paper, we examine the role of talin, an adapter protein at cell–matrix attachment sites, in outside-in signaling. We used lentiviral small hairpin ribonucleic acid to deplete talin in mammary epithelial cells. These cells still attached to the ECM in an integrin-dependent manner and spread. They had a normal actin cytoskeleton, but vinculin, paxillin, focal adhesion kinase (FAK), and integrin-linked kinase were not recruited to adhesion sites. Talin-deficient cells showed proliferation defects, and reexpressing a tail portion of the talin rod, but not its head domain, restored integrin-mediated FAK phosphorylation, suppressed p21 expression, and rescued cell cycle. Thus, talin recruits and activates focal adhesion proteins required for proliferation via the C terminus of its rod domain. Our study reveals a new function for talin, which is to link integrin adhesions with cell cycle progression.

## Introduction

Cells sense their location and respond to the environment through integrin-containing adhesion complexes located at the plasma membrane (Zaidel-Bar et al., 2007). Adhesion complexes control cell architecture and migration and integrate microenvironmental signals with those from soluble factors to influence cell fate decisions (Streuli and Akhtar, 2009). However, how integrin signaling determines cell phenotype is not fully understood. This problem is compounded by the complexity of the assembly and the variety of adaptor proteins that bind to integrin cytoplasmic tails as well as differences between adhesions among cell lineages (Zaidel-Bar et al., 2007; Legate and Fässler, 2009).

Most adherent cells require integrins to progress through the cell cycle. Genetic deletion of integrins in vivo and culture has revealed their key role for the proliferation of many cell types (Wickström et al., 2011). In the mammary gland, $\beta1$ integrin is needed for efficient proliferation in both development and cancer (Li et al., 2005; Lahlou et al., 2007). Integrins control growth factor signaling pathways in some cell types, whereas in others, they activate enzymes that are necessary for

the G1 phase of the cell cycle (Giancotti and Tarone, 2003; Bustelo et al., 2007). Integrins therefore provide an adhesion checkpoint for cell cycle progression (Streuli, 2009). However, the proximal adhesion complex proteins that link integrins with proliferation are not known. Here, we ask whether a core protein of adhesion complexes, talin, might be directly involved in linking integrins with cell cycle progression.

Talin is a ubiquitous integrin-interacting scaffold protein at cell–matrix attachment sites, containing N-terminal globular head and C-terminal rod domains. Talin activates integrins and connects integrins to the actomyosin machinery (Critchley, 2009). It provides inside-out signals by interacting with the integrin cytoplasmic region through its head domain causing $\alpha/\beta$ integrin chain separation. This activity allows talin to regulate the ECM-binding activity of integrins (Tadokoro et al., 2003; Simonson et al., 2006; Nieswandt et al., 2007; Watanabe et al., 2008; Anthis et al., 2009; Lee et al., 2009; Ye et al., 2010). Talin also connects integrins with the cytoskeleton via the adaptor protein vinculin. This imparts mechanical stability to the adhesions between muscle cells and tendons (Gingras et al., 2008; Löer et al., 2008;

Correspondence to Charles H. Streuli: cstreuli@manchester.ac.uk

Abbreviations used in this paper: ABS, actin-binding site; Cas, Crk-associated substrate; DS, dimerization site; Erk, extracellular signal–regulated kinase; FN, fibronectin; IBS, integrin-binding site; ILK, integrin-linked kinase; MEC, mammary epithelial cell; myrFAK, myristoylated FAK; Rhrx, Rhodamine Red-X; shRNA, small hairpin RNA; VBS, vinculin-binding site.

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Critchley, 2009; Carisey and Ballestrem, 2011). In addition, talin transmits forces from the ECM to the cytoskeleton, which enables focal adhesion formation and cell spreading (Giannone et al., 2003; Zhang et al., 2008). We now demonstrate that talin is necessary for transducing integrin-regulated pathways to control cell cycle progression in mammary epithelial cells (MECs) and that this activity is contained within the C-terminal portion of its rod domain.

## Results

### Talin links integrins to epithelial cell proliferation

To determine the function of talin in epithelia, we took advantage of FSK7, MEC strain isolated from virgin mice that synthesizes talin1 but not its homologue talin2 (Fig. S1 A). Lentiviral small hairpin RNA (shRNA) directed against talin1 (shTln1) depleted talin by ∼90% in MECs as detected by immunoblotting (Fig. 1 A) and to nearly undetectable levels in adhesion complexes when analyzed by immunofluorescence (Fig. 1 B, top). Similar effects were seen with two separate shTln1 sequences (Fig. S1 B, top).

In mouse embryonic fibroblasts, talin depletion led to a complete inhibition of cell spreading, confirming that our shRNA sequences for talin1 have a similar efficacy as previously shown by others (Fig. S2; Zhang et al., 2008). In contrast to fibroblasts, MECs lacking talin remained spread on the tissue culture dishes (Fig. 1 B, bottom). However the density of these spread cells did not increase over several days, and because apoptotic cells were not present, we hypothesized that talin depletion caused a proliferation block. To test this possibility, we stained subconfluent cultures with BrdU, EdU, or phospho-Histone3 and found that shTln1 reduced the proportion of cells undergoing DNA synthesis (Fig. 1 C). To confirm that talin depletion was specifically involved with cell cycle arrest, we coexpressed a human talin cDNA that could not be targeted by mouse shTln1. This vector rescued cell cycle arrest caused by shTln1 (Fig. 1 D). Talin depletion also caused a proliferation defect in another mammary epithelial line that causes malignant lesions in vivo, 4T1 (Fig. 1 E). A scrambled version of the shTln1 sequence did not deplete talin in MECs nor did it prevent proliferation (Fig. S1 C).

These data suggest that talin provides a link between integrins and downstream cellular processes that control the proliferation of MECs. In the subsequent experiments, we dissected the mechanism for outside-in signaling via talin.

### Talin coordinates the assembly of core epithelial adhesion complex proteins

Because β1 integrins are required for MEC proliferation (Li et al., 2005; Naylor et al., 2005), we tested whether shTln1 simply prevented integrin activation. To determine the contribution of β1 integrin to MEC adhesion, endogenous β1 integrin was depleted with a microRNA-adapted shRNA (shRNAmir). Approximately 80% of the attachment was prevented, reflecting the key role of β1 integrins to MEC adhesion (Fig. 2 A, left and middle). In contrast, adhesion was only marginally affected by talin1 depletion with shTln1. Similarly, knocking down talin had no effect on MEC adhesion to a variety of other ECM substrata, including laminin, vitronectin, or collagen I (Fig. 2 A, right).

These results suggest that integrins are still active in talin-depleted MECs. Indeed, β1 integrin–containing adhesions were present in both control and talin-depleted MECs by immunostaining with 9EG7, a monoclonal antibody to a conformation-specific epitope that is only exposed on activated β1 integrin (Fig. 2 B; Lenter et al., 1993). Thus, although the extent to which talin was knocked down did not prevent integrin-mediated attachment, it did impair cell cycle progression.

We therefore examined the possibility that the cell cycle defects might be related to altered cytoskeleton or cell shape. Adhesion complexes are sites of cytoskeleton assembly, where microfilaments are built through interactions with integrin- and actin-binding proteins, such as talin (Carisey and Ballestrem, 2011). Interestingly, we found that in MECs, talin depletion had no apparent effect on the presence of stress fibers (Fig. 2 C, top and bottom left). Quantifying the intensity of F-actin fluorescence per micrometer squared revealed that levels of microfilaments were similar in the presence and absence of talin (Fig. 2 C, bottom right). Because one of talin's functions is to link integrins with the cytoskeleton, we assessed whether other proteins might substitute for the lack of talin. One possible linker is tensin because some of the GFP-tensin expressed in MECs relocated from stress fibers in control cells to adhesions in talin1-depleted cells (Fig. S3).

The cell cycle regulation of many cell types is dependent on cell size, and proliferation can be blocked when spreading is compromised (Chen et al., 1997). Thus, although microfilaments were still present in talin-depleted MECs, proliferation might be prevented if the cells cannot spread appropriately. To examine the possibility that talin depletion inhibited cell cycle progression by preventing a full degree of spreading, we measured the sizes of several hundred GFP- or shTln1 virus-infected MECs and compared this with the ability of the same cells to incorporate BrdU. There was a broad range of cell spread area in these particular cultures, and their spreading was marginally affected in talin-depleted cells (Fig. 3 A, left). However, regardless of cell spread area, in each case, their cell cycle was similarly inhibited (Fig. 3 A, right). To confirm that defective cell spreading was not the cause of the proliferation defect, MECs expressing GFP or shTln1 were spread on adhesive 225-μm² islands. Under these conditions of identical spreading, talin1 was still required for the cells to transduce the adhesion pathways for DNA synthesis (Fig. 3 B).

