Cellular/Molecular

Erbin Controls Dendritic Morphogenesis by Regulating Localization of δ-Catenin

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The LAP [leucine-rich and postsynaptic density-95/Discs large/zona occludens-1 (PDZ)] protein erbin and δ-catenin, a component of the cadherin–catenin cell adhesion complex, are highly expressed in neurons and associate through PDZ-mediated interaction, but have incompletely characterized neuronal functions. We show that short hairpin RNA-mediated knockdown of erbin and knockdown or genetic ablation of δ-catenin severely impaired dendritic morphogenesis in hippocampal neurons. Simultaneous loss of erbin and δ-catenin does not enhance severity of this phenotype. The dendritic phenotype observed after erbin depletion is rescued by overexpression of δ-catenin and requires a domain in δ-catenin that has been shown to regulate dendritic branching. Knockdown of δ-catenin cannot be rescued by overexpression of erbin, indicating that erbin is upstream of δ-catenin. δ-Catenin-null neurons have no alterations in global levels of active Rac1/RhoA. Knockdown of erbin results in alterations in localization of δ-catenin. These results suggest a critical role for erbin in regulating dendritic morphogenesis by maintaining appropriate localization of δ-catenin.

Key words: **dendrite; δ-catenin; erbin; LAP proteins; localization; PDZ**

Introduction

The formation and maintenance of dendrites is a complex process and needs to be precisely regulated because neuronal function is critically dependent on correct arborization of dendrites, the major sites of information input to neurons (Parrish et al., 2007). Dendrites serve critical roles in establishing neuronal circuits and integration of synaptic signals, so mechanisms that regulate their arborization have significant impact on brain function. This is emphasized by the existence of human disorders, including Fragile X, Rett, and Down's syndromes and other mental retardation disorders, in which dendritic structure is abnormal (Newey et al., 2005).

Intrinsic and extrinsic cues, including growth factors (Jin et al., 2003) and transcription factors (Aizawa et al., 2004; Gomez-Ospina et al., 2006), coordinate dendritic arborization and refinement, both during development and in mature neurons. However, our knowledge of the cellular and molecular mechanisms that promote the formation and maintenance of dendritic arbors remains incomplete.

Erbin is a member of the leucine-rich and postsynaptic density-95 (PSD-95)/Discs large/zona occludens-1 (PDZ) (LAP) family of proteins, which in mammals includes erbin, densin-180 (Apperson et al., 1996), scribble (Murdoch et al., 2003), and lano (Saito et al., 2001). These proteins share common features including N-terminal (N-term) leucine-rich repeats and one to four PDZ domains, with the exception of lano that lacks a PDZ domain. LAP family of proteins have been implicated in establishment and maintenance of epithelial cell polarity in *Drosophila* (Bilder and Perrimon, 2000) and *Caenorhabditis elegans* (Legouis et al., 2003). Erbin was originally identified through its interaction with the ErbB2 receptor and has been shown to regulate its surface expression and basolateral targeting (Borg et al., 2000; Huang et al., 2001). Erbin is widely expressed in brain, localized at postsynaptic densities and interacts with components of the PSD, including PSD-95 (Huang et al., 2001). However, its major functional roles in neurons are not well characterized.

In addition to ErbB2, erbin binds several additional proteins with PDZ domain interaction motifs (Boisguerin et al., 2004; Calin-Jageman et al., 2007). Recent studies have shown that constituents of the cadherin–catenin cell adhesion complex, in particular δ-catenin, p0071, and armadillo repeat protein deleted in velo-cardio-facial syndrome (ARVCF), directly bind to the PDZ domain of erbin (Laura et al., 2002). Indeed, the affinity of erbin for δ-catenin is higher than its affinity for ErbB2 (Laura et al., 2002). The cadherin–catenin cell adhesion complex, comprising cadherins and associated cytosolic catenins, is localized at central synapses (Uchida et al., 1996). Components of the complex have

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been implicated in different aspects of neuronal morphogenesis (Bamji et al., 2003; Yu and Malenka, 2003; Elia et al., 2006), implying that this complex has functional roles that extend beyond its ability to regulate cell adhesion.

δ-Catenin belongs to the p120 catenin (p120ctn) family of catenins and binds to the cytoplasmic domains of type 1 and 2 cadherins at a membrane proximal region that is not involved in binding of either β-catenin or plakoglobin. Each member of the p120ctn family consists of a central domain consisting of nine armadillo (arm) repeats broken by a wedge between repeats 5 and 6 plus N- and C-terminal sequences that differ substantially between family members (Pokutta and Weis, 2007). In addition, δ-catenin, ARVCF, and p0071 also contain a PDZ binding motif at the C terminus.

In neurons, overexpression studies have indicated a role for δ-catenin in promoting dendritic branching (Martinez et al., 2003; Kim et al., 2008). The mechanisms that underlie the ability of δ-catenin to promote dendritic branching have not been completely clear, although several mechanisms have been proposed including regulation of cortactin (Martinez et al., 2003), p190RhoGEF (Kim et al., 2008), RhoA (Martinez et al., 2003), presenilin-1 (Kim et al., 2006), and actin (Kim et al., 2002). Loss of a related member of the δ-catenin family, p120ctn, leads to reduced dendritic branching in neurons with decreased Rac1 and increased Rho activity (Elia et al., 2006). Upstream activators that coordinate δ-catenin/p120ctn-mediated regulation of dendritic branching remain unclear.

