Microtubule plus-end binding protein EB1 is necessary for muscle cell differentiation, elongation and fusion

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Accepted 5 January 2009
Journal of Cell Science 122, 1401-1409 Published by The Company of Biologists 2009
doi:10.1242/jcs.039255

Summary

During muscle differentiation, microtubule stability, nucleation and orientation all undergo profound changes, which are simultaneous with and possibly necessary for the elongation and fusion of muscle cells. We do not yet understand these events, but they present similarities with the polarized migration of fibroblasts, in which EB1 is necessary for microtubule stabilization. However, it was recently reported that EB3, not EB1, is involved in muscle cell elongation and fusion, and that neither of these two proteins influences microtubule stabilization. To re-examine the role of EB1, we have generated C2 cell lines permanently expressing EB1-targeted shRNAs. In these lines, EB1 is specifically knocked down by more than 90% before any differentiation-related changes can take place. We find that differentiation (assessed by myogenin expression), elongation and fusion are prevented. In addition, two early events that normally precede differentiation – microtubule stabilization and the accumulation of cadherin and β-catenin on the plasma membrane – are inhibited. Re-expression of EB1 as EB1-GFP restores all aspects of normal differentiation, whereas overexpression of EB3-GFP restores elongation but not fusion. We conclude that EB1 is necessary for the early stages of muscle differentiation.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/9/1401/DC1

Key words: EB1, Microtubules, Skeletal muscle differentiation

Introduction

Skeletal muscle cell differentiation involves remarkable changes in morphology as myoblasts differentiate, elongate and fuse into multinucleated myotubes. As part of these changes, the microtubule cytoskeleton and the organelles associated with it are reorganized (Tassin et al., 1985; Ralston, 1993; Lu et al., 2001). The classic network of microtubules nucleated at the centrosome changes into an array of mostly parallel longitudinal microtubules that are not focused on centrosomes (Tassin et al., 1985). In addition, an increasing fraction of the microtubules is stabilized; they become resistant to depolymerization by cold or by nocodazole treatment (Gundersen et al., 1989), and show post-translational modifications, for example, an increase in detyrosinated α-tubulin (glu-tubulin), resulting from removal of the C-terminal tyrosine of tubulin by tubulin carboxypeptidase (Gundersen et al., 1989; Bulinski and Gundersen, 1991). Other post-translational modifications of microtubules such as acetylation (Gundersen et al., 1989) also take place. We know little of the role and regulation of microtubule organization and post-translational modifications in muscle development, but the few available results suggest a need for tight regulation. For instance, highly organized microtubules provide directional cues for the positioning and organization of myosin filaments during sarcomere formation (Pizon et al., 2005). Massive pharmacological stabilization of microtubules causes formation of abnormal myotubes (Holtzer et al., 1985; Saitoh et al., 1988) and chemical alteration of the C-terminal amino acid of tubulin, preventing the action of tubulin carboxypeptidase, inhibits differentiation of L6 muscle cells (Chang et al., 2002). This latter result suggests that microtubules could have a signaling role as well as a structural one.

The signaling pathways controlling stabilization of microtubules have been studied in great detail in neurons (Lee et al., 1998; Krylova et al., 2000; Arevalo and Chao, 2005) and in fibroblasts, in which local microtubule stabilization is necessary for polarized migration into a wound (Wen et al., 2004; Eng et al., 2006). These and other studies (Nakagawa et al., 2000; Su and Qi, 2001; Komarova et al., 2002; Stepanova et al., 2003) have highlighted the role of the protein EB1 (also known as MARE1) and its relatives. EB1 was first identified as an adenomatous polyposis coli (APC)-interacting protein whose mutations are implicated in colon cancer (Su et al., 1995). EB1 binds preferentially to the extreme plus-end of growing microtubules (Morrison et al., 1998). It is also found at the centrosome (Berrueta et al., 1998; Morrison et al., 1998; Askham et al., 2002; Yan et al., 2006). Its roles in the regulation of microtubule dynamics, cell polarity, chromosome stability and cell migration have been studied extensively (reviewed by Tirnauer and Bierer, 2000; Akhmanova and Hoogenraad, 2005; Vaughan, 2005; Morrison, 2007). Importantly, EB1 is necessary and sufficient for the stabilization of microtubules in fibroblasts responding to lysophosphatidic acid

stimulation or to lithium chloride, which is a model for polarized migration (Wen et al., 2004; Eng et al., 2006). In addition, a potent microtubule elongation effect of EB1 has been revealed in an in vitro assay (Ligon et al., 2003).

There is thus a large body of data showing that EB1 is involved in microtubule stabilization in different cellular systems, and that microtubule stabilization is important and probably necessary for muscle differentiation. But whether EB1 is involved in microtubule stabilization in differentiating muscle cells has not been shown. In fact, it was recently reported that EB3 (also known as MARE3), but not EB1, has a role in myoblast elongation and fusion in the mouse muscle C2 cell line and that microtubule stabilization is independent of both EB1 and EB3 (Straube and Merdes, 2007).

We examined EB1 expression and distribution in differentiating C2 cells and investigated its function by RNA interference to knock down EB1, and by overexpression of EB1-GFP constructs. Cell lines in which EB1 is selectively and permanently knocked down (EB1 KD cells) have the advantage of lacking EB1 at the earliest stages of differentiation. We find that EB1 KD cells fail to show the normal increase in glu-tubulin, the hallmark of microtubule stabilization. Under differentiation conditions, EB1 KD cells neither elongate nor fuse, and the upregulation of the differentiation marker myogenin is reduced. In addition, the translocation of the cadherin-catenin complex to the plasma membrane, a key event in myogenic induction and myoblast fusion (Takeichi, 1991; Knudsen et al., 1998; Goichberg et al., 2001; Charrasse et al., 2002), is inhibited in the EB1 KD cells. The defects of the EB1 KD cell lines are rescued by reintroduction of EB1-GFP.