These results indicate that the requirement of talin for mammary cell cycle progression is not dependent on cell architecture. We therefore reasoned that talin might be required to recruit additional adhesion complex proteins, which are important for proliferation. The ability of MECs to spread on tissue culture dishes after talin knockdown enabled us to search for adhesion components that are affected by the absence of talin and that potentially link integrins to cell proliferation pathways. Talin binds vinculin, which is a core adaptor protein within integrin adhesions (Critchley, 2009). Vinculin was not present
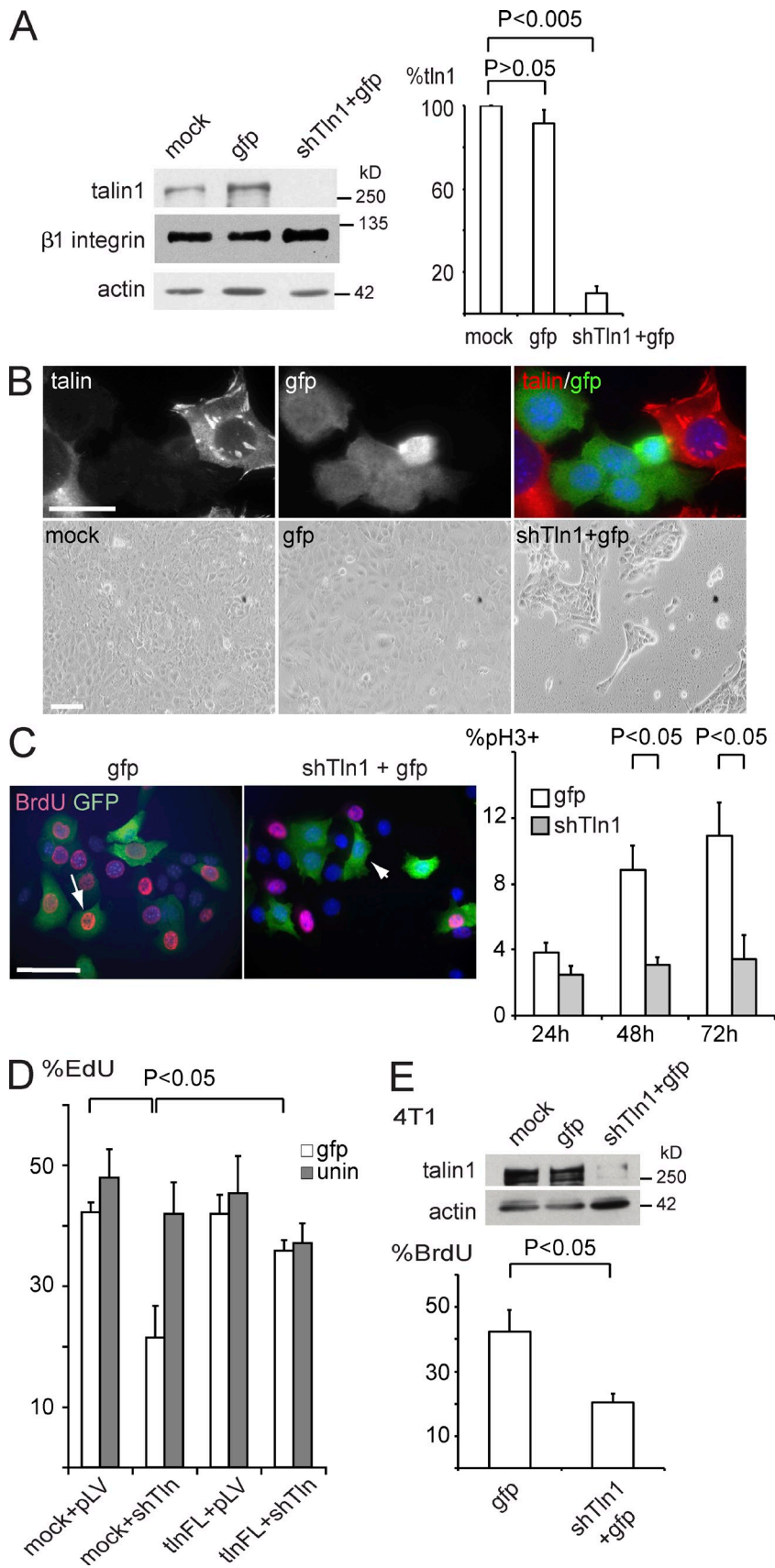
Figure 1. **Talin1 is required for MEC proliferation.** (A, left) MECs were infected with either shTln1 or GFP-only lentivirus or mock infected (3 h), cultured (48 h), FACS sorted to isolate GFP+ cells, and immunoblotted with antibodies to talin1, $\beta1$ integrin, and actin. (right) Relative talin levels were quantified by an infrared imaging system (Odyssey; LI-COR Biosciences; actin is the loading control). Note that in this and subsequent figures, $n = 3$ or more independent experiments. (B) Mock, GFP-only, or shTln1-infected cells were cultured for 48 h, replated, cultured a further 72 h, and then examined by fluorescence (top) and phase-contrast microscopy (bottom). The talin-depleted cells spread but did not become confluent. Talin localized to adhesions of uninfected MECs (red) but was absent in shTln1-infected cells (green). (C) GFP-only or shTln1-infected cells were replated, cultured overnight, and labeled with BrdU (2 h) or stained for phospho-Histone3. (left) Representative field after BrdU labeling. The arrow points to GFP-positive cells in S phase, whereas the arrowhead shows that the GFP-positive cells in talin knockdown cells did not incorporate BrdU into their DNA. Quantification of proliferation in infected cells was GFP only = 60 ± 5.9% and shTln1/GFP = 21 ± 3.6%. (right) Quantification of proliferation by phospho-Histone3 (pH3+) in infected cells. (D) MECs were cotransfected with either shTln1 or empty pLVTHM plasmid (pLV) together with shRNA-resistant human full-length talin1 (tlnFL), cultured (48 h), replated (overnight), and labeled with EdU (2 h). EdU+ cells were quantified in both the infected (GFP) and uninfected (unin) control cells on the same dishes. (E) GFP-only or shTln1-infected 4T1 cells were cultured (48 h), FACS sorted to isolate GFP+ cells, and either immunoblotted with antibodies to talin1 and actin or cultured overnight and labeled with BrdU (2 h). Error bars indicate SEM. Bars: (B, top) 20 µm; (B, bottom) 100 µm; (C) 50 µM.
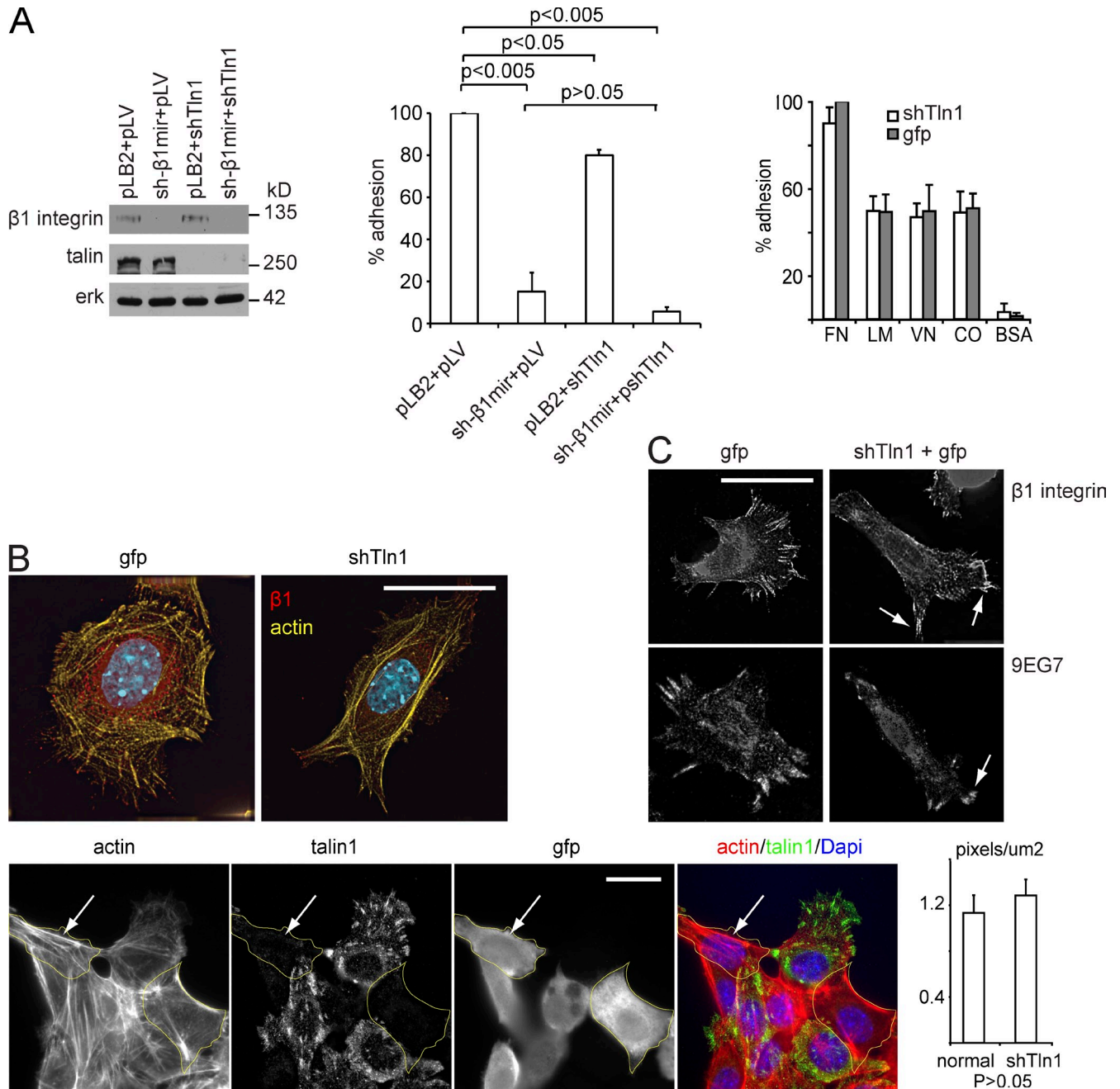
Figure 2.  **Depletion of talin1 does not affect β1 integrin activation or the MEC cytoskeleton.** (A, left and middle) Cells were cotransfected with shRNAmir for β1 integrin (sh-β1mir), shRNA for talin1 (shTln1), and the empty vectors (pLB2 and pLVTHM [pLV]). After 48 h, GFP-positive cells sorted by FACS were used in an adhesion assay on FN. (left) Blot showing that both β1 integrin and talin1 proteins were effectively deleted by using shRNA. (middle) Depletion of β1 integrin almost completely blocked cell attachment, but in contrast, talin knockdown only slightly reduced adhesion. (right) GFP-only or shTln1-infected MECs were FACS sorted to isolate GFP+ cells and used for adhesion assays on FN, laminin (LM), vitronectin (VN), or collagen I (CO). (B, top) FACS-sorted GFP-only or shTln1-infected cells were cultured overnight and stained for β1 integrin and phalloidin. (bottom) Costaining of talin with F-actin in shTln1-infected cells. (bottom left) Representative images. (bottom right) The intensity of total actin fluorescence per micrometer squared in control GFP-negative cells and in cells with talin knockdown (GFP positive). The GFP-positive cells depleted of talin showed normal microfilament distribution (arrows). The yellow lines demarcate the cell periphery. (C) FACS-sorted GFP-only or shTln1-infected cells were cultured overnight, briefly detergent extracted before fixation, and stained for either total (top) or active (bottom) β1 integrin (9EG7). Arrows point to β1 integrin–containing adhesion complexes in talin-depleted cells. Error bars indicate SEM. Bars, 15 µm.