To characterize the function of erbin in neurons, we used short hairpin RNA (shRNA)-mediated knockdown to deplete erbin in hippocampal neurons. Our results indicate that depletion of erbin results in reduced dendritic branching, a phenotype similar to that observed after depletion of δ-catenin. Furthermore, our results indicate that erbin and δ-catenin control dendritic morphogenesis through a common pathway in which erbin is upstream of δ-catenin. Interestingly enough, hippocampal neurons from δ-catenin-null mice do not show any significant alterations in the levels of active Rac and Rho. Knockdown of erbin results in altered localization of δ-catenin. Our results therefore suggest that erbin regulates dendritic morphogenesis through control of the localization of δ-catenin, which in turn promotes dendritic morphogenesis through a pathway independent of global alterations in active Rac1 and RhoA.

Materials and Methods

Antibodies. Antibodies to erbin have been described previously (Borg et al., 2000; Huang et al., 2001; Ohno et al., 2002). The mouse monoclonal anti-δ-catenin antibody was purchased from BD Transduction Laboratories. Anti-β-tubulin antibody (E7) was obtained from the Hybridoma Bank at the University of Iowa. Anti-MAP2 antibody was obtained from Millipore.

DNA constructs. Green fluorescent protein (GFP) δ-catenin constructs have been described previously and were kind gifts from Dr. Qun Lu (East Carolina University, Greenville, NC) and Dr. Kenneth Kosik (University of California, Santa Barbara, Santa Barbara, CA). Full-length mouse δ-catenin construct was a kind gift from Dr. Werner Franke (German Cancer Research Center, Heidelberg, Germany). The C-terminal erbin construct has been described previously (Huang et al., 2003) and includes amino acids 965–1371 of human erbin. The N-term, N-arm, and δ-PDZ constructs of δ-catenin were generated from the full-length δ-catenin construct by site-directed mutagenesis to introduce stop codons at amino acids 529, 1035, and 1243, respectively, corresponding to the mouse cDNA sequence, respectively. Full-length human erbin with an N-terminal myc tag and the red fluorescent protein (RFP)-tagged PDZ domain construct were kind gifts from Dr. Richard Laura (Neurocrine Biosciences, San Diego, CA). The enhanced GFP (EGFP) δ-catenin Δ-PDZ was generated from the full-length construct by introducing a stop codon before the last four amino acids.

ShRNA constructs. shRNA 1 for erbin and shRNA for δ-catenin were generated in the pSuper-GFP construct. shRNA 2 for erbin was generated in the lentivirus vector (pLLX3.1). The sequences for the erbin shRNA have been described previously (Huang et al., 2003; Rangwala et al., 2005; Dai et al., 2006) and are TAG ACT GAC CCA GCT GGA ATC TCT TGA ATT CCA GCT GGG TCA GTC TA for shRNA 1 and TGC ATC CCT CTA GAG AAC AAC TTT CAA GAG AAC TTG TTC TCT AGA GGG ATG C for shRNA 2. The sequence for the δ-catenin shRNA was GCA ACT ATG TCG ACT TCT A TCT CTT GAA TAG AAG TCG ACA TAG TTG C. The EGFP in pSuper vector and shRNA 1 was replaced with mOrange for the experiments in Figure 8.

Transient transfection of tsa201 cells. tsa201 cells were maintained in culture and transfected using calcium phosphate. For examining shRNA-mediated knockdown, cells were collected 4 d after transfection, lysed, and immunoblotted.

Rat hippocampal culture and transfection. Embryonic day 18 (E18) rat hippocampal neurons were cultured and maintained as described previously (Bamji et al., 2003; Elia et al., 2006). Neurons were transfected with 1–2 μg of DNA using the calcium phosphate or Effectene (Qiagen) or Lipofectamine technique. The appropriate empty vector (pSuper GFP, pSuper mOrange, or pLLX3.1) was transfected as a control in each experiment. Cells were fixed with 4% paraformaldehyde/sucrose, washed with PBS, and mounted in the Antifade mounting medium or were processed for immunofluorescence with the appropriate primary antibodies and fluorescently conjugated secondary antibodies. For transfection of cortical neurons for biochemical studies, neurons were transfected using the Amaxa electroporator before plating using the manufacturer's instructions. To examine shRNA-mediated knockdown, cells were collected 4 d after transfection, lysed, and immunoblotted.

Mouse hippocampal culture and transfection. Hippocampal cultures were obtained from postnatal day 0 (P0)/P1 hippocampi from *δ-catenin*+/+ and *δ-catenin*−/− mice as described previously (Elia et al., 2006). Cells were transfected using the Effectene (Qiagen). δ-Catenin mice have been described previously (Israely et al., 2004). For experiments in Figure 5*A*, wild-type C57BL6 neurons and neurons from *δ-catenin*−/− animals were used to examine the effect of erbin knockdown on mouse neurons.