Results

EB1 level is stable throughout C2 differentiation but its distribution changes

Immunoblotting of extracts from undifferentiated C2 myoblasts in growth medium (GM) and from myotubes in fusion medium (FM) (Fig. 1A) shows that EB1 is easily detected in freshly plated cells, and its level remains relatively stable throughout differentiation. By contrast, EB3 is barely detected before but increases during C2 cell differentiation, which is reflected in the appearance of myogenin. Glu-tubulin is detectable in myoblasts before myogenin and EB3 (Fig. 1A). The pattern of EB1 in myoblasts, determined by immunofluorescence, resembles that in other cell types: EB1 is predominantly found at the far end of growing microtubules in a comet-like pattern (Fig. 1B) and, less prominently, at the centrosome. Mitotic cells in the same cultures show EB1 along the mitotic spindle and at the split centrosome (Fig. 1C). After 2 days in FM, C2 cultures contain multinucleated myotubes and mononucleated cells. Both cell types show the microtubule plus-end staining (Fig. 1D,E). In the central part of myotubes, the stained EB1 comets point in different directions and are sparse compared with myoblasts but at the myotube ends, they are denser and uniformly point outwards, suggesting a bipolar organization of microtubules. In addition, a weaker punctate staining along microtubules (Fig. 1D,E) is observed in myotubes and in unfused cells in FM, but not in young myoblasts (compare Fig. 1B,D). In areas that contain few microtubules, this staining is well resolved (arrow in Fig. 1D) but in thicker areas of the cell, in which microtubules are more numerous, it is not so well resolved (because these images are projections through the whole cell). We did not detect an accumulation of EB1 along the nuclear membrane of myotubes, which is the site of redistribution of centrosomal proteins and microtubule nucleation during myogenesis (Tassin et al., 1985; Bugnard et al., 2005).

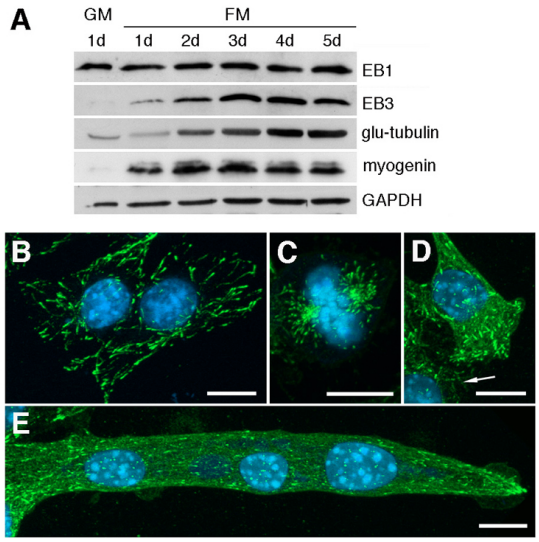

Fig. 1. EB1 is expressed throughout C2 differentiation. (A) Immunoblotting of extracts from C2 cultures in GM and FM for EB1, EB3, glu-tubulin and myogenin, with GAPDH as loading control, shows EB1 at the earliest time point, whereas EB3 is only detected after differentiation. Data shown here are representative of three different experiments. (B) Immunostaining of myoblasts in GM with mouse anti-EB1 shows the typical comet-like pattern at microtubule plus-ends, pointing from the center of the cell outwards. (C) Mitotic myoblasts, by contrast, show strong centrosomal EB1. In FM, both mononucleated cells (D) and multinucleated myotubes (E) show the comet-like pattern and a finer punctate staining along microtubules (arrow). The microtubule plus-end EB1 comets are uniformly oriented outwards at the myotube ends. Each image is the projection of a *z*-series of confocal images taken with identical imaging parameters. EB1 staining is shown in green; nuclei stained with Hoechst 33342 are shown in blue. Scale bars: 10 μm (B,D,E) and 5 μm (C).

Silencing of EB1 prevents myotube formation

Considering that EB1 is the isoform necessary for microtubule stabilization in fibroblasts and that microtubule stabilization takes place early during muscle differentiation, we decided to test the function of EB1 by knocking it down in C2 cultures using shRNAs. To ensure that EB1 was sufficiently knocked down before differentiation started, we avoided transient expression of shRNAs and instead established stable shRNA-transfected C2 cells. We used two different shRNAs targeting mRNA encoding EB1 (shRNA-2 and shRNA-3) and a control non-targeting shRNA (Fig. 2A). First, we examined pooled puromycin-selected EB1 KD (C2-sh2 and C2-sh3) and control cells (C2-shC). Compared with C2-shC cells, EB1 was knocked down by 80% and 70% in C2-sh2 and C2-sh3 cells, respectively, whereas EB3 was not affected (Fig. 2B). Immunofluorescence of C2 myoblasts confirmed, at the single cell level, that EB1 could be knocked down to below a detectable level without affecting the concentration or localization of EB3 (Fig. 2C). This was also observed in NIH3T3 cells stably expressing shRNA-2 or shRNA-3 (supplementary material Fig. S1). Transmitted light images of the cultures showed that after 2 days in FM, both C2-sh2 and C2-sh3 have strong defects in elongation and fusion compared with C2-shC cells (Fig. 2D). We also studied the role