in the adhesion complexes of talin-depleted MECs (Fig. 4 A, top), even though its total levels were similar to control cells infected with GFP-only virus or mock-infected cells (Fig. 4 B). To obtain a semiquantitative estimate of vinculin and talin within adhesions, we compared their fluorescence intensity

profiles simultaneously in control and talin-depleted MECs. The colocalization of vinculin with talin coincided in uninfected control cells, whereas the neighboring infected (i.e., GFP expressing) cells contained virtually no vinculin or talin (Fig. 4 A, top graph). We confirmed the data with total internal reflection
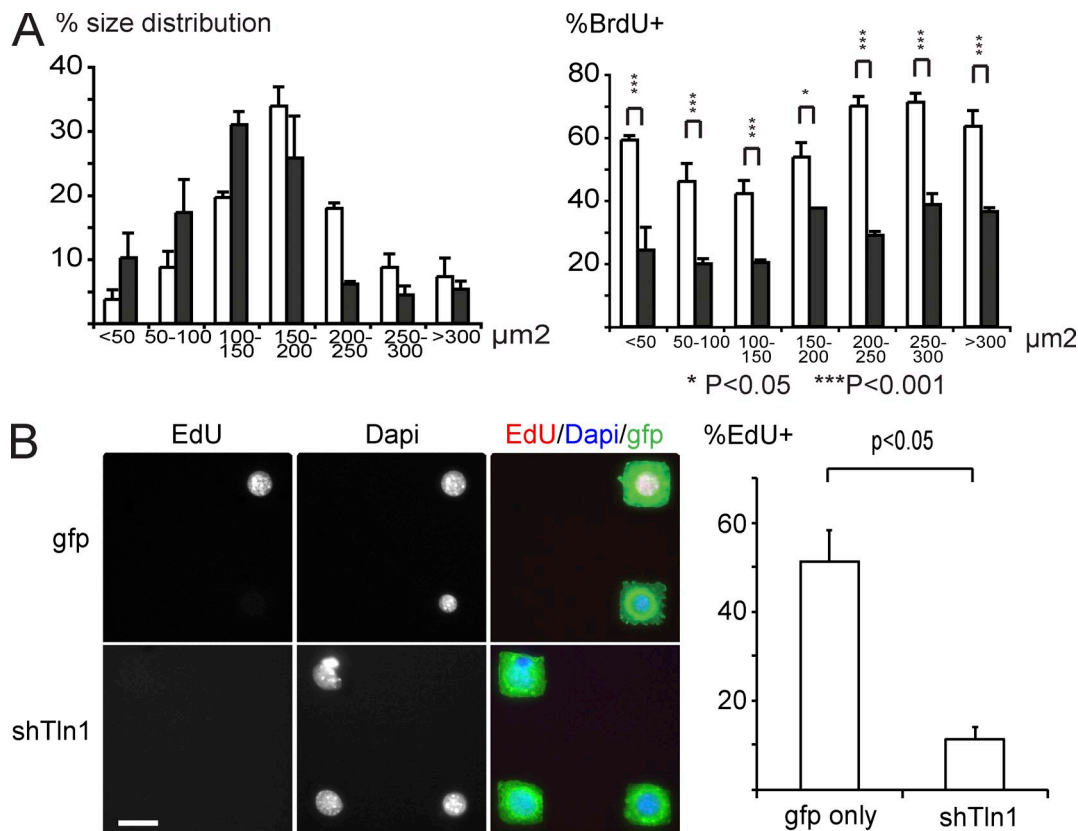
Figure 3. **Cell cycle is defective in talin-depleted MECs regardless of their spread size.** (A) GFP-only or shTln1-infected MECs were replated, cultured overnight, and labeled with BrdU. Infected cells were visualized by GFP fluorescence, and the area of cell spreading was quantified and divided into seven groups ranging from <50 to >300 μm². (left) Size distribution. (right) Quantification of BrdU staining. White bars: GFP-only infected cells. Black bars: GFP/shTln1-infected cells. (B) GFP-only or shTln1-infected MECs were replated on FN-coated micropatterned coverslips and then cultured overnight so that the cells spread to the same extent. They were then labeled with EdU. (left) Representative images. Note that the cells expressing shTln1 were defective in proliferation. (right) Quantification of proliferation data. Error bars indicate SEM. Bar, 15 μm.

fluorescence microscopy, which eliminates background fluorescence by detecting epitopes only within 150 nm from the substratum, thus enhancing the chances to detect potentially hidden adhesion structures (unpublished data). As with vinculin, paxillin and integrin-linked kinase (ILK) were also absent in talin-depleted adhesions (Fig. 4 A, second and third row). Moreover, ectopically expressed GFP-paxillin expressed in talin-depleted cells was cytosolic and unable to localize to adhesion complexes (Fig. 4 C). We examined recruitment of adhesion complex proteins in cells spread on a specific ECM protein, fibronectin (FN), and found that they were absent in talin-depleted cells (Fig. 4 D).

In addition to the lack of adapter proteins, FAK, a classical adhesion signaling enzyme known to participate in proliferation, was also affected by talin depletion. The talin-depleted cells did not show FAK in any large adhesion complexes (Fig. S4). pY397-FAK was absent from the adhesions of talin-depleted cells as were other tyrosine-phosphorylated proteins (Fig. 4 A, fourth and fifth row). shTln1-expressing cells contained similar levels of FAK to controls as determined by immunoblotting, but FAK failed to become phosphorylated on Y397, indicating that it could not be activated (Fig. 4 B). Talin-depleted adhesions remained free of phosphoproteins, such as p-paxillin, even over a period of several days.

Together, these results show that knockdown of talin in MECs does not compromise β1 integrin activation nor does it prevent cell adhesion and spreading and the accompanying formation of actin stress fibers. It remains possible that there is sufficient residual talin after knockdown to permit integrin activation. However, our data highlight the essential role for talin in coordinating the assembly of core regulatory proteins into epithelial adhesion complexes. The mechanism of cytoskeletal assembly and adhesion complex formation in talin-depleted cells will be followed up in the future, but for this study, we continued to focus on the role of talin in cell cycle progression.