Image analysis and statistical methods. Images were taken on an inverted Zeiss Pascal confocal microscope, using a 40× or 63× oil-immersion lens. In some cases, a digital zoom of 0.7× was used. Images were analyzed using the ImageJ software or manually. Images were minimally processed using Adobe Photoshop. The numbers of neurons counted for each experiment are indicated in the figure legends. For the rat hippocampal cultures, each number represents the sum total of neurons analyzed from three or more independent cultures. For the mouse cultures, the number of neurons indicates the total number of neurons analyzed from three or more control and mutant animals. Statistical significance was assessed using the Student's *t* test, using a two-tailed test assuming unequal variances. *p* values obtained from this test are indicated in the figure legends.

Scholl analysis. For Scholl analysis, neurons were imaged with a 20× objective and the estimation of the number of intersections versus distance from cell body was estimated using ImageJ with a plugin available online (http://www.biology.ucsd.edu/labs/ghosh/software/index.html). The images were imported into Adode Photoshop and the axons were removed from the images. The axons were identified by morphology. The resulting images were then imported into ImageJ and used for Scholl analysis. Neurons from two independent cultures were used for the estimations.

Estimation of active Rac1 and RhoA. Hippocampal neurons from control and *δ-catenin* mutant mice were cultured and lysed at day *in vitro* (DIV) 17, after which active Rac1 and RhoA levels in lysates measured using solid phase effector domain binding assays with reagents provided in a kit (Cytoskeleton) according to the manufacturer's instructions.