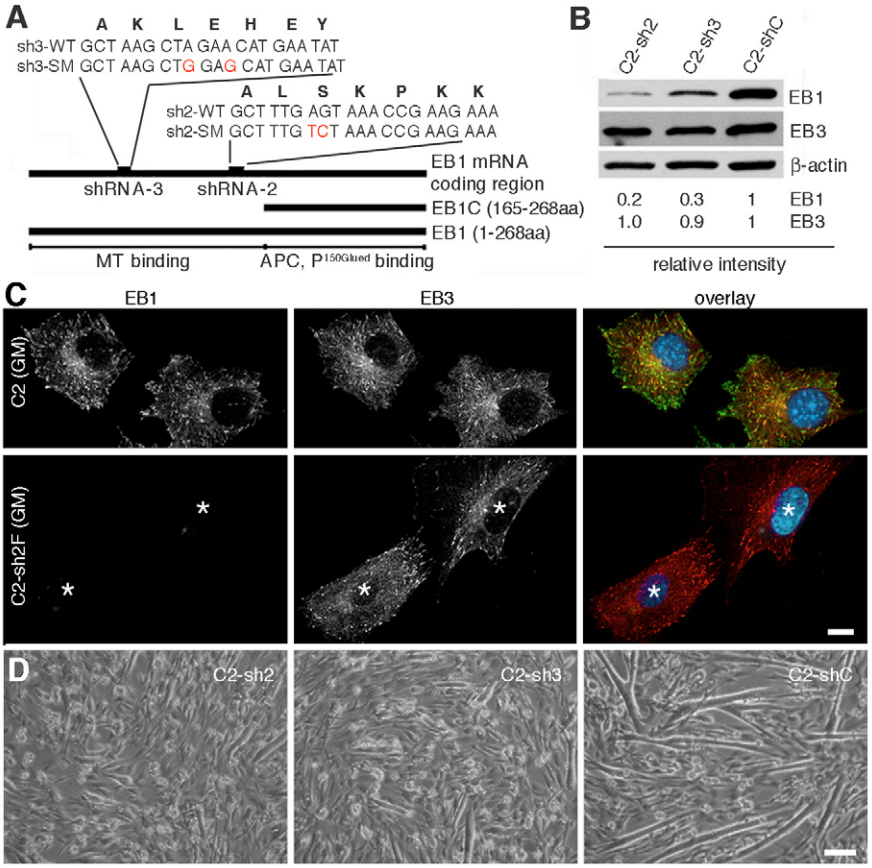

Fig. 2. Specific knockdown of EB1 with shRNAs inhibits C2 myotube formation. (A) Schematic representation of EB1 mRNA coding region with shRNA targeting sequences (sh2-WT, sh3-WT) used for knockdown of EB1. Silent mutations introduced in cDNAs used for rescue (sh2-SM, sh3-SM) are indicated in red. The lower bars show schematic representations of EB1 and EB1C proteins. (B) After 2 days in FM, extracts were prepared from pooled C2 cells stably transfected with shRNA-2 (C2-sh2), shRNA-3 (C2-sh3) or non-targeted control shRNA (C2-shC). Immunoblot from one of two independent experiments shows that the shRNAs are specific for EB1 because it is knocked down by both sh2 and sh3 whereas EB3 is unaffected. β-actin is shown as loading control. (C) Immunofluorescence staining of EB1 and EB3 in control and sh2-transfected C2 shows that EB3 is not affected in the EB1 KD cells (indicated by asterisks). Confocal images were taken with identical imaging parameters. (D) Phase-contrast images show that the level of fusion is reduced in C2-sh2 or C2-sh3 cells after 2 days in FM, compared with C2-shC. Scale bars: 10 μm (C) and 100 μm (D).

of EB1 in C2 by overexpressing a dominant-negative GFP-tagged EB1 C-terminal fragment (Fig. 2A), which is reported to inhibit microtubule stabilization in fibroblasts (Wen et al., 2004). Both C2 elongation and fusion were affected by EB1C-GFP (supplementary material Fig. S2). Thus, targeting of EB1 by shRNA-2 and shRNA-3 is specific and EB1 has a role in elongation and fusion of C2 cells during differentiation.

Knocking down EB1 affects the whole differentiation program of C2 myoblasts

We noticed that after a longer time in culture (5 days in FM), the difference in fusion between C2-shC and C2-sh2 or C2-sh3 cells was less apparent than after 2 days in FM. The decreased effect of EB1 KD after 5 days in FM might be due to a growth advantage of cells with a higher residual level of EB1. To obtain more homogeneous EB1 KD cell populations we established several shRNA-2, shRNA-3 and shRNA-C clonal cell lines and studied in more detail five EB1 KD lines with low residual EB1 (C2-sh2C, C2-sh2F, C2-sh2K, C2-sh2L and C2-sh3I) and two control lines (C2-shCA, C2-shCG). When extracts from these lines were immunoblotted for EB1 and for proteins that normally increase during differentiation (myogenin, glu-tubulin, cadherin, β-catenin and EB3), we found that all were reduced (Fig. 3A). EB1 depletion thus appears to affect the whole C2 differentiation program; in particular, EB3 is reduced. In all of the EB1 KD lines, fusion was inhibited to the point that when living cultures were observed by phase-contrast microscopy, most fields did not show a single myotube (see examples in Fig. 3B) and the absence of fusion persisted at later time points (3-5 days in FM; data not shown). By contrast, all the control lines fused normally (Fig. 3B). The effects of knocking down EB1 were quantified in several ways: the percentage of myogenin-positive cells was calculated by immunofluorescence and the level of myogenin expression by immunoblotting; cell elongation was estimated by calculating the average length of mononucleated cells after 2 days in FM; and fusion was quantified by calculating the percentage of nuclei in myotubes. All quantifications revealed significant differences between knockdown and control cells (Fig. 3C).

Microtubule stabilization is inhibited in EB1 KD C2 myoblasts

Generation of a stable, post-translationally modified microtubule array is an early event in myogenic differentiation (Gundersen et al., 1989). Determination of whether EB1 has a role in the stabilization of microtubules during muscle differentiation was one of our goals at the outset of this work. Such a role is suggested by the immunoblot shown in Fig. 3A, but the effect could be secondary to differentiation inhibition. Therefore we repeated the immunoblotting on cultures of undifferentiated myoblasts. The basal glu-tubulin level is reduced to 12-28% of control levels in different EB1 KD cell lines (Fig. 4A). Immunofluorescence confirmed this result, and the staining of glu-tubulin in the primary cilia was reduced in EB1 KD cells (Fig. 4B). We also verified that the glu-tubulin-containing microtubules were stabilized by showing their resistance to either cold or nocodazole treatment (supplementary material Fig. S3). Neither the level nor organization of the dynamic tyrosinated microtubules appears grossly affected in EB1 KD cells (data not shown). Thus EB1 is necessary for the basal microtubule stabilization taking place before C2 differentiation.