### Talin links integrins with cell cycle progression via FAK

From the results obtained thus far, we reasoned that talin is required in order to recruit the integrin-associated adaptor proteins and signaling enzymes for transducing downstream physiological processes, such as proliferation. To test this hypothesis, we examined the role of FAK because genetic knockout has shown that it is required for efficient MEC proliferation in primary MEC cultures (unpublished data) as well as in mammary epithelial tumors in vivo (Lahlou et al., 2007; Provenzano et al., 2008; Pylayeva et al., 2009).
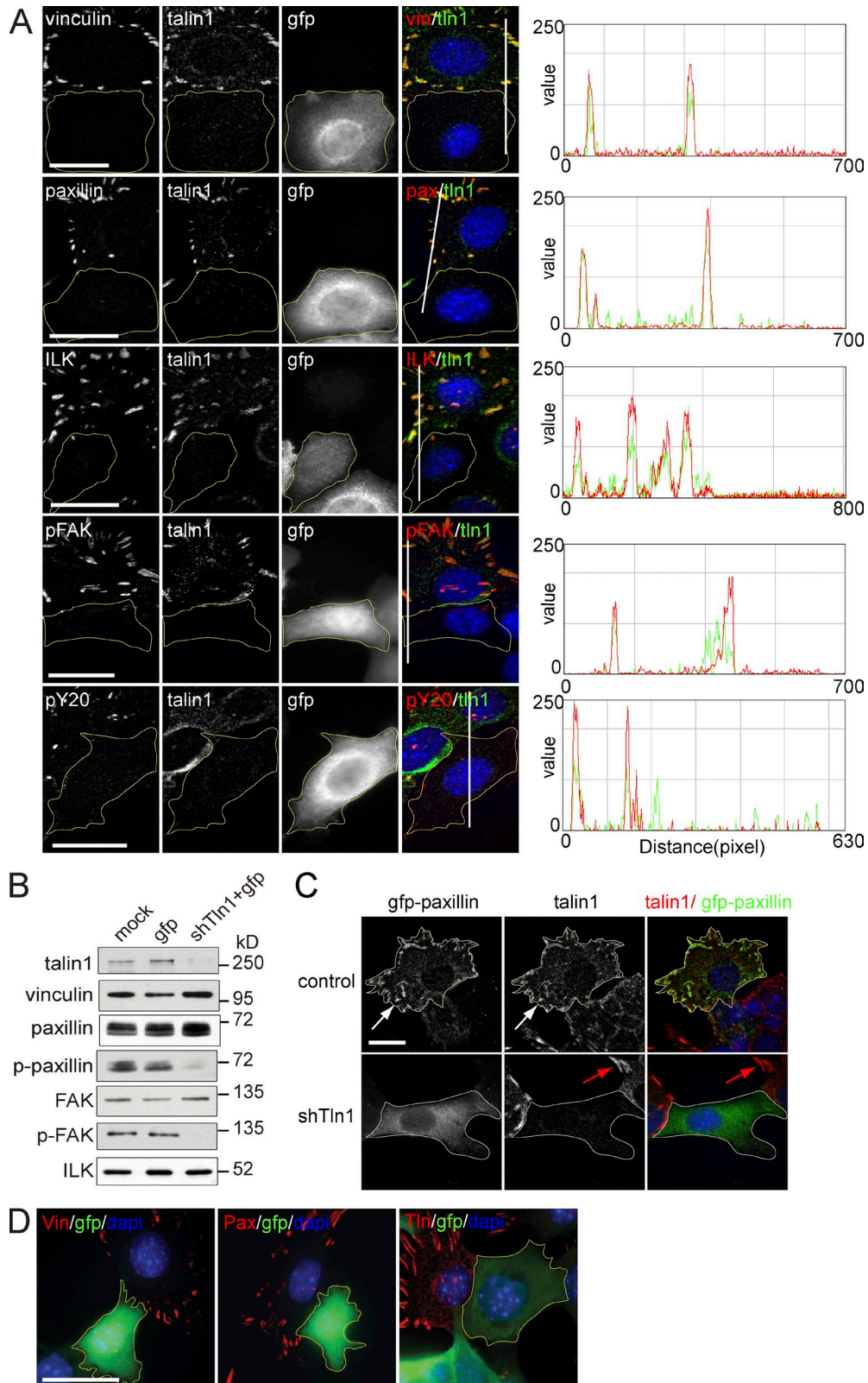
Figure 4. **Talin1 is required for recruitment of adhesion complex proteins.** (A) GFP-positive talin1-deficient cells were replated (overnight) and coimmunostained for vinculin (vin), paxillin (pax), ILK, pFAK, or pY20 together with antitalin–Alexa Fluor 568. GFP-expressing cells lack talin (encircled by yellow lines), whereas the adjacent uninfected cells contain talin adhesions. Fluorescence intensity profiles, to the right of the fluorescence images, depict the area of the line drawn in image overlays. (B) FACS-sorted mock-infected, GFP-only, or shTln1-infected cells were immunoblotted for adhesion proteins. (C) GFP-paxillin was expressed in MECs together with either shTln1 or control pSilencer. GFP-paxillin localizes to talin-containing adhesions in control cells (white arrows). In talin-depleted cells, GFP-paxillin does not localize to adhesions and is cytosolic. Red arrows point to talin in an adjacent untransfected cell. (D) MECs transduced with shTln1 were replated onto FN-coated coverslips, and after 48 h, the cells were immunostained for vinculin, paxillin, or talin. Note the absence of vinculin and paxillin in talin (Tln)-depleted cells. Bars: (A) 15 µm; (C and D) 10 µm.

To investigate whether talin depletion affects cell cycle progression via FAK, we expressed in talin-deficient cells, a myristoylated FAK (myrFAK), which targets FAK to membranes. myrFAK localized to MEC focal adhesions and also associated with integrin adhesions in talin1-depleted cells (unpublished data). Because endogenous FAK was unable to be recruited to adhesions of talin-depleted cells, this strategy allowed us to determine whether FAK, artificially recruited to adhesion sites in the absence of talin, might be involved in cell cycle control. We found that wild-type myrFAK (wt-myrFAK), but not an autophosphorylation site mutant, mutant myrFAK (mu-myrFAK), rescued the defect in DNA synthesis as measured by EdU incorporation (Fig. 5 A).

To identify possible downstream mechanisms linking talin to DNA synthesis, we tested whether talin depletion affected either growth factor signaling and/or cell cycle proteins. MECs respond to growth factors via extracellular signal–regulated kinase (Erk) and Akt, both of which are necessary for cell cycle progression. However, neither Erk nor Akt phosphorylation were compromised in talin-depleted cells (Fig. 5 B). Src also remained phosphorylated, even though FAK was dephosphorylated in the same cells (Fig. S5). Similarly, the levels of most of the cell cycle regulators that we were able to measure by immunoblotting were talin independent (Fig. 5 C, top two sections). However, one protein, p21, did stand out as being upregulated in the talin-depleted cells (Fig. 5 C, third section).

p21 inhibits Cdks, thereby directly suppressing progression through the G1/S phases of the cell cycle, and indeed, cyclin D1 was present at lower levels after talin depletion (Fig. 5 C, bottom section). We therefore reasoned that p21 might provide the mechanistic link between talin and cell cycle regulation. To test this possibility, we determined whether depleting p21 in shTln1-expressing cells could rescue the proliferation defect. We identified and tested the efficacy of an shRNA sequence for p21 (Fig. 5 D, left; Fasano et al., 2007). When sh-p21 was cotransfected into MECs along with shTln1, it rescued the proliferation defect observed with shTln1 alone (Fig. 5 D, right).

We next tested whether FAK connects talin and p21 in cell cycle control of MECs. wt-myrFAK, but not mu-myrFAK, prevented p21 up-regulation in talin-depleted cells (Fig. 5 E). To confirm that this was a causal relationship, we again examined the ability of FAK to rescue the shTln1-induced proliferation defect, but this time in the presence of overexpressed p21. As already noted, shTln1 blocked cell cycle progression in MECs, and this was alleviated by wt-myrFAK (Fig. 5 F). However, overexpressing p21 reversed the combined effects of shTln1 and wt-myrFAK, resulting in cell cycle arrest. These results correlate with a previous study showing that p21 mediates cell cycle arrest in MECs lacking β1 integrins and suggest that in the absence of talin, FAK cannot be activated, leading to p21 up-regulation and cell cycle arrest (Li et al., 2005).

### Talin's C-terminal tail domain is required for epithelial cell cycle progression

To study the cell cycle role for talin in more detail, we examined whether specific regions of talin could rescue pFAK recruitment to adhesions and DNA synthesis. Talin's head contains a FERM (4.1 protein, ezrin, radixin, and moesin) domain that mediates integrin binding as well as an actin-binding site (ABS), whereas the rod comprises vinculin-binding sites (VBS), additional ABSs, a second integrin-binding site (IBS2), and a dimerization motif (dimerization site [DS]; Fig. 6 A; Critchley, 2009; Gingras et al., 2010). To determine whether either the head or rod domains link integrins to proliferation, V5-tagged shTln1-resistant human talin1 fragments were expressed in talin-deficient MECs.

Expression of full-length talin in talin-deficient MECs rescued the phosphorylation of FAK within adhesions (Fig. 6 B, left) and restored DNA synthesis (Figs. 1 D and 6 B, right). By expressing talin fragments, we found that the talin head domain (TlnHD; aa 1–435) had no effect on proliferation, whereas the rod domain (aa 435–2,541) rescued both pFAK phosphorylation within adhesions and DNA synthesis (Fig. 6 B). To further dissect the regions within talin's rod domain that are involved with cell cycle regulation, we prepared additional talin constructs. The N-terminal half of talin (HDVBS; aa 1–1,975) did not localize to adhesion complexes, it did not lead to FAK phosphorylation, and neither did it rescue cell cycle progression (Fig. 6 C). In contrast, the C-terminal end of talin's rod (talinC; aa 1,974–2,541) was able to rescue both FAK phosphorylation and DNA synthesis. These results suggested that talin transduces the downstream effects of integrins through the C-terminal region of its rod domain.

To obtain a more mechanistic insight into the regions within talinC that are required for proliferation, we performed detailed analysis using specific point mutations (Fig. 7 A). First, we mutated amino acids within talinC helix 50, which is involved with β integrin binding (Gingras et al., 2009). L2094A/I2095A disrupts the α-helical structure of IBS2 and prevents talin recruitment to adhesions of Tln$^{-/-}$ fibroblasts (Moes et al., 2007). K2085/K2089 binds two conserved outward-facing glutamines in the membrane proximal region of β integrin tails, and the KK/DD double mutation prevents integrin binding and adhesion complex localization in CHO cells (Rodius et al., 2008). However, in MECs, neither of these pairs of mutations prevented talinC localization to adhesions, vinculin recruitment, or FAK phosphorylation, and they both rescued DNA synthesis in talin-depleted cells (Fig. 7, B [third and fourth row] and C).

The talinC, KK/DD, and LI/AA mutants that rescued FAK phosphorylation and cell cycle progression also recruited vinculin to adhesions (Fig. 7 B). However, TlnHD and HDVBS were not able to recruit vinculin (unpublished data). This suggests that talin's ability to bind vinculin might be required for FAK phosphorylation and cell cycle progression. TalinC contains VBSs within helices 50 and 58, and a V2087G;L2091S;I2352G mutation reduces binding to the vinculin head (Himmel et al., 2009). This triple mutation prevented talinC's ability to recruit vinculin, precluded FAK phosphorylation, and failed to rescue the cell cycle defect (Fig. 7, B [fifth row] and C). Thus, the ability of talinC to bind vinculin is required for both FAK phosphorylation and proliferation.