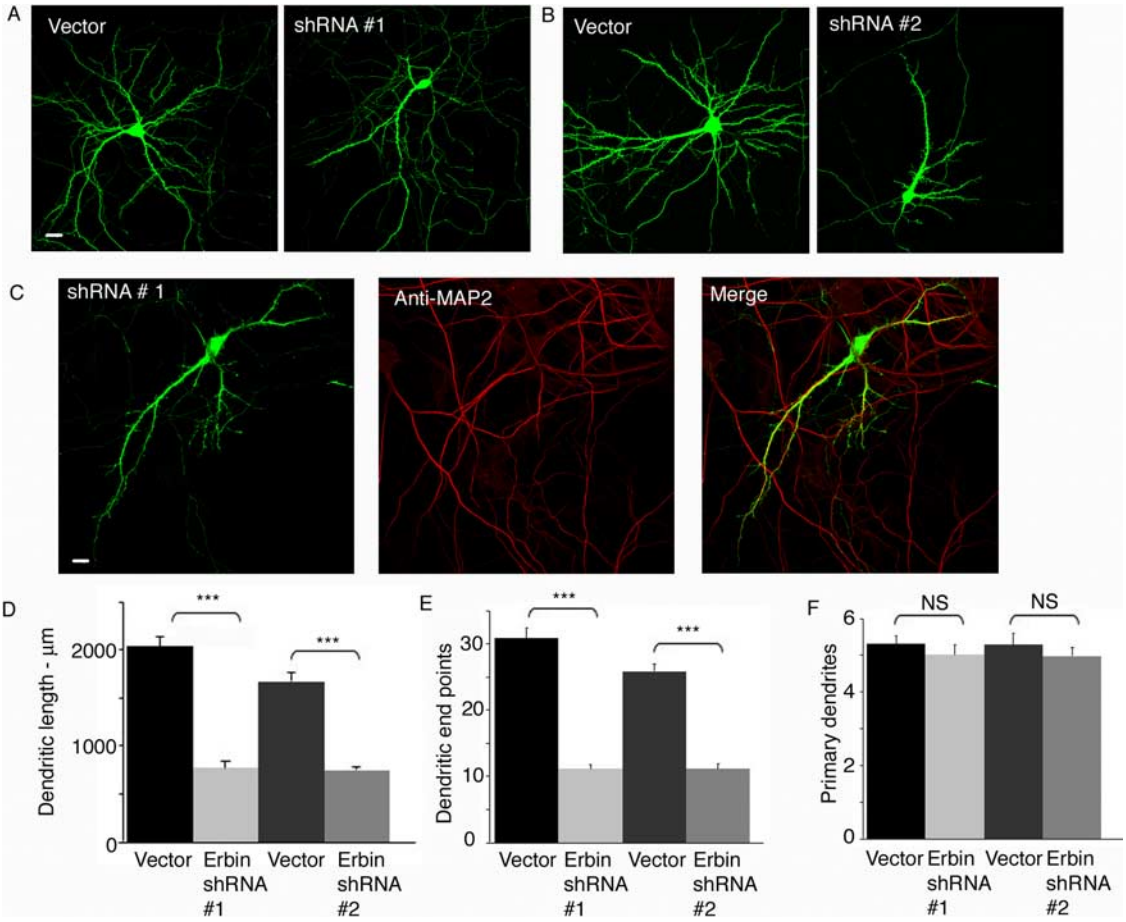

Figure 1. Knockdown of erbin impairs dendritic morphogenesis in hippocampal neurons. ***A***, ***B***, Rat hippocampal neurons were transfected at DIV 11 with vector or shRNA 1 to erbin (***A***) or vector or shRNA 2 to erbin and examined at DIV 17 (***B***). Representative images show dendritic morphology. ***C***, Anti-MAP2 staining at DIV 17 of cultured rat hippocampal neurons expressing erbin shRNA 1. ***D–F***, Quantification of total average dendritic length (vector 1, 2019.99 ± 99.87 μm; shRNA 1, 760.56 ± 70.13 μm; $p < 0.0001$; vector, 1663.59 ± 91.10 μm; shRNA 2, 741.23 ± 30.89 μm; $p < 0.0001$; ***D***), dendritic end points (vector, 30.78 ± 1.6; shRNA 1, 11.13 ± 0.668; $p < 0.0001$, vector 2, 25.8 ± 1.16; shRNA 2, 13.79 ± 0.72; $p < 0.0001$; ***E***), and primary dendrites (vector, 5.3 ± 0.22; shRNA 1, 5 ± 0.29; $p = 0.41$; vector, 5.28 ± 0.31; shRNA 2, 4.97 ± 0.24; $p = 0.43$; ***F***) from rat hippocampal neurons transfected at DIV 11 with vector, shRNA 1, vector or shRNA 2, and examined at DIV 17 ($n > 21$ neurons for each; ***$p < 0.0001$). NS, Not significant. Scale bars, 20 μm. Error bars indicate SEM.

Results

Knockdown of erbin impairs dendritic complexity in hippocampal neurons

In initial experiments, we observed that erbin is robustly expressed in the late embryonic (E18), early postnatal (P7), and adult hippocampus (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material), suggesting that it may have important roles in both hippocampal development and function. To examine potential effects of erbin depletion on neuronal development, two different GFP expressing vector plasmids were used to generate two nonoverlapping shRNAs (1 and 2) (for details, see Materials and Methods), each of which had been used previously by other groups to deplete erbin expression (Huang et al., 2003; Rangwala et al., 2005; Dai et al., 2006). To confirm their effectiveness, erbin expression was examined by immunoblot 4 d after each shRNA-expressing vector was cotransfected with myc-tagged erbin into tsa201 cells. Results showed that each shRNA was highly effective in reducing erbin expression (supplemental Fig. 1*B*,*C*, available at www.jneurosci.org as supplemental material). Each of the shRNA-expressing vectors was also effective at inhibiting expression of endogenous erbin in rat cortical neurons (supplemental Fig. 1*D*,*E*, available at www.jneurosci.org as supplemental material). The apparent efficiency of inhibition of endogenous erbin in cortical neurons was less than that of coexpressed erbin in tsa201 cells. This was not unexpected given the lower transfection efficiency of cortical neurons. These results indicate that each of the two shRNAs to erbin is effective in knocking down its expression, consistent with published reports (Huang et al., 2003; Rangwala et al., 2005; Dai et al., 2006).

To examine the effects of erbin knockdown on hippocampal neuron morphogenesis, we transfected cultured rat hippocampal neurons with either a vector control or one of the two erbin-specific shRNAs at DIV 11 and examined consequences of erbin depletion 6 d later at DIV 17. Gross assessment of neuronal morphology indicated that erbin knockdown significantly reduced dendritic length and branching (Fig. 1*A*,*B*, supplemental Fig. 2*A*,*B*, available at www.jneurosci.org as supplemental material), although dendrites retained their identity as examined by expression of MAP2 (Fig. 1*C*). These effects were similar in neurons expressing either shRNA. Expression of an shRNA-resistant construct of erbin significantly restored the total length of dendrites in shRNA-treated neurons (supplemental Fig. 2*B*,*C*, available at www.jneurosci.org as supplemental material), confirming the specificity of the shRNA knockdown. The rescue with the shRNA resistant construct was not complete, possibly because of the difficulty in expressing such a large protein. Alternatively, more than one of the several isoforms of erbin present may need to be expressed to promote optimal dendritic growth (Favre et al.,

2001). The rescue construct we used expresses only one of several human isoforms of erbin. Because expression of each of the nonoverlapping shRNAs results in a similar phenotype, these effects are almost certainly specific. Quantification of dendritic parameters indicated erbin depletion reduced the total length of dendrites (Fig. 1*D*) and number of dendritic end points (Fig. 1*E*), with no significant effect on the number of primary dendrites (Fig. 1*F*). Because of the greater growth rate and length of axons, it was not possible to compare the axonal lengths of these same neurons. To examine possible effects of erbin depletion on axonal growth, neurons were transfected at DIV 1 and examined at DIV 4 (supplemental Fig. 1*F*, available at www.jneurosci.org as supplemental material). Results indicated that, in contrast to its striking effects on dendritic growth, erbin depletion did not significantly reduce axon growth (total average axon lengths: vector, 692.4 ± 70.2 μm; shRNA 1, 774.6 ± 78.1 μm; $p = 0.43$). In summary, these results indicate that loss of erbin results in severe reductions in growth and branching of dendrites.