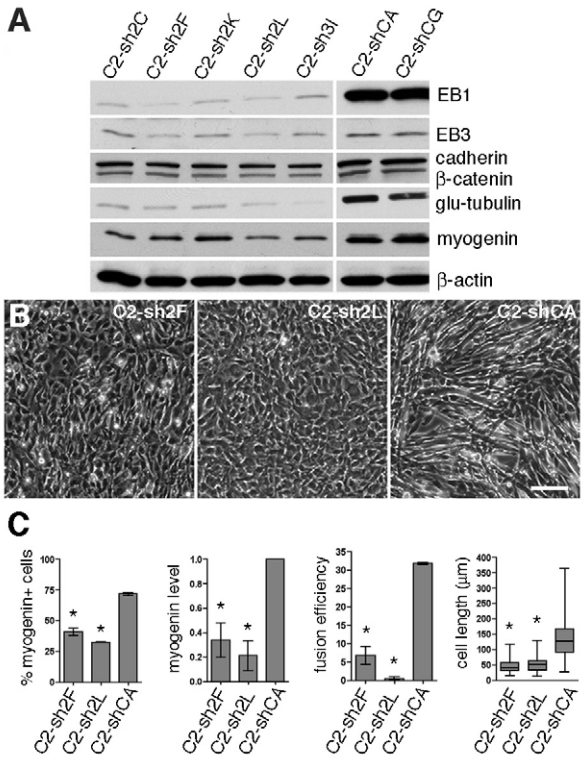

Fig. 3. Differentiation, fusion and elongation are inhibited in EB1 KD C2 cell lines. (A) Extracts were prepared from the indicated stable EB1 KD and control cell lines after 2 days in FM. The level of EB1 and EB3, and cadherin, β-catenin, glu-tubulin and myogenin, which normally increase during muscle differentiation, were examined by immunoblotting, with β-actin as a loading control. (B) Representative phase-contrast images of C2-sh2F, C2-sh2L and C2-shCA cultured for 2 days in FM show practically complete inhibition of fusion in the EB1 KD cells. Scale bar: 100 μm. (C) Quantification of myogenin-positive cells from immunofluorescence (n=335, 350 and 345, respectively); myogenin expression from immunoblots was normalized to β-actin (n=3); fusion efficiency is shown as the percentage of nuclei in myotubes (n=3 and a total of 1125, 729 and 716 cells counted, respectively), and cell elongation as the average length of mononucleated cells (n=129, 122 and 115, respectively). In final graph, the boundaries of the boxes indicate the 25th and the 75th percentile, and the whiskers represent the minimum and maximum values respectively. The mean is shown by the straight line in the box. Values are mean ± s.d. Significant differences (unpaired Student's t-test): *P≤0.001.

Cadherin and β-catenin fail to accumulate at the plasma membrane of EB1 KD C2 cells

Interactions between microtubules and the cadherin-catenin complex have been implicated in myoblast fusion (Kaufmann et al., 1999) and accumulation of this complex at cell-cell contacts is involved in myogenic induction (Goichberg et al., 2001; Charrasse et al., 2002). In EB1 KD cultures kept for 2 days in FM, the level of both cadherin and β-catenin was reduced to about 50% of that in control cells (Fig. 3A), and the accumulation of the cadherin-catenin complex at the plasma membrane was reduced (Fig. 5A). Confocal images of control C2 myoblasts stained for β-catenin show a weak cytoplasmic staining with some concentrations at the zipper-shaped early cell-cell contacts. After only 1 day in FM, β-catenin concentrates at the plasma membrane, even in cells without cell-cell contacts. By contrast, in EB1 KD cells, there is little β-catenin at the plasma membrane and the staining resembles that seen in control myoblasts. A similar distribution was observed for cadherin (supplementary material Fig. S4). These results suggest that EB1 is necessary for the plasma membrane accumulation of the cadherin-catenin complex. The possibility of a direct or indirect interaction between EB1 and the cadherin-catenin complex was then investigated by immunoprecipitation. We used anti-EB1 antibodies to precipitate endogenous EB1 and used anti-GFP antibodies to precipitate EB1-GFP from C2 cells overexpressing EB1-GFP. Both cadherin and β-catenin were present in these precipitates (Fig. 5B,C), suggesting that cadherin and β-catenin form a complex with EB1.

EB1-GFP rescues elongation and fusion of EB1 KD C2

To verify that the defects observed are specifically related to EB1 depletion, we transfected the EB1 KD cells with several constructs: wild-type EB1-GFP; a silent mutated EB1-GFP designed to resist either shRNA-2 or shRNA-3 (see Fig. 2A, mutations in red); EB3-GFP; and a control farnesylated-GFP (GFP-f). After 2 days in FM, both EB1-GFP and EB3-GFP restored cell elongation compared with GFP-f, but only the EB1-GFP was able to rescue fusion to normal levels (Fig. 6B). Immunofluorescence staining confirmed that all nuclei in EB1-GFP-rescued myotubes were positive for myogenin (data not shown), as expected given the sequence of events in myogenesis. Rescued myotubes were also positive for glu-tubulin (Fig. 6C). Rescue by EB1-GFP was observed in three out of four EB1 KD cell lines tested. In these experiments, all constructs were expressed at grossly similar levels except for the wild-type EB1-GFP, which was completely knocked down, again reflecting the specificity of shRNAs for EB1. The mutated EB1-GFP decorated microtubule plus-ends or the whole length of the microtubules depending on the expression level (supplementary material Fig. S5). This is not unique to the EB1-GFP construct, because we noticed that rabbit anti-EB1, which is more sensitive than mouse anti-EB1 showed endogenous EB1 along the whole microtubule. EB3-GFP showed similar patterns of microtubule decoration. We also verified that overexpression of EB1-GFP or EB3-GFP does not on its own affect C2 differentiation or fusion by transfecting the control C2-shCA line (Fig. 6A) and the parent C2 cells (data not shown). Fusion and elongation took place regardless of the level of overexpression. The rescue data therefore confirm that EB1 is necessary for C2 fusion.

Discussion

The present study establishes that EB1 is necessary for the microtubule stabilization that takes place in skeletal myoblasts at the onset of differentiation, thereby extending to muscle the role that EB1 is known to play in fibroblasts (Wen et al., 2004). In addition, we unexpectedly found that all aspects of differentiation were affected when EB1 was knocked down permanently. In particular, cadherin and β-catenin failed to accumulate at the plasma membrane in EB1 KD cells and they coprecipitated with EB1. Therefore, a direct or indirect interaction between EB1 and the cadherin-catenin complex might mediate some of the downstream effects of EB1.

Straube and Merdes (Straube and Merdes, 2007) found that EB3, but not EB1, has a role in myoblast elongation in C2 and that neither EB3 nor EB1 has a role in microtubule stabilization. In view of these results, it was important to verify that our results do not reflect nonspecific knockdown of EB3 by EB1 shRNA. The specificity of EB1 shRNAs is shown by both immunoblotting (Fig. 3B) and immunofluorescence, in C2 myoblasts (Fig. 3C), as well as in NIH3T3 cells (supplementary material Fig. S1). Additional evidence that the shRNAs do not target EB3 was found in the rescue experiments: wild-type EB3-GFP was expressed