We also examined the C-terminal end of talinC. R2526 faces outwards in the C-terminal helix 62 and is necessary for
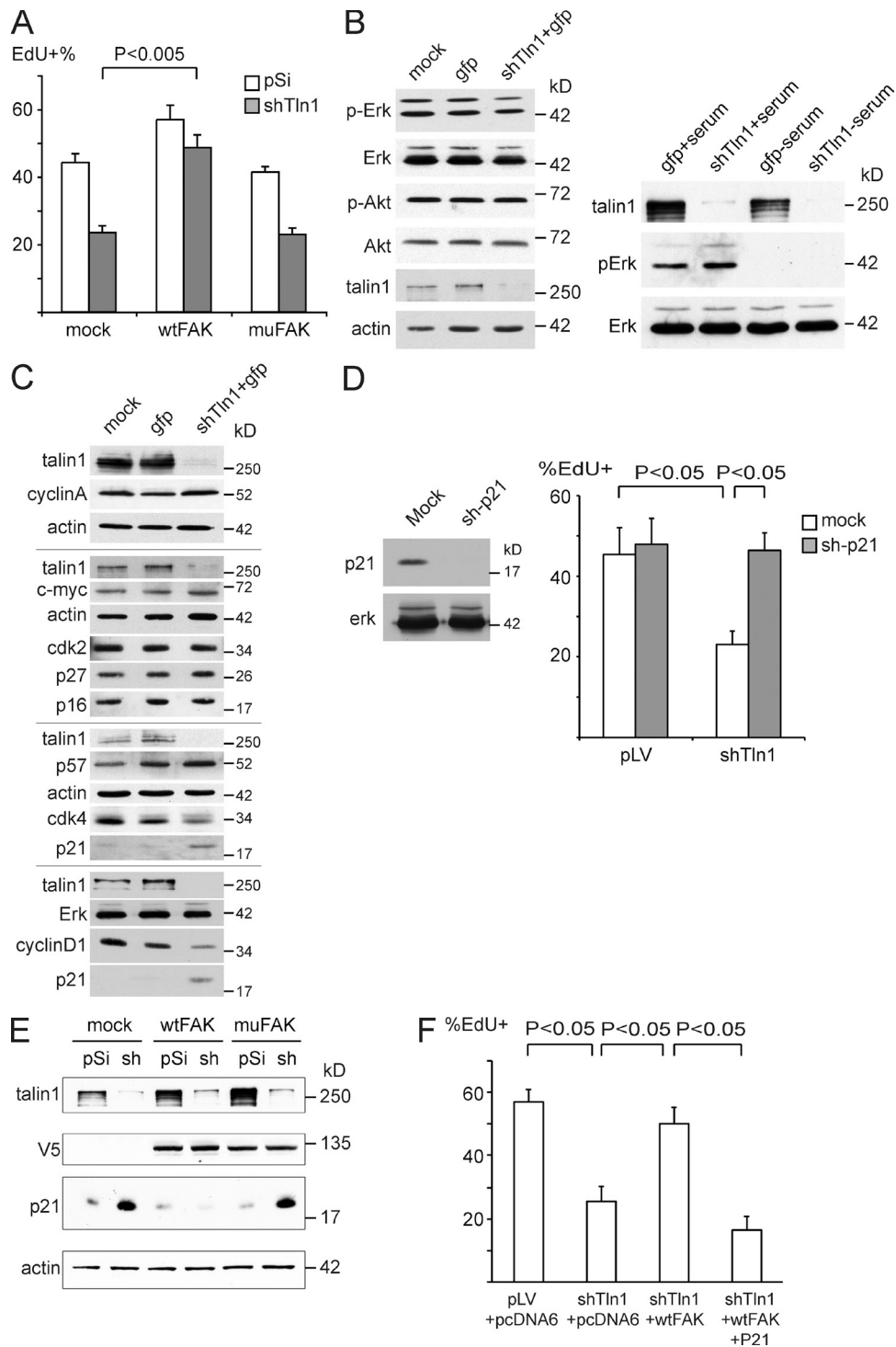
Figure 5. **FAK links talin with the cell cycle.** (A) MECs were cotransfected with shTln1 or empty vector (pSilencer [pSi]) together with vectors encoding wt-myrFAK-V5 (wtFAK) or an autophosphorylation site mutant, mu-myrFAK (muFAK). Replated cells were then labeled with EdU to evaluate proliferation. (B, left) FACS-sorted mock-infected, GFP-only, or shTln1-infected cells cultured in the continuous presence of serum were immunoblotted for signaling proteins Erk and Akt. Actin is the loading control. (right) Alternatively, cells were serum starved, then treated with or without serum for 6 h, and immunoblotted for talin, pErk, and total Erk. (C) Cells infected as in B (left) were blotted for positive and negative Cdk regulators, the cyclins, and the Cdk inhibitors. The lines separate independent blots, each showing talin knockdown and loading controls for either actin or Erk. (D, left) To deplete MECs of p21, they were transfected with either shRNA for p21 (sh-p21) or empty pLVTHM (pLV; mock) and cultured (48 h). After sorting for GFP-positive cells, whole-cell lysates were immunoblotted for p21. Erk was using as a loading control. The p21 band was confirmed by virtue of its strong up-regulation in aphidicolin-treated cells (not depicted). (right) MECs were cotransfected with either sh-p21 or mock (pLVTHM) together with either shTln1 or pLVTHM (control for shTln1), cultured (48 h), and labeled with EdU. Note that shTln1 blocks proliferation, but this is rescued by cotransfection with sh-p21. (E) MECs transfected as in A were extracted and immunoblotted for p21. Note that shTln1 induces expression of p21, which is reversed by cotransfection with wt-myrFAK but not mu-myrFAK. (F) MECs were transfected with controls (pLV + pcDNA6) or with vectors for either just talin knockdown (shTln1 + pcDNA6), talin knockdown and wt-myrFAK rescue (shTln1 + wtFAK), or talin knockdown and FAK rescue together with excess p21 (shTln1 + wtFAK + p21). Note that talin depletion blocked proliferation, which was rescued by wt-myrFAK, but the rescue was prevented by overexpressed p21. Error bars indicate SEM.
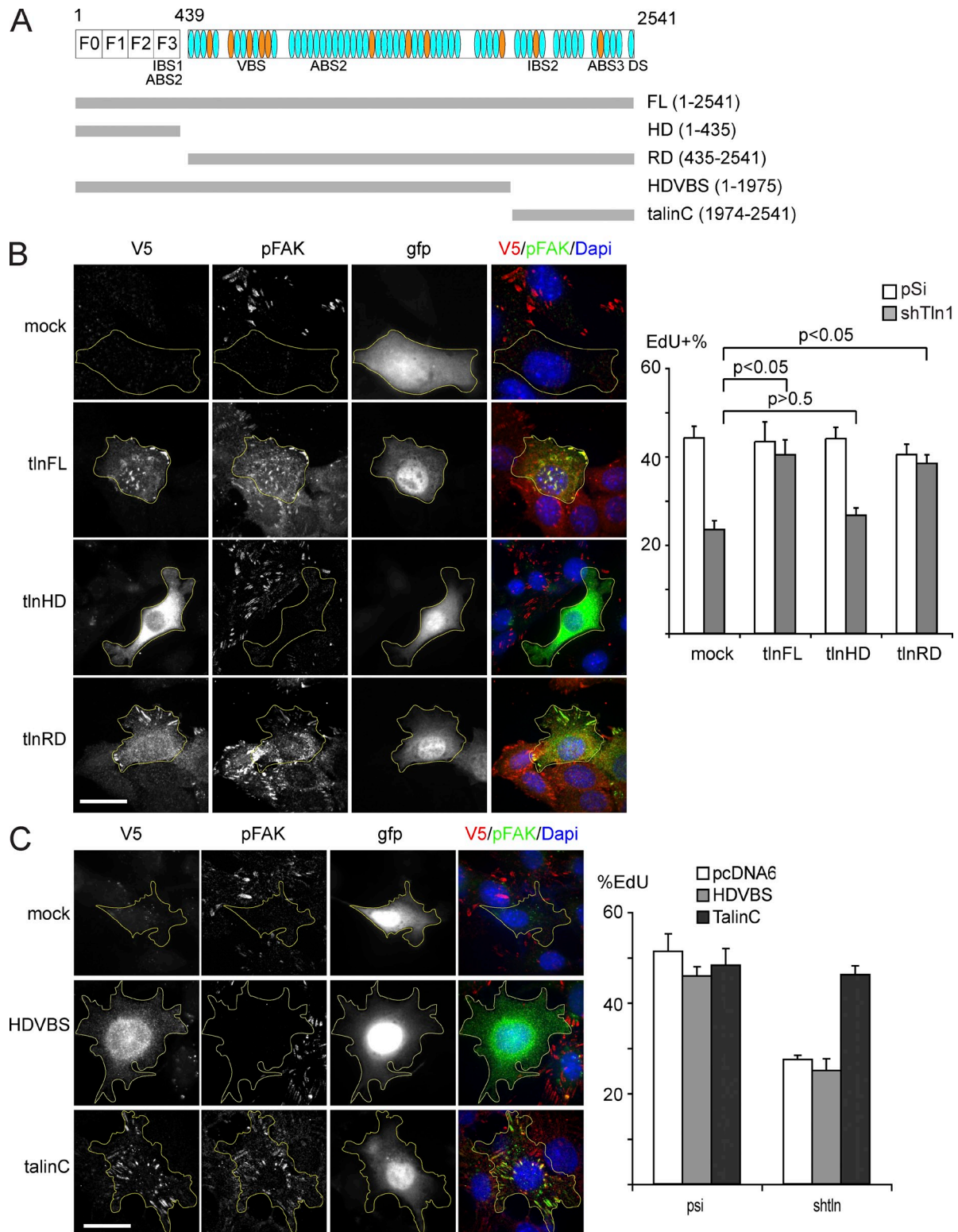
Figure 6. **The talin rod domain is required for efficient cell cycle in MECs.** (A) Map of human talin1 domains, with amino acid numbers. The gray boxes denote constructs used for rescue experiments. The N-terminal head contains four FERM domains F0–F3. The C-terminal rod contains 62 amphipathic α helices, shown as ovals. The vinculin binding sites (VBS) are in orange. The positions of actin-binding sites (ABS), integrin binding sites (IBS), and the dimerization domain (DS) are shown. The figure was adapted from Critchley (2009). FL, full length. (B, left) MECs were cotransfected with shTln1 and vectors encoding human talin1-V5 tag fusion proteins for full-length talin (tlnFL), the talin head (tlnHD), and rod domain (tlnRD). After 48 h, cells were replated (overnight) and costained for pFAK and V5. (right) Cells expressing human talin1 constructs with either shTln1 or empty plasmid (pSilencer [pSi]) were labeled with EdU to evaluate proliferation. (C, left) MECs were cotransfected with shTln1 and vectors encoding human talin1-V5 tag fusion proteins for the N-terminal half of talin (HDVBS) or the C-terminal portion (talinC). After 48 h, cells were replated (overnight) and costained for pFAK and V5. (right) Cells expressing human talinC, HDVBS, or control pcDNA6 together with either shTln1 or pSilencer were labeled with EdU to evaluate proliferation. The yellow lines demarcate the cell periphery. Error bars indicate SEM. Bars, 15 μm.
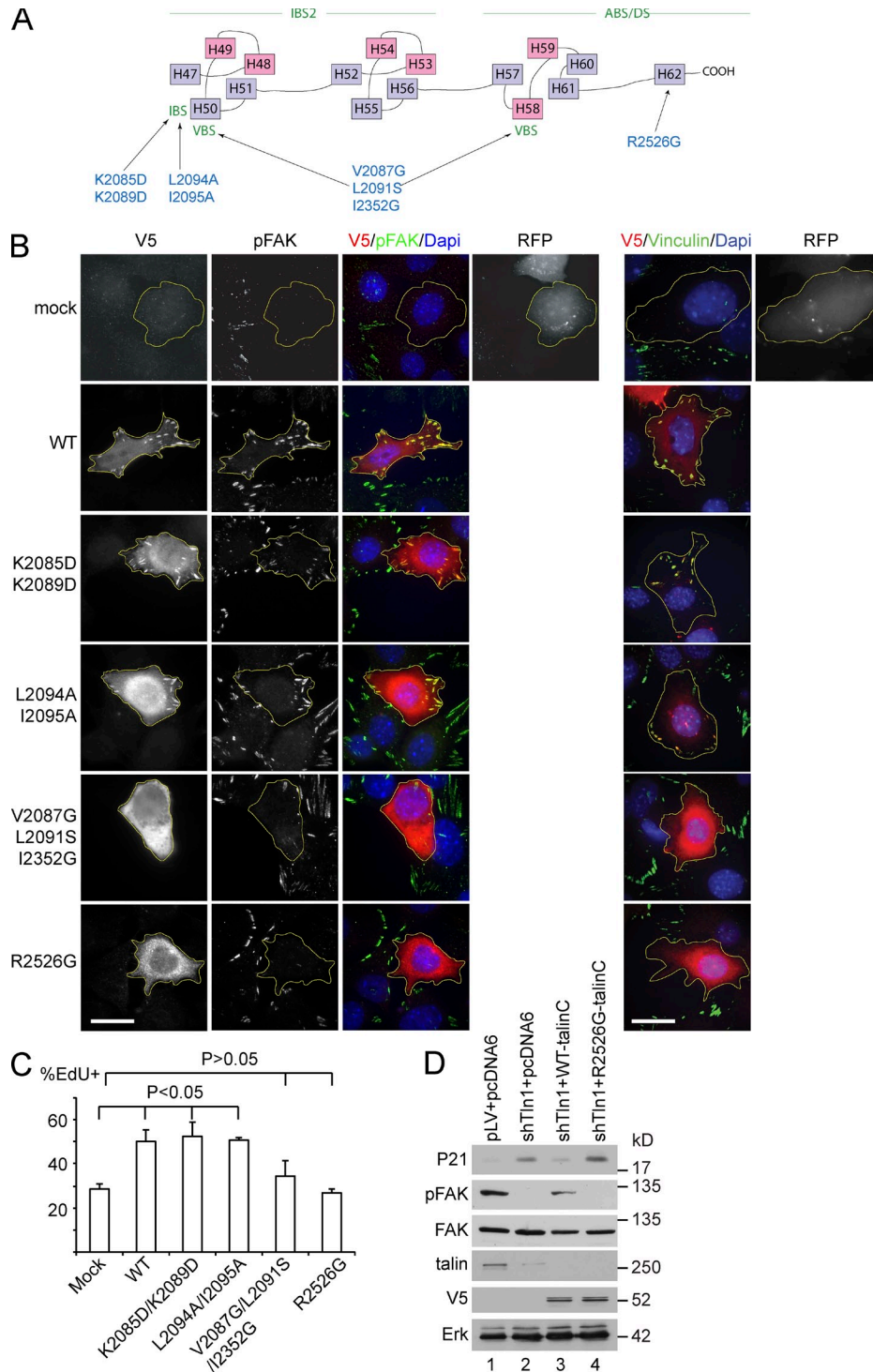
Figure 7. **The C-terminal talin dimerization motif is necessary for cell cycle.** (A) Map of talin1 C-terminal α-helical bundles, with specific helix numbers (shown in pink if they run antiparallel), and locations of the IBS2 double helical bundle and the C-terminal ABS helical bundle coupled to the DS domain. Mutations within the IBS and VBS sites and the dimerization domain are as shown. Each mutant was constructed as a V5 tag fusion in pcDNA6. (B) MECs were cotransfected with shTln1 and vectors encoding wild-type (WT) talinC or talinC mutations in ΔIBS (K2085D/K2089D and L2094A/I2095A), ΔVBS (V2087G/L2091S/I2352G), and ΔDS (R2526G). shTln1 together with empty pcDNA6 was used as a mock. Cells were costained for V5 (to identify location of transfected talinC constructs) and either pFAK (left) or vinculin (right). Note that wild-type talinC (second row) and the ΔIBS mutants (third and fourth rows) localized to adhesion complexes and recruited pFAK and vinculin, whereas ΔVBS and ΔDS (fifth and sixth rows) did not. The yellow lines demarcate the cell periphery. (C) Similar cultures to B were stained for EdU, and the percentage of EdU staining in the transfected cells was determined. Note that there was no significant difference in proliferation between MECs with talin1-only knockdown (i.e., mock) and those expressing shTln1 together with talinC ΔVBS or ΔDS. P-values are compared with mock. (D) MECs were transfected similarly to those in B, but instead of immunostaining, GFP+ cells were FACS sorted, and whole-cell lysates were blotted for p21, pFAK, total FAK, talin, the V5 epitope, and Erk as a loading control. Note that the cells expressing shTln1 with control pcDNA6 vector (lane 2) contained no pFAK and expressed p21 (these cells are equivalent to mock in C, in which cell cycle was suppressed). FAK was phosphorylated, and p21 was largely abolished by rescue with wild-type talinC (lane 3) but not with talinC-ΔDS (lane 4; these cells are equivalent to wild type and R2526G in C). Error bars indicate SEM. Bars, 15 µm.