Overexpression of erbin promotes dendritic growth in hippocampal neurons

Because knockdown of erbin resulted in a reduction in dendritic growth and branching, we sought to examine whether its overexpression leads to alterations in dendritic morphogenesis. To this end, we transfected hippocampal neurons at DIV 11 with a plasmid encoding full-length erbin or a vector control. We chose to examine total dendritic length as a measure of dendritic morphogenesis. In neurons overexpressing erbin, there was a small, but significant increase in total dendritic length compared with neurons expressing the vector only (supplemental Fig. 2*D*,*E*, available at www.jneurosci.org as supplemental material). These results indicate that elevated erbin promotes dendritic length.

Overexpression of the C terminal of erbin or of the PDZ domain reduces dendritic length in hippocampal neurons

Erbin includes a C-terminal PDZ domain that is known to interact with several neuronal proteins, including δ-catenin, a component of the cadherin–catenin complex that has been implicated in dendritic branching (Borg et al., 2000; Laura et al., 2002; Martinez et al., 2003). To examine the functional role of this domain, we used a previously characterized construct (Huang et al., 2003) to overexpress the C-terminal region of erbin (amino acids 965–1371), including the PDZ domain (amino acids 1280–1368), in cultured hippocampal neurons at DIV 11 and examined neuronal morphology at DIV 17 (Fig. 2*A*). Protein expression was assessed by immunoblots of transiently transfected tsa201 cells and immunocytochemistry of transiently transfected hippocampal neurons (data not shown). We chose to examine changes in total dendritic length as a measure of alterations in dendritic morphogenesis because our previous results above demonstrated that this parameter was significantly affected by either knockdown or overexpression of erbin. Overexpression of the erbin C-terminal domain resulted in a slight, but significant decrease in dendritic length (Fig. 2*A*) (total average dendritic length: vector, 1839.5 ± 104.6 μm; vector plus C terminus, 1448.3 ± 88.6 μm; $p < 0.01$; $n > 22$ neurons). These results indicate that the C-terminal domain of erbin interacts with proteins that control dendrite growth and branching. To further confirm that this phenotype can be ascribed to the function of the PDZ domain, we performed a similar experiment by overexpressing only the PDZ domain of erbin. In this case, we observed a reduction in dendritic length in neurons overexpressing the PDZ domain (Fig. 2*B*) (total average dendritic length: vector, 1802.9 ± 206.9 μm; vector plus RFP PDZ erbin, 1102 ± 117.9 μm, SEM; $p < 0.01$; $n = 13$ neurons). The phenotype is similar to but weaker than that observed in neurons in which erbin levels have been depleted by shRNA expression (Fig. 1). Overall, these results indicate that erbin regulates dendritic morphogenesis in hippocampal neurons and are consistent with the possibility that its PDZ domain may mediate, at least in part, these functions.

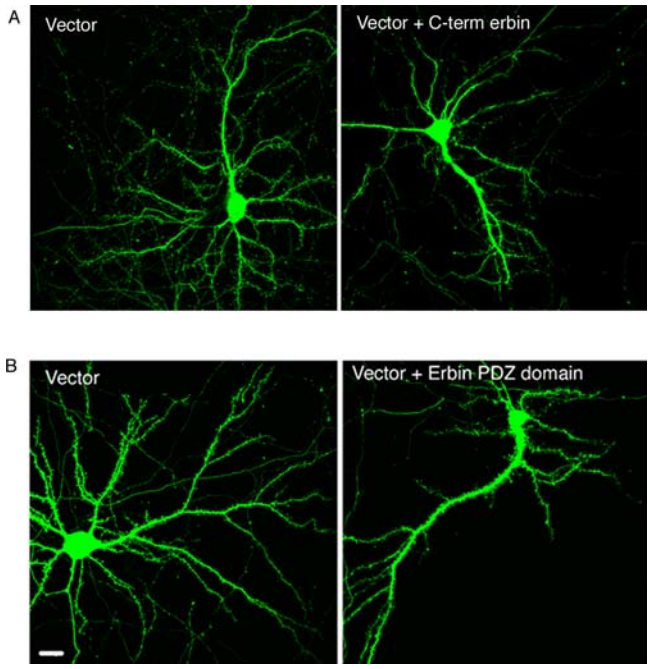

Figure 2. Overexpression of the C terminus or the PDZ domain of erbin reduces dendritic length in rat hippocampal neurons. ***A***, Rat hippocampal neurons were transfected with vector that also encodes GFP or vector and the C terminus of erbin. Representative images show dendritic morphology of neurons transfected at DIV 11 and examined at DIV 17. ***B***, Rat hippocampal neurons were transfected with vector or vector and the PDZ domain of erbin. Representative images show dendritic morphology of neurons transfected at DIV 11 and examined at DIV 17. Scale bar, 20 μm.