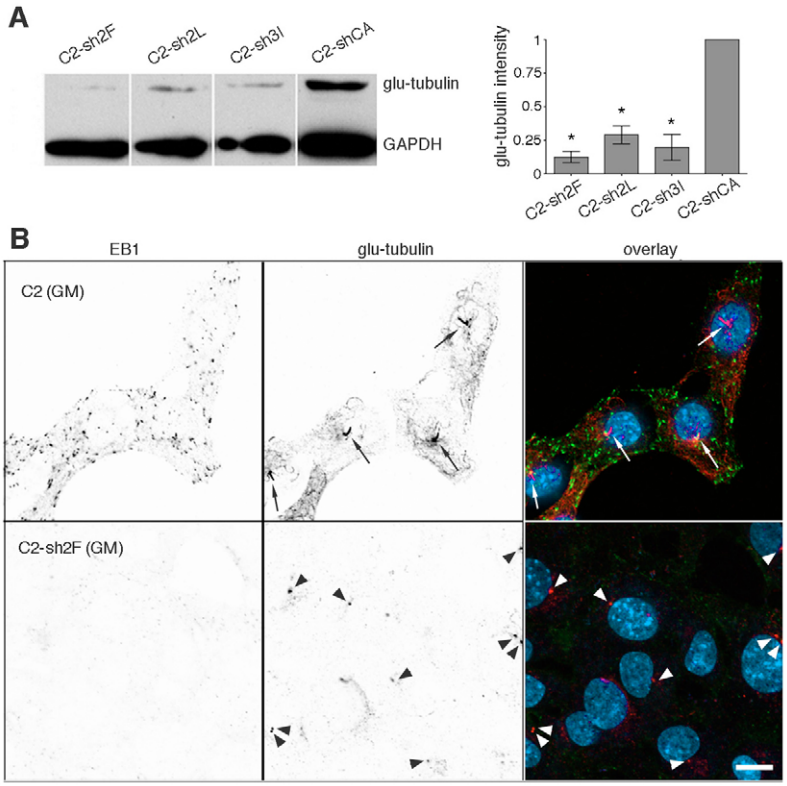

Fig. 4. The basal level of glu-tubulin microtubules is reduced in EB1 KD cells. (A) The level of glu-tubulin was measured by immunoblotting extracts from several stable EB1 KD cell lines (C2-sh2F, C2-sh2L and C2-sh3I) and control C2-shCA cells, all in GM. Relative glu-tubulin levels were normalized to GAPDH. Values are means ± s.d. (n=3). Significant differences (unpaired Student's t-test): *$P \leq 0.0001$. (B) Immunofluorescence staining of C2-sh2F and control C2 cells for glu-tubulin (red) and EB1 (green). To make the visualization of the weak fluorescence easier, the individual confocal images are shown with the gray scale inverted. The imaging parameters were identical for each antibody staining. These representative images show that microtubules containing glu-tubulin, except for those in the centrioles (arrowheads), are barely detectable in EB1 KD cells. In addition, primary cilia (arrows), which are abundant in glu-tubulin, are only detected in control C2. Nuclei were counterstained with Hoechst 33342 (blue). Scale bar: 10 µm.

normally after transfection in the EB1 KD cells (Fig. 6), whereas wild-type EB1-GFP was completely knocked down and only EB1 mutants designed to resist the shRNAs could be expressed. We are therefore confident that the shRNAs used in this study affect EB1 specifically.

The divergence in the relative importance of EB1 compared with EB3 and in their role in microtubule stabilization might then be at least partially reconciled by considering experimental differences: the isolation of permanently knocked down cell lines (this work) compared with transient transfections (Straube and Merdes, 2007). Given the usual timeline of C2 culture and differentiation (2 days in GM and then switch to FM), transient transfection might not be able to knock down EB1 fast enough to prevent any effect it might have in early differentiation. Microtubule stabilization, in particular, is an early event in muscle differentiation (Gundersen et al., 1989) and could become immune to EB knockdown if it initiates an irreversible cascade of events (Eng et al., 2006; Ciani and Salinas, 2007; Onishi et al., 2007). Consistently with this explanation, when we overexpressed EB1 constructs transiently, we did not observe any effects on glu-tubulin levels (data not shown).

Elongation of myoblasts, which occurs before fusion, was blocked in the EB1 KD cell lines. We also found that elongation was prevented by an EB1-C-terminal construct, but this construct might interact with EB3-binding partners as well as EB1-binding partners. EB3 rescued elongation of EB1 KD cell lines as well as EB1 did. It is therefore possible that both EB1 and EB3 have the capacity to cause elongation of myoblasts; EB1 would be dominant during differentiation, but in a rescue situation, both are effective. C2 elongation even takes place outside the context of differentiation, for instance, when microtubules are massively stabilized by taxol (data not shown). This shows that the cell shape responds to microtubule stabilization regardless of the pathway.

Fusion of myoblasts is a later event, and we found that EB1 is also necessary for fusion. Since our experiments were done on a background of normal, low EB3 expression in myoblasts, and since EB1-GFP re-expression, which restores normal differentiation, would increase the level of EB3, we cannot rule out the idea that EB3 has a role in fusion, as proposed by Straube and Merdes (Straube and Merdes, 2007). However, we restore very little fusion when we overexpress EB3-GFP in the EB1 KD cell lines. Fusion of myoblasts into myotubes was inhibited in all five EB1 KD clones, demonstrating that these effects were not clone specific. We therefore demonstrate that EB1 is necessary for the later events of differentiation, such as fusion, but our experimental design does not allow us to conclude whether or not EB1 is sufficient. Since we find that EB3 alone is not sufficient, we propose that fusion is facilitated by EB1 alone or by EB1 and EB3. A model is presented in Fig. 7.

Although a C-terminal GFP tag has been found to affect certain roles of EB1 (Komarova et al., 2005), EB1-GFP labeled microtubules, as expected (see supplementary material Fig. S5), and restored C2 differentiation to the full extent. Rescue by EB1-GFP led to more than 50% fusion, which is in the normal range, given that maximum fusion in control C2 cells in similar conditions is ~65%. It is possible, but not very probable, that the GFP tag affects EB3 more than EB1. Straube and Merdes (Straube and Merdes, 2007) used constructs labeled with internal double Flag tags. The fact that control C2 cells with high levels of EB1-GFP or EB3-GFP overexpression show normal fusion indicates that overexpression of GFP-tagged EB1 or EB3 itself does not affect fusion.