talin both to bind F-actin and to form dimers (Gingras et al., 2008). An R to G mutation at this site prevented the ability of talinC to localize to adhesions and to rescue DNA synthesis in talin-depleted MECs (Fig. 7, B [bottom row] and C). This experiment suggests that talin dimerization and actin binding are necessary for vinculin recruitment, FAK phosphorylation, and p21 suppression (Fig. 7 D).

Together, the results distinguish two separate functions for talin. Talin's head binds and activates integrin (Ye et al., 2010). However, the TlnHD failed to cause FAK phosphorylation within MEC adhesions or to permit proliferation, indicating that this domain does not provide the essential link for other proteins to deliver sustained downstream signaling. In contrast, provided it is able to dimerize, the C-terminal region of talin's tail recruits vinculin and leads to FAK phosphorylation, which activates downstream events required for proliferation. We suggest that the ability of talinC to become localized to adhesion complexes and, thereby, influence cell cycle progression is mediated through a cooperative binding to vinculin and actin and self-dimerization, which in turn is required for FAK phosphorylation.

## Discussion

Previous studies have demonstrated that talin is important for integrin activation, transducing traction forces, and for sustained cell spreading and adhesion (Priddle et al., 1998; Giannone et al., 2003; Tadokoro et al., 2003; Zhang et al., 2008; Kopp et al., 2010). Here, we identified two key roles of talin in MECs. First, we found that talin coordinates the recruitment of the adhesion proteins vinculin, FAK, paxillin, and ILK to adhesion complexes in these cells. Second, we discovered that the C-terminal tail region of talin controls cell cycle progression.

Thus, talin has several roles, all of which are central to integrin function. One part of the molecule, its head, mediates outward conformational changes to activate integrins, whereas another part, the rod, both links to the cytoskeleton and intracellular pathways required for cell cycle progression (Fig. 8).