Hippocampal neurons from δ-*catenin*−/− mice exhibit reduced dendritic complexity

To explore possible mechanisms through which erbin regulates dendritic growth and branching, we chose to examine the role of one of its known binding partners, δ-catenin, which interacts with the PDZ domain of erbin (Laura et al., 2002). δ-Catenin is an attractive candidate to function in a signaling pathway with erbin because it has also been shown to regulate dendritic growth and branching (Kim et al., 2002, 2007; Martinez et al., 2003) although no deficits in hippocampal architecture have been observed in adult δ-*catenin*−/− mice (Israely et al., 2004). In initial experiments, we compared dendritic growth and branching in cultured hippocampal neurons from δ-*catenin*+/+ and δ-*catenin*−/− mice, using EGFP transfection to visualize the dendritic trees of individual neurons (Fig. 3). Results indicate that absence of δ-catenin significantly impairs the length and number of dendritic end points of dendritic arbors without reducing the number of primary dendrites. (total average dendritic length: δ-*catenin*+/+, 1495.29 ± 102.1 μm; δ-*catenin*−/−, 1197.02 ± 83.59 μm; $p < 0.03$; dendritic end points: δ-*catenin*+/+, 22.5 ± 2; δ-*catenin*−/−, 16.9 ± 1; $p < 0.05$; primary dendrites: δ-*catenin*+/+, 3.4 ± 0.2, δ-*catenin*−/−, 3.2 ± 0.2, SEM; $p = 0.6$; $n > 22$ neurons). These results indicate that loss of δ-catenin results in a phenotype that is grossly similar to the phenotype observed after knockdown of erbin.

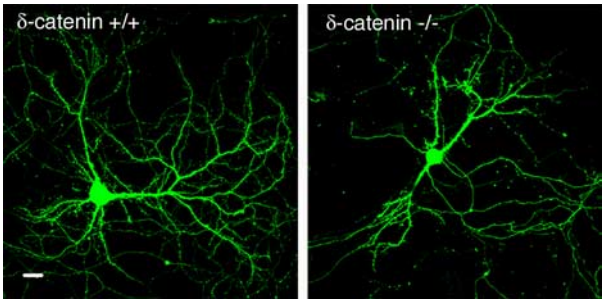

Figure 3. Hippocampal neurons from δ-catenin-null mice exhibit reduced dendritic complexity. Mouse hippocampal neurons from δ-*catenin*+/+ and δ-*catenin*−/− neurons were cultured, transfected with a plasmid encoding GFP, and examined at DIV 17. Representative images show dendritic morphology. Scale bar, 20 μm.

Acute shRNA-mediated knockdown of δ-catenin impairs dendritic complexity

To further examine the role of δ-catenin in cultured rat hippocampal neurons, we used a δ-catenin-specific shRNA to acutely deplete this protein. A basic local alignment search tool search indicated no significant homology of this sequence to any sequence in the database. Hippocampal neurons expressing the δ-catenin-specific shRNA also had strikingly reduced expression of δ-catenin, as assessed by immunocytochemistry (supplemental Fig. 3*A*, available at www.jneurosci.org as supplemental material). This shRNA was highly effective at reducing expression of δ-catenin in transiently transfected tsa201 cells and in cultured rat cortical neurons, as assessed by Western blot analysis (supplemental Fig. 3*B*,*C*, available at www.jneurosci.org as supplemental material). Thus, this δ-catenin-specific shRNA efficiently reduces δ-catenin expression.

To assess effects of acute δ-catenin depletion on hippocampal neuron development, neurons were transfected with vector or shRNA at DIV 11 and examined at DIV 17 (Fig. 4*A*). Quantification of results shows that depletion of δ-catenin results in reduced dendritic length and number of dendritic end points without affecting the number of primary dendrites (total average dendritic length: vector, 1841.5 ± 124.4; shRNA, 806.6 ± 79.9 μm; $p < 0.0001$; dendritic end points: vector, 28.9 ± 1.6; shRNA, 19.9 ± 1.2; $p < 0.0001$; primary dendrites: vector, 5.6 ± 0.3; shRNA, 5.7 ± 0.3; $p = 0.89$, SEM; $n > 22$ neurons) (supplemental Fig. 3*D*, available at www.jneurosci.org as supplemental material), similar to the phenotypes observed in neurons cultured from δ-catenin-null mice. Expression of an shRNA-resistant construct of δ-catenin significantly restored the total length of dendrites in shRNA-treated neurons (supplemental Fig. 3*E*,*F*, available at www.jneurosci.org as supplemental material), confirming the specificity of the shRNA knockdown. The dendrites of shRNA-transfected neurons continued to express the dendritic marker, MAP2 (Fig. 4*B*), indicating that depletion of δ-catenin did not perturb dendritic identity. These results indicate that acute knockdown of δ-catenin results in inhibitory effects on dendritic growth and branching similar to effects of genetic deletion of δ-catenin in cultures from mutant mice.