Fusion could be indirectly affected by EB1 removal through microtubule reorganization, because cell motility is involved in C2 fusion (Dedieu et al., 2004), yet our preliminary tracking of living cells for 3 hours (data not shown) did not show any gross differences in motility between EB1 KD cells and control C2 cells. However,

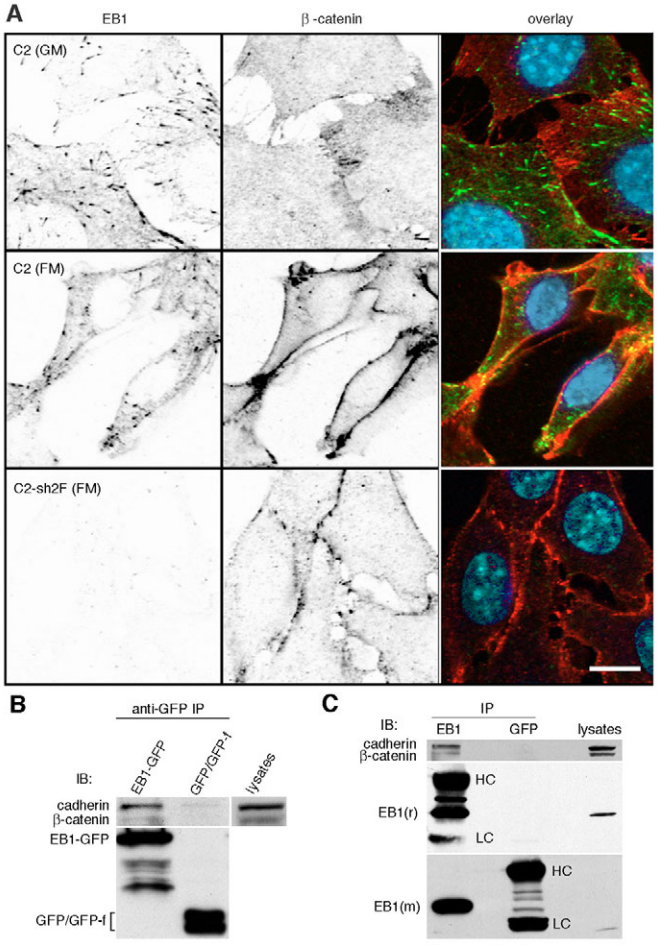

Fig. 5. In the absence of EB1, the accumulation of β-catenin on the plasma membrane is reduced. (A) Immunofluorescence of EB1 and β-catenin is shown in control C2 cells in GM (top row) or FM (middle row), and in C2-sh2F after 1 day in FM (bottom row). As in Fig. 4, the gray scale is inverted and the imaging parameters were identical for each antibody staining. Notice that the β-catenin staining at the plasma membrane of C2-sh2F after 1 day in FM is patchy and weak compared with that in control cells. Scale bar: 10 μm. (B) Immunoprecipitation with anti-GFP from extracts of cultures transfected with either EB1-GFP or doubly transfected with GFP and GFP-f. Immunoblotting shows that cadherin and β-catenin coimmunoprecipitate with EB1-GFP. (C) Immunoprecipitation of endogenous EB1 from C2 cells in FM. Immunoblotting shows the presence of cadherin and β-catenin in the immunoprecipitates of anti-EB1-coated beads but not in that of anti-GFP coated beads. Immunoprecipitation efficiency was verified with mouse anti-EB1 [EB1(m)] or rabbit anti-EB1 [EB1(r)]. In addition, both antibody heavy (HC) and light (LC) chains were detected and used as loading controls.

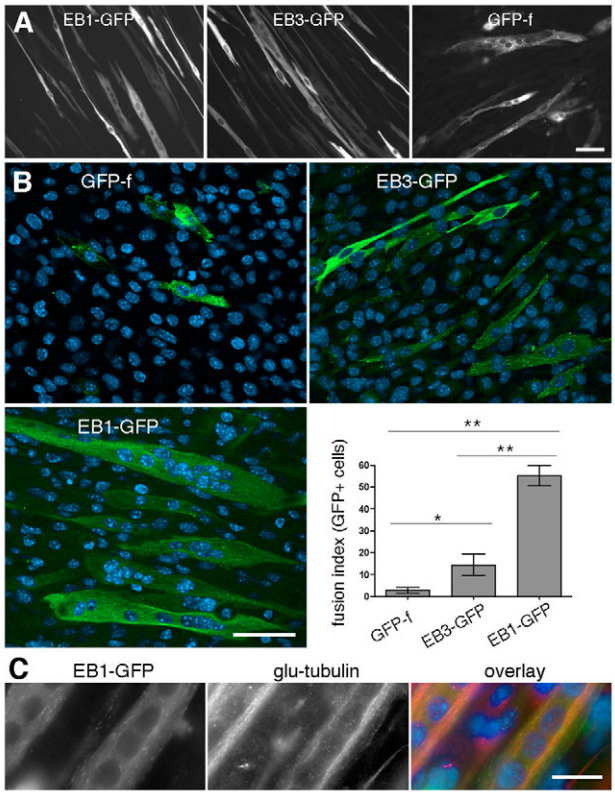

Fig. 6. EB1-GFP restores fusion of the EB1 KD cell lines. (A) Representative fluorescence images show that overexpression of EB1-GFP, EB3-GFP and GFP-f (as control) does not inhibit control C2-shCA elongation or fusion. (B) Representative fluorescence images show C2-sh2F EB1 KD cultures in FM 2 days after transfection with the same constructs. GFP-f-overexpressing cells show practically no elongation or fusion rescue. EB3 overexpression rescues cell elongation but only occasionally rescues fusion. EB1-GFP (with a silent mutation in the shRNA-2 targeting sequence) leads to restoration of both elongation and fusion. The bar graph shows the fusion index (percentage of nuclei in GFP-positive cells that are part of myotubes). Values are means ± s.d. (n=3 and a total of 2014, 1283 and 946 nuclei counted, respectively). Significant differences (unpaired Student's t-test): $^{**}P \leq 0.0001$; $^{*}P < 0.02$. (C) Immunofluorescence staining shows that glu-tubulin microtubules are present in EB1-GFP-rescued myotubes. Scale bars: 50 μm (A,B) and 20 μm (C).

individual cells did show changes in cell shape and ruffling, suggesting that there might be differences in actin organization. A reduction in the level of EB1 might also increase the free pool of its binding partners, such as APC, overexpression of which also leads to cell shape changes (Kroboth et al., 2007).

Staining of C2 with anti-EB1 revealed a change in overall distribution as cells differentiate: the staining appears to spread from the microtubule plus-ends to the total length of microtubules. We do not know whether this change reflects differences in microtubule properties, such as the closure of the tubulin sheets (Vitre et al., 2008), differences in microtubule dynamics, or in competition of EB1 with other plus-end proteins, particularly EB3. The absence of staining around myotube nuclei, a site of microtubule nucleation (Bugnard et al., 2005), is compatible with the absence of microtubule tethering at that site of microtubule nucleation.