### Talin coordinates adhesion complex assembly

Talin is widely viewed as a key structural protein of integrin adhesion complexes that both regulates the affinity of integrin for ECM ligands and links adhesions to the actin cytoskeleton (Anthis et al., 2009; Critchley, 2009; Ye et al., 2010). In MECs, knocking talin1 down to low levels has surprisingly minor effects on adhesion and spreading, and the cells retain stress fibers. This is in contrast to MEFs, suggesting that the spreading of epithelial cells is less sensitive than fibroblasts to talin knockdown (Zhang et al., 2008). It is possible that the low levels of talin remaining after knockdown are sufficient for integrin activation and for cell spreading. Alternatively, the results may reflect different mechanisms of integrin–actin linking in different cell types (Otey et al., 1990; Calderwood et al., 2003). For example, in MECs, interactions between adhesion complexes and the cytoskeleton might be compensated for by other integrin-binding proteins, such as tensin (Liu et al., 2000; Legate and Fässler, 2009).
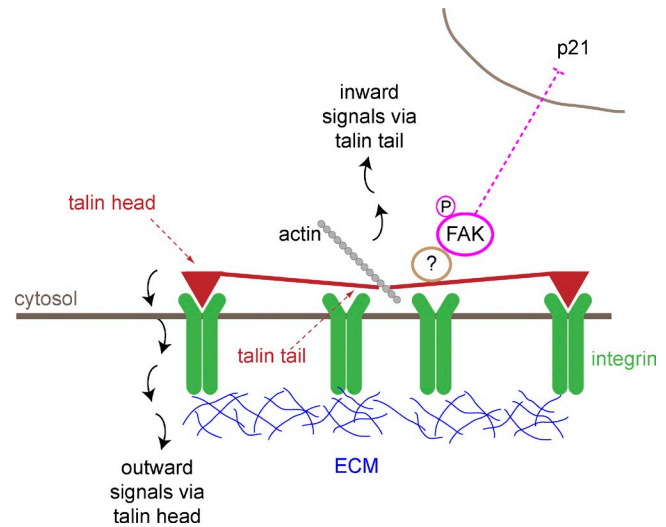


Figure 8. **Talin is a bidirectional transducer of integrin signals.** Our study reveals that talin is a bidirectional transducer of integrin signals, both activating adhesion and regulating cell behavior. Talin's head activates integrins, providing outward conformational changes that lead to high affinity interactions of the extracellular portion of integrins with ECM proteins. This has been well established in the literature. Our new results show that talin's C-terminal tail is involved in recruiting vinculin and FAK to adhesion complexes and that this domain of talin is involved with cell cycle progression via p21 suppression. P, phosphorylation.

Because MECs remain spread after talin knockdown, we were able to identify which adhesion complex proteins are directly recruited by talin. This includes the cytoskeletal adaptor proteins, vinculin, paxillin, and ILK, and the adhesion signaling enzyme, FAK. Talin is the earliest component to be recruited to adhesions after integrin activation, suggesting that it has a key scaffolding function to recruit adhesion complex adaptors and enzymes (Partridge and Marcantonio, 2006). In myotendinous junctions of skeletal muscle, talin also has a role for recruitment of vinculin and ILK (Conti et al., 2009).

### Differential targeting of the talin head and tail domains to adhesions of talin-depleted cells

The adhesions of talin-depleted MECs are able to recruit exogenous full-length V5-tagged talin but not its head domain. Others have found, similarly, that the talin head does not localize to adhesions and cannot rescue adhesion complex formation in Tln$^{-/-}$ cells (Zhang et al., 2008; Himmel et al., 2009). This may be caused by structural constraints because the lack of FAK activation precludes PIPK1-γ–talin-F3 interactions or because the Rap1-GTP–interacting adaptor molecule cannot recruit the talin head to integrins in the absence of other signals (Barsukov et al., 2003; Lee et al., 2009; Elliott et al., 2010). Similarly, the N-terminal head/rod HDVBS fragment of talin was not recruited to adhesions in talin-depleted cells. This may be caused by an internal interaction between one of the head's FERM domains and a 5-α-helical bundle in the rod, which precludes integrin binding if talin's C-terminal domain is absent (Goksoy et al., 2008; Goult et al., 2009). Alternatively, the lack of a C-terminal ABS could prevent HDVBS from being stretched by the cytoskeleton, preventing exposure

of the cryptic VBS at helix 4 and thereby recruitment to adhesions (del Rio et al., 2009).

In contrast to the N terminus, the talin C-terminal tail domain was readily recruited to adhesion sites in cells depleted of endogenous talin. Candidate regions for adhesion targeting include an IBS, two VBSs, and the dimerization/F-actin–binding C terminus. Because talinC-containing mutations within the IBS region still targeted to adhesions of talin-depleted MECs, we made subtle mutations either within the C-terminal VBS helices 50 and 58 to prevent vinculin binding or within helix 62 to inhibit dimerization and actin binding (Gingras et al., 2008). Both of these mutations prevented the ability of talinC to target to adhesions and to recruit vinculin. Vinculin has a key role in linking adhesions with F-actin, suggesting that the vinculin-binding ability of talinC might contribute to adhesion targeting (Humphries et al., 2007). Being that talinC binds to F-actin within adhesion complexes, rather than broadly decorating stress fibers, our data suggest that talinC requires cooperative binding to vinculin, integrin, and actin to be targeted to adhesion sites.

### Talin links integrins with cell cycle progression via FAK

The observation that talin knockdown inhibits cell cycle progression reveals a key role for talin in controlling cell signaling. The experiments in which we forced cells to spread on FN-coated islands of defined size indicate that the inhibition of proliferation in talin-depleted cells is not a result of the cells being unable to adhere to ECM substrata via integrins or to spread appropriately on the ECM (Chen et al., 1997). By depleting talin in MECs to such a level that integrins remain active and the cells can spread on ECM, we were able to uncover a correlation between recruitment of adhesion complex proteins and proliferation.

Integrins are crucial for cell cycle in the mammary gland. $\beta 1$ integrin gene deletion in vivo inhibits the proliferation of MECs, with a concomitant decrease of FAK activity and an increase of p21 expression (Li et al., 2005). The link between integrin, p21, and cell cycle was confirmed genetically in that study, and we have now extended those conclusions by identifying a pathway connecting integrin with p21 expression.

Several lines of evidence in our study suggest that FAK might be a candidate for linking talin with proliferation. First, endogenous FAK is phosphorylated in the adhesions of shTln1 cells expressing only the talin constructs that rescue cell cycle progression. Second, membrane-targeted FAK colocalizes with integrin adhesions, and the wild type, but not an autophosphorylation mutant, rescues proliferation in the absence of talin. Third, the rescue of proliferation by myrFAK is suppressed by additional expression of p21. Fourth, p21 is up-regulated in talin-depleted cells, and this is reversed by wild type, but not mutant, myrFAK. Fifth, shTln1-induced p21 expression is suppressed by talinC but not a mutant that cannot rescue proliferation, and finally depleting p21 rescues the proliferation defect in the absence of talin. Together, these results delineate an integrin-mediated pathway via talin and FAK that is required in MECs to suppress p21 and permit cell cycle progression in the presence of growth factors.

The mechanism of FAK recruitment to integrin adhesions is potentially complex because it may be targeted there by a variety of possible FAK-interacting proteins, which could vary among different cell types. Our data suggest that in MECs, the talin C terminus is required to recruit FAK to adhesion sites. There are no known FAK-binding sites in the rod domain of talin, so it is likely that FAK binds indirectly. We have found that vinculin depletion in MECs largely precludes FAK phosphorylation within adhesion sites without affecting endogenous talin localization, so vinculin may have an indirect role (unpublished data).

FAK supports proliferation in many cell types (Lim et al., 2010; Zhao et al., 2010). In the mammary gland, its role has been studied in tumor initiation and growth, in which FAK overexpression correlates with a majority of human breast cancers, and FAK deletion slows proliferation and cancer formation in animal models. The requirement for FAK in mammary tumor formation is linked to phosphorylated Erk, Src, and Crk-associated substrate (Cas; Provenzano et al., 2008; Pylayeva et al., 2009). In our study, the autophosphorylation FAK mutant did not rescue proliferation in MECs, indicating a likely requirement for a pY397-binding, SH2-containing protein. Neither Erk nor Akt phosphorylation were altered in talin-defective MECs, suggesting that it is unlikely that the control point is via these pathways. This is supported by other studies in MECs, in which $\beta 1$ integrin gene deletion or suspension culture does not alter growth factor–induced Erk and Akt signaling (Wang et al., 2004; Naylor et al., 2005). An alternate possibility is that Cas, which is activated by FAK-pY397–bound Src and binds prolines within FAK's C terminus, provides the proximal link between FAK and proliferation. Cas overexpression causes mammary hyperplasias, and Cas–FAK interactions are required for the latter's proliferative ability in primary MEC culture (Cabodi et al., 2006; Pylayeva et al., 2009). Src phosphorylation was not altered in talin-depleted cells, but this may not be surprising as it remains phosphorylated in FAK$^{-/-}$ MECs and can be activated independently by growth factor receptors (Yeatman, 2004; van Miltenburg et al., 2009). p190RhoGEF also connects to FAK and has a role in fibroblast proliferation, so this could also be involved together with Pyk2 (Lim et al., 2008b).

Downstream of components within the adhesion complex, the current literature has not fully clarified how FAK might control p21 levels and thereby cell cycle progression. A possible mechanism is via ubiquitination. For example, in endothelial cells, FAK causes the degradation of p27 and p21, which for p27 is Skp2 dependent, and FAK inhibition up-regulates both these Cdk inhibitors (Bryant et al., 2006). Our data indicate a role for p21 in the integrin/talin control of MEC proliferation, so FAK may determine the levels of p21 either through Skp2-independent degradation or via p53-mediated transcription (Lim et al., 2008a).

### In summary

Our work pinpoints a central role for the C terminus of talin in recruiting core components to the adhesion complexes of MECs. This is crucial for cellular physiology because some of

these proteins are adaptors that link ECM–integrin interactions to distal cell fate decisions. In the current study, we uncovered a role for talin in linking integrins with proliferation, and our data delineate an integrin–talin–FAK pathway required for p21 suppression. Talin also has a role in cell cycle regulation of a metastatic mammary cancer line, 4T1, lending credence to the idea that targeting proteins downstream of integrins might represent a therapeutic route of intervention in cancer patients. Future studies will focus on the molecular mechanisms by which talinC is recruited to adhesion complexes, how vinculin and FAK are recruited to adhesions by talinC, and the pathways downstream of adhesion complexes that determine p21 levels and cell cycle progression in MECs.