Nonadditive effects of erbin and δ-catenin depletion on dendritic length

To determine whether erbin and δ-catenin may act in the same pathway to promote dendritic length, we compared the consequences on dendritic arbor growth of simultaneous depletion of both erbin and δ-catenin to depletion of either protein alone, using both rat and mouse hippocampal neurons. We chose to examine the total dendritic length as a measure of dendritic morphogenesis, because our results in Figures 1–4 indicate that this parameter was significantly affected by loss of either erbin or δ-catenin. Results in Figure 5, *A* and *B*, show that total dendritic arbor length in murine neurons is reduced to similar extents by erbin-specific shRNA treatment of wild-type neurons, by genetic deletion of δ-catenin, and by erbin-specific shRNA treatment of δ-*catenin*-null neurons. We confirmed these results using shRNA-mediated knockdown of erbin and δ-catenin in rat hippocampal neurons. To this end, we examined neuronal morphology at DIV 17 in cultured rat hippocampal neurons that were transfected at DIV 11 with a vector control, erbin-specific, or δ-catenin-specific shRNA, or both erbin-specific and δ-catenin-specific shRNAs. Similar to the data in Figures 1 and 4, loss of either erbin or δ-catenin resulted in a highly significant decrease in total dendritic arbor length (Fig. 5*C*,*D*). The reductions in dendritic arbor length observed after depletion of either one or both proteins were not significantly different from each other. Because the double depletion of erbin and δ-catenin results in a similar phenotype to depletion of either protein alone in both rat and mouse hippocampal neurons, these results suggest that both erbin and δ-catenin regulate dendritic length via the same pathway.

Overexpression of δ-catenin, but not p120ctn, can rescue the loss of erbin phenotype

We next sought to examine whether the reduction in dendritic arbor growth resulting from depletion of erbin can be rescued by overexpression of δ-catenin by examining neurons that had been transfected with a vector control, erbin-specific shRNA, or erbin-specific shRNA plus δ-catenin. Visual examination indicated that transfection with δ-catenin restored significantly dendritic arbor growth in neurons lacking normal levels of erbin (Fig. 6*A*). Quantification showed that expression of EGFP-δ-catenin in the presence of erbin-specific shRNA 1 increased dendritic arbor length significantly (Fig. 6*B*) Similarly, overexpression of a WT δ-catenin in the presence of erbin-specific shRNA 2 resulted in an increase in total dendritic length (Fig. 6*B*). In each case, the inhibitory effect of erbin-specific shRNA transfection was significantly reduced, but not eliminated by coexpression of δ-catenin.

We then sought to examine whether this erbin deficiency phenotype could be restored by overexpression of p120ctn, a member of the δ-catenin family does not bind erbin because it lacks a PDZ domain interaction motif similar to that present in δ-catenin (Laura et al., 2002). Similar to δ-catenin, p120ctn has also been shown to regulate dendritic branching (Elia et al., 2006). In contrast to overexpression of δ-catenin, overexpression of p120ctn in neurons depleted of erbin did not result in enhanced dendritic arbor growth (Fig. 6*C*). Thus, loss of erbin can be partially compensated for by overexpression of its binding partner δ-catenin, but not by overexpression of the δ-catenin homolog, p120ctn, that is unable to interact with erbin. We also examined the levels of expression of erbin in the cortex of δ-catenin-null mice at P0/P1 by immunoblotting cortical lysates. There were no obvious changes in the level of expression of erbin in δ-*catenin*−/− cortex compared with δ-*catenin*+/+ cortex (data not shown). Together, the results in this section are consistent with a model in which the PDZ domain-mediated interaction of erbin and δ-catenin regulates dendritic arbor length with δ-catenin acting downstream of erbin in this pathway.

δ-Catenin shRNA-induced reduction in dendritic length is not restored by overexpression of erbin

To further confirm the directionality of the pathway by which erbin and δ-catenin regulate dendritic morphogenesis, we sought

to examine whether the dendritic length phenotype generated by loss of δ-catenin could be rescued by overexpression of erbin. To this end, we transfected rat hippocampal neurons at DIV 11 with constructs expressing vector, δ-catenin shRNA alone, or δ-catenin shRNA and a construct mediating overexpression of erbin (Fig. 6*D*). Expression of the δ-catenin shRNA significantly reduced dendritic length, as estimated by total dendritic length. However, dendritic length was not restored in neurons expressing both δ-catenin shRNA and a plasmid expressing full-length erbin. These results, together with previous results, provide additional evidence that erbin and δ-catenin regulate dendritic length via a common pathway in which erbin is upstream of δ-catenin.