The cadherin-catenin complex has a key role in myogenic induction and myoblast fusion (Takeichi, 1991; Knudsen et al., 1998; Goichberg et al., 2001; Charrasse et al., 2002). In addition, cadherin signaling has also been reported to stabilize microtubules in centrosome-free CHO cytoplasts (Chausovsky et al., 2000), which mimic some aspects of terminally differentiated cells (such as epithelial and muscle cells) in which most microtubules are not tethered to a centrosome. The cadherin-catenin complex then appeared as a potential mediator of EB1 effects and we did indeed observe a reduced level and cell surface localization of cadherin and β-catenin in the EB1 KD cell lines (Fig. 3A, Fig. 5A; supplementary material Fig. S4). Coimmunoprecipitation of EB1 with cadherin and β-catenin indicates that they are associated, but we do not know whether this association is direct or where in the cell it takes place. Both β-catenin and EB1 are found in the centrosome (Berrueta et al., 1998; Morrison et al., 1998; Askham et al., 2002; Yan et al.,

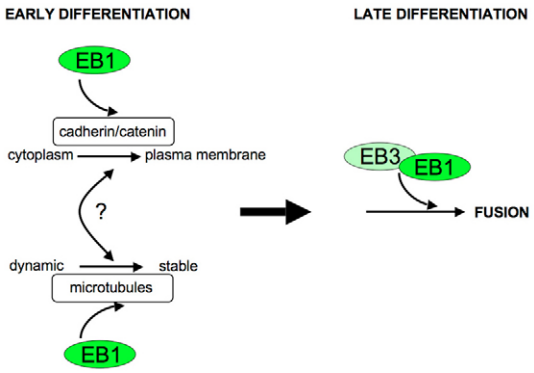

Fig. 7. A model demonstrating the essential roles of EB1 during early C2 differentiation. Early in muscle differentiation, microtubules become stabilized and cadherin and β-catenin translocate from the cytoplasm to the plasma membrane. We have shown that EB1 is necessary for both events, but we do not know whether the two events are causally related. In the absence of EB1, differentiation is largely inhibited and later events, especially fusion, are blocked. Rescue experiments show that EB1 is necessary for the events of late differentiation, but we do not know whether it is sufficient. Since the level of EB3 becomes elevated at this stage, it is possible that EB3 also contributes.

2006; Bahmanyar et al., 2008) in addition to the cytoplasm, microtubules (EB1) and plasma membrane (cadherin-catenin). The contribution of the centrosome could be determined by overexpressing partial constructs of the protein CAP350, which has been reported to displace EB1 from the centrosome but not from microtubule plus-ends (Yan et al., 2006). It will also be interesting to determine how glu-tubulin expression and cadherin-catenin translocation are related during muscle differentiation. The canonical Wnt/β-catenin pathway, which is involved in cell migration into a wound and involves microtubule stabilization might be a good frame of reference (Nelson and Nusse, 2004).

The EB family of proteins, with three isoforms differentially expressed over time during muscle differentiation (Straube and Merdes, 2007), can be compared with other families of proteins. The caveolin family, for example, also has three members, with caveolin-3 replacing caveolin-1 during muscle development (Galbiati et al., 2001); the Murf proteins, which have an important role in muscle differentiation (Spencer et al., 2000) also comprise three isoforms, one of which (Murf1) predominates in muscle. None of the EB proteins is muscle-specific but each of them is regulated during muscle differentiation. Cell context will thus most likely affect and shape different roles for EB1 and EB3. For example, EB1 knockdown does not affect cadherin and β-catenin localization at cell-cell contacts in HeLa cells (Shaw et al., 2007) and nitrotyrosination of tubulin, which prevents its detyrosination and decreases the association of EB1 with microtubules, stops proliferation of vascular smooth muscle cells (Phung et al., 2006). However, the reduced glu-tubulin level in EB1 KD C2 myoblasts (Fig. 4) and fibroblasts (Wen et al., 2004; Schroder et al., 2007) implies a conserved role of EB1 in microtubule stabilization. It is likely that the mitotic status of the cell is one of the factors that influences EB function, because exit from the mitotic cycle must assign new roles to many microtubule-associated proteins and their interacting partners.

Materials and Methods

Reagents and antibodies

Mouse anti-EB1 was from BD Transduction Laboratories (Franklin Lakes, NJ), rabbit anti-EB1, rabbit anti-β-catenin and mouse anti-α-tubulin were from Sigma (St Louis, MO). Mouse anti-myogenin F5D was from DAKO (Carpinteria, CA). Rabbit anti-pan-cadherin was from Abcam (Cambridge, MA), rabbit anti-GAPDH and anti-GFP were from Cell Signaling (Danvers, MA) and mouse anti-GFP was from Roche (Indianapolis, IN). Rabbit anti-glu-tubulin was kindly provided by George Cooper IV (Medical University of South Carolina, Charleston, SC). Rabbit anti-EB3 was a kind gift from Anna Akhmanova (Erasmus Medical Center, Rotterdam, Netherlands).

Alexa Fluor 488- or Alexa Fluor 568-conjugated anti-mouse or anti-rabbit IgG were from Invitrogen (Carlsbad, CA). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies were from Pierce (Pierce, Rockford, IL). Protein A/G beads were from Santa Cruz (Santa Cruz, CA).

Cell culture, cDNAs and shRNA transfection

The mouse skeletal muscle cell line C2 was cultured as described previously (Lu et al., 2001). Briefly, undifferentiated myoblasts are plated on tissue culture dishes or on glass coverslips coated with 0.5% gelatin in growth medium (GM) which consists of Dulbecco's modified Eagle's Medium (DMEM, 1 g/l glucose) containing 20% FBS (Atlanta Biologicals, Atlanta, GA), 0.5% chick embryo extract (MP Biomedicals, Aurora, OH) and 2 mM Glutamax (Invitrogen). When cells reached 70% confluence, the medium was replaced by fusion medium (FM). FM consists of DMEM containing 4% horse serum and 2 mM Glutamax. The cultures thereafter receive a daily half-feed. NIH3T3 fibroblasts, a kind gift from Wing-Hang Tong (NICHD, NIH, Bethesda, MD), were cultured in DMEM containing 10% FBS and 2 mM Glutamax. The C-terminally GFP-tagged EB1 (EB1-GFP) or EB1C (EB1C-GFP) cDNA have been described (Wen et al., 2004). Farnesylated-GFP (GFP-f) and GFP cDNA were from Clontech (Mountain View, CA). EB3-GFP cDNA was a kind gift from Anna Akhmanova.

To knock down EB1 expression, we used MISSION plasmids (Sigma), which encode short hairpin RNAs targeting mouse EB1 mRNA, and non-targeting shRNA controls. The target and non-target short hairpin sequences are as follows: shRNA-2, CCGGGCTTTGAGTAAACCGAAGAAACTCGAGTTTCTTCGGTTTACTCAAAGCTTTTT; shRNA-3, CCGGGCTAAGCTAGAACATGAATATCTCGAGATATTCATGTTCTAGCTTAGCTTTTT; and control shRNA, CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT.