## Materials and methods

### Cell culture
FSK7 is a mouse MEC strain isolated from luminal epithelial cells and was used at a low passage number (Kittrell et al., 1992). FSK7 cells were cultured in DF12 medium (BioWhittaker; Lonza) supplemented with 5 ng/ml EGF, 880 nM insulin, and 2% FCS at 37°C in a humidified atmosphere of 5% $CO_2$. Primary MECs were extracted from 19-d pregnant mice and cultured in growth medium containing 5 µg/ml insulin, 1 µg/ml hydrocortisone, 3 ng/ml EGF, 10% FCS, 50 U/ml penicillin/streptomycin, 0.25 µg/ml fungizone, and 50 µg/ml gentamycin in F12 medium (Pullan and Streuli, 1996). For growing lentivirus, 293T was cultured in DME supplemented with 10% FCS.

### DNA constructs
shRNA for talin1 was designed with siRNA Target Finder (Invitrogen), and the sequences used were shTlnA, 5′-AAGAAGCACAGAGCCGATTGA-3′, and shTlnB, 5′-AAGAACAAGATGGATGAATCA-3′. The scrambled shRNA sequence for talin1 contained the same nucleotides as those for shTlnA, 5′-GACAGATGAGGAGCAATCACA-3′, and this sequence did not reveal any known targets by a BLAST (basic local alignment search tool) search. The shRNA sequence for p21 was 5′-TTAGGACTCAACCGTAATA-3′. The shRNAmir for β1 integrin was 5′-GGCTCTCAAACTATAAAGAAA-3′ (provided by P. Stern, Massachusetts Institute of Technology, Cambridge, MA).

Doubled-stranded oligonucleotides were cloned into the shRNA transfer vectors pSilencer 3.1 (Invitrogen) or pLVTHM (Tronolab). pLVTHM was used in most of the experiments and was used to generate lentiviruses. In a few experiments, the same sequences in pSilencer, which does not also express GFP, were used in transfection experiments, and the empty vector was used as a control (Figs. 5, A and E; and 6, B and C, pSi). The extent of knockdown with pSilencer is the same as for pLVTHM. In the case of sh–β1 integrin-mir, the transfer vector was pLB2Cap2Gm (pLB2).

For rescue vectors, human full-length wild-type talin1 cDNA was obtained from Source BioScience LifeSciences. To avoid deletion by shTln1, specific mutations were created by using site-directed PCR and mutagenic oligonucleotides (QuikChange; Agilent Technologies). The shRNA target sequence for mouse talin1 is nucleotides 3,707–3,728, which contains two variations from the corresponding human talin1 sequence (A → T and A → G), and additional G → A and C → T mutations were placed in the human talin1 sequence (no amino acid change). The plasmids containing shTln1-resistant human talin1 or FAK constructs were cotransfected with pLVTHM-shTln1 using Lipofectamine plus (Invitrogen).

Full-length talin (aa 1–7,623), head domain (aa 1–1,305), and rod domain (aa 1,305–7,623) as well as HDVBS (aa 1–5,925) and talinC (aa 5,922–7,623) were cloned into pcDNA6/V5-His(A) (Invitrogen). Additional talinC mutants described in this paper were created by using site-directed PCR and mutagenic oligonucleotides.

wt-myrFAK or an autophosphorylation-site Y397F mutant form of FAK (mu-myrFAK) was generated by cloning the viral Src myristoylation sequence N terminal to the FAK coding sequence in pcDNA6/V5-His(A) and provided by A. Gilmore (University of Manchester, Manchester, England, UK). Full-length p21 cDNA (Thermo Fisher Scientific) was generated in pCMV-SPORT6.

### Lentiviral infection
The lentiviral shRNA system was provided by D. Trono (University of Geneva, Geneva, Switzerland). For lentivirus production, the transfer vectors were cotransfected with the envelope plasmid pMD2G and the packaging plasmid psPAX2 by the calcium phosphate method into 293T cells that had been precultured for 8–16 h. Media were replaced after 8–10 h. 10 ml viral supernatants was harvested 48–60 h after transfection, passed through a 0.45-µm filter, and further concentrated by centrifugation at 25,000 rpm at 4°C for 2.5 h. Viral pellets were resuspended in 0.1 ml fresh DF12 medium.

Lentiviral infection was performed by adding the appropriate amount of lentiviral particles directly to precultured 75% confluent cells. Dishes were spun at 3,000 rpm at 37°C for 20 min to increase the infection rate followed by incubation for 3 h. The infected cells were cultured for 48–72 h before being replated for further experiments. This protocol was necessary to ensure turnover of preexisting talin.

Depending on the batch of lentivirus, we normally obtained a 70–100% efficiency of infection in MECs. For some experiments, we FACS sorted the GFP+ cells to enrich to ∼100% infected cells for the endpoint assays.

### Immunofluorescence
Cells were fixed with 4% formaldehyde for 10 min, permeabilized for 5 min with 0.5% Triton X-100 (Sigma-Aldrich), and subsequently incubated for 1 h with primary antibodies directed against the proteins indicated in this section followed by the appropriate conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). For actin fluorescence staining, Alexa Fluor 647–phalloidin (Invitrogen) was added to cells together with the secondary antibody. For integrin staining, cells were briefly treated with 0.5% CHAPS before fixing.

In the case of costaining for talin1 with the other monoclonal antibodies for vinculin, paxillin, ILK, and Py20, the antitalin antibody was preconjugated to Alexa Fluor 548 by using a monoclonal antibody-labeling kit (Invitrogen). Cells were incubated with monoclonal antibodies followed by a Cy5-conjugated donkey anti–mouse fragment antigen-binding region (Jackson ImmunoResearch Laboratories, Inc.), fully washed with PBS, and then the Alexa Fluor 548–antitalin antibody was added to stain talin1. Antibodies used for immunostaining were as follows: β1 integrin (Klinowska et al., 1999); FAK (a gift from L. Romer, Johns Hopkins University School of Medicine, Baltimore, MD); 9EG7, paxillin, and py20 (BD); pFAK-Y397 (Invitrogen); V5 and GFP (Invitrogen); secondary mouse Cy2 and Rhodamine Red-X (Rhrx), rabbit Cy2 and Rhrx, and rat Rhrx (Jackson ImmunoResearch Laboratories, Inc.); talin and vinculin (Sigma-Aldrich); and ILK (Millipore).

After staining, coverslips were mounted with antifade reagent (ProLong; Invitrogen). Images were acquired on a restoration microscope (DeltaVision RT; Applied Precision) using either a 100×/1.40 Uplan S Apochromat or a 60×/1.42 Plan Apochromat objective and the Sedat filter set (89000; Chroma Technology Corp.). The images were collected using a camera (CoolSNAP HQ; Photometrics) with a z optical spacing of 0.2 µm. Raw images were then deconvolved using the softWoRx software (Applied Precision), and maximum intensity projections of these deconvolved images are shown in the Results and followed by analysis with ImageJ 3.4 (National Institutes of Health) or Openlab 4 software (PerkinElmer).

### Immunoblotting
Equivalent amounts of protein were separated by SDS-PAGE and immunoblotted for proteins. Detection was achieved with peroxidase-conjugated anti–rabbit, anti–goat, and anti–mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) followed by enhanced chemiluminescence (GE Healthcare). Antibodies different to those used in immunostaining were as follows: talin1- and talin2-specific antibody (a gift from D.R. Critchley, University of Leicester, Leicester, England, UK); FAK (a gift from A. Ziemiecki, University of Bern, Bern, Switzerland); p21 (BD); pY31-paxillin (Invitrogen); pT202/Y204-p42/44-MAPK, Akt, pS473-Akt, pY416-Src, and pY527-Src (Cell Signaling Technology); c-myc (Roche); cyclin A, cyclin D1, Cdk2, Erk2, Src, p16, and p27 (Santa Cruz Biotechnology, Inc.); and actin and p57 (Sigma-Aldrich).

### Adhesion assay
Cell adhesion experiments were performed as previously described (Edwards and Streuli, 1999). In brief, wells of 96-well plates were precoated with ECM proteins as indicated 4°C overnight. After blocking with 0.5% bovine serum albumin, $10^5$ cells were added into wells in triplicate and incubated for 2 h at 37°C. Wells were washed with PBS, and the adherent cells were quantified by labeling with 5 mg/ml Crystal violet (Sigma-Aldrich) and reading the absorbance at 595 nm.

### dU incorporation
Replated cells were cultured for 16 h and then labeled with either 10 µM BrdU or EdU for 2 h before fixation. Cells were stained with either anti-BrdU antibodies or with the EdU Alexa Fluor 647 imaging kit (Click-iT;

Invitrogen). BrdU/EdU staining was quantified within the GFP-positive, infected/transfected cells. 100–200 cells were counted per experimental point. Error bars are SEM. Statistical data were obtained using Student's *t* test.

For the spreading on micropatterned substrata, coverslips were provided by C. Chen, M. Wozniak, and G. Lin (University of Pennsylvania, Philadelphia, PA). The pattern was etched into a polydimethylsiloxane surface, and the $25 \times 25$–µm adhesive areas were coated with FN. $10^5$ lentiviral infected cells were plated into 35-mm dishes containing micropatterned coverslips for 16 h to allow cell spreading and then fixed after EdU staining as in the previous paragraph.

### Online supplemental material
Fig. S1 shows control experiments. Fig. S2 shows that talin1 depletion causes a severe attachment deficiency in fibroblasts. Fig. S3 shows that tensin may provide a link between adhesion complexes and F-actin in talin1-deficient cells. Fig S4 shows staining for total FAK. Fig S5 shows that Src remains phosphorylated in talin-depleted MECs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201104128/DC1.

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