Transfections were done with FuGENE 6 (Roche, Indianapolis, IN). Permanently transfected colonies were selected with puromycin (3 μg/ml, Clontech). EB1-GFP plasmids containing silent mutations in the shRNA target sequence were generated by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA). Primer sequences were as follows: Sh2-SM forward, 5′-GTCGCCCCAGCTTTGTCTAAACCGAAGAAACC-3′ and Sh2-SM reverse, 5′-GGTTTCTTCGGTTTAGACAAAGCTGGGGCGAC-3′; Sh3-SM Forward, 5′-GTGAAATTCCAAGCTAAGCTGGAGCATGAATATATCCAGAACTTC-3′ and Sh3-SMand reverse, 5′-GAAGTTCTGGATATATTCATGCTCTAGCTTAGCTTGGAATTTCAC-3′. The following protocol was used for each reaction: initial denaturation at 95°C for 1 minute, three-step cycling with 18 cycles consisting of denaturation at 95°C for 50 seconds, annealing at 60°C for 50 seconds and extension at 68°C for 7 minutes, and a final extension at 68°C for 7 minutes. Mutations were confirmed by sequencing (MTR Scientific, Ijamsville, MD).

Microscopy and image analysis

Cells cultured on coverslips were fixed in methanol at –20°C for 6 minutes. For some experiments (see figure legends), cells were extracted for 1 minute at room temperature with 1% Triton X-100 in PHEM buffer (60 mM PIPES, 10 mM EGTA, 2 mM $MgCl_2$, 25 mM HEPES, pH 6.9) before fixation. Cells were then rehydrated in PBS and incubated for 1 hour at room temperature in blocking buffer consisting of PBS with 1% bovine serum albumin (Sigma), 2% horse serum (Hyclone, Logan, UT), and 3% normal goat serum (Sigma). They were then labeled at room temperature with primary and secondary antibodies for 2 and 1 hour(s), respectively, counterstained with Hoechst 33342, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). For cell length calculation, EB1 KD and control cells were transfected with GFP-f and cultured in FM for 2 days. Cells were then fixed and stained with Hoechst 33342 and mounted. We then calculated the length of GFP-f positive, mononucleated cells with ImageJ (http://rsb.info.nih.gov).

Widefield immunofluorescence images were taken on a Leica DMR microscope with a Hammamatsu C4742-95 digital camera (Bridgewater, NJ) and phase-contrast images of live cultures were taken on a Leica DMRI microscope with a CoolSNAP CCD camera (Roper Scientific, Tucson, AZ) and IPLab software (BioVision Technologies, Exton, PA). Confocal images were recorded on a Zeiss LSM 510 or a Leica SP5 equipped with a 63× 1.4 NA oil-immersion lens. We collected single optical sections or generated maximum intensity projections of *z*-series (0.6 μm slice spacing) through the cell. Digital image files were transferred to Photoshop 7.0 to assemble montages and enhance images for presentation. In some figures (Figs 1, 2, 4, 5, 6; supplementary material Figs S1, S3, S4 and S5), the hue and saturation of nuclear staining was adjusted. Images shown are representative examples from three independent experiments.

Cell and tissue lysates

Cultures were rinsed with cold PBS and lysed on ice in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) with complete mini protease inhibitor cocktail

(Roche, Indianapolis, IN) and homogenized with a pellet pestle (Kontes, Vineland, NJ). Homogenates were centrifuged at 4°C at 16,000 × ***g*** and supernatants were stored at –20°C. Protein concentrations were determined with the Bio-Rad DC assay (Hercules, CA).

Coimmunoprecipitation of EB1 or EB1-GFP with cadherin and β-catenin in C2 cells

C2 cells were cultured in FM for 48 hours or were transfected with EB1-GFP or GFP and/or GFP-f in six-well plates for 24 hours and cultured in FM for another 2-3 days. After washing once with PBS, cells were incubated on ice for 1 hour with 1 ml RIPA buffer supplemented with complete mini protease inhibitor cocktail (Roche) and then were harvested with a rubber policeman. Tubes containing the cell extracts were spun for 15 minutes at 16,000 × ***g*** in a microcentrifuge at 4°C. The supernatants were combined with 20 µl of a 50% slurry of protein A/G agarose beads (Invitrogen), and kept rotating for 2 hours at 4°C to clear any protein that binds non-specifically to the beads. Another batch of 40 µl beads was incubated for 8 hours at 4°C with 10 µl mouse anti-GFP. These GFP antibody-coated beads were combined with the cleared supernatant, and left on a rotator for 8 hours at 4°C. Beads were washed five times, and bound material was eluted in SDS-PAGE sample buffer. Samples were boiled and separated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-pan-cadherin, anti-β-catenin, anti-GFP and mouse or rabbit anti-EB1. Anti-EB1-coated beads were used for immunoprecipitation of endogenous EB1 from untransfected C2 cultured in FM, anti-GFP-coated beads were used as control.

Electrophoresis and immunoblotting

Western blot analysis was done as follows: 40 µg of cell extract was loaded on 12% pre-cast SDS-PAGE gels (Bio-Rad), separated in Tris-glycine buffer, and transferred onto nitrocellulose membranes. The membranes were blocked in TBST, (25 mM Tris, 140 mM NaCl, 3 mM KCl, 0.05% Tween-20, pH 7.4) with 5% non-fat milk, incubated for 16 hours at 4°C with primary antibodies, and for 1 hour with horseradish-peroxidase-conjugated secondary antibodies. Peroxidase activity was revealed with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). X-ray films were scanned and the bands were measured with ImageJ.

Statistical analysis

All graphs were made with Prism 4.0a (Graphpad Software) the statistical analysis was done with Prism or Excel. Data are expressed as means ± s.d. The unpaired Student's *t*-test was used two compare between two groups.

We thank colleagues who provided us with reagents and cells. We are also thankful to Ericka Reid (LIS, NIAMS) for technical help, Vittorio Sartorelli (NIAMS) for useful discussions, Shajia Lu (NIAMS), Adrian Lobito (NIAMS), Ming Zhao (NIAID), Mary Ann Robinson (NIAID), Raynaldo Martin (NIAID), and Kirsten Remmert (NHLBI) for help with different experiments. This work was funded by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health. Deposited in PMC for release after 12 months.

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