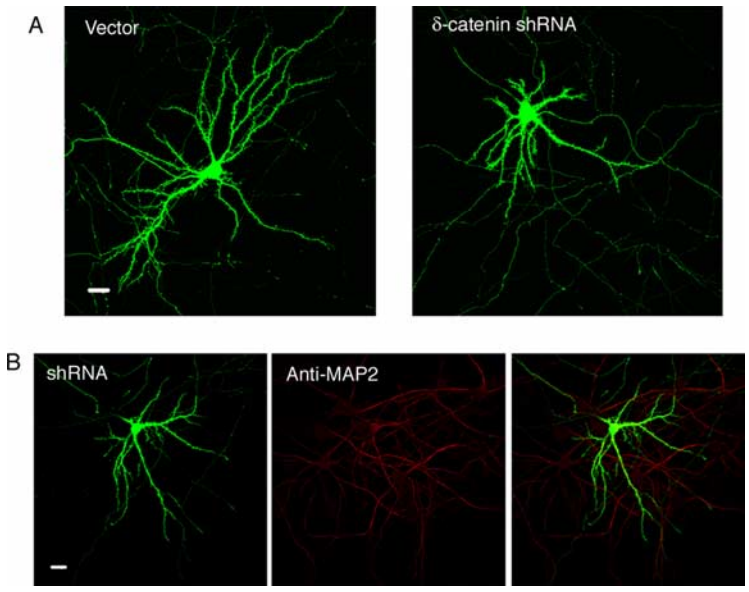

Figure 4. Acute knockdown of δ-catenin impairs dendritic morphogenesis. ***A***, Rat hippocampal neurons were transfected at DIV 11 with vector or δ-catenin shRNA and examined at DIV 17. Representative images show dendritic morphology. ***B***, Anti-MAP2 staining at DIV 17 of cultured rat hippocampal neurons expressing δ-catenin shRNA. Scale bars, 20 μm.

The C-terminal region of δ-catenin is necessary for δ-catenin to rescue the loss of function of erbin phenotype

Overexpression of δ-catenin has been shown to promote dendritic branching in hippocampal neurons. A region in the C terminus of δ-catenin adjacent to the arm repeats, but not including the PDZ domain, has been implicated in this functional role (Martinez et al., 2003; Kim et al., 2008). To delineate the mechanism by which δ-catenin regulates dendritic length, we generated a series of C-terminal deletions of δ-catenin (Fig. 7*A*), including the N terminus alone (amino acids 1–529), N terminus plus arm repeats (amino acids 1–1035), and full-length lacking the C-terminal 4 aa (δ-PDZ interaction motif; amino acids 1–1243). Robust expression of each construct was observed in Western blots of transfected tsa201 cells (Fig. 7*B*).

To examine the ability of these δ-catenin truncations to promote dendritic arbor growth in neurons lacking erbin, each construct was cotransfected with erbin shRNA 1 into hippocampal neurons at DIV 11 and dendritic arbor morphologies were examined 6 d later at DIV 17. Consistent with our previous data (Fig. 1), expression of erbin shRNA caused a severe reduction in total dendritic arbor length compared with cells that express the control vector (Fig. 7*C*). Overexpression of the N terminus or the N terminus-arm constructs had no significant effect on the total dendritic length in neurons that also expressed the erbin shRNA. However, the expression of the δ-PDZ construct did partially restore the total dendritic length in cells that expressed the erbin shRNA.

In previous work, it has been shown that the C-terminal region of δ-catenin,

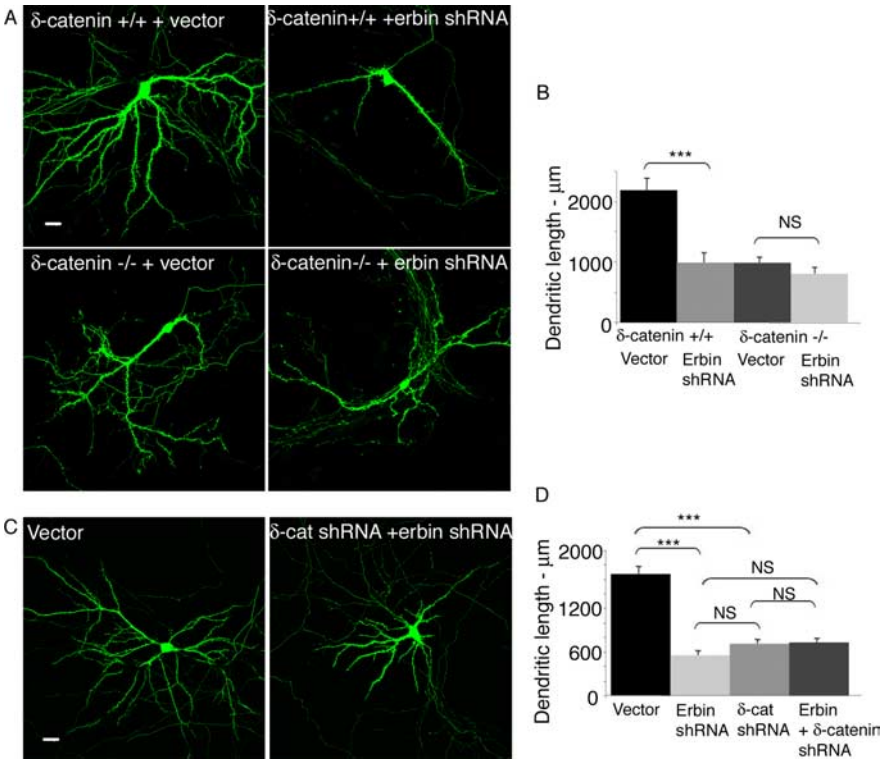

Figure 5. Nonadditive effects of erbin and δ-catenin depletion on dendritic length. ***A***, Hippocampal neurons from wild-type (*δ-catenin*+/+) or δ-catenin-null (*δ-catenin*−/−) mice were transfected with a vector or erbin shRNA at DIV 11 and examined at DIV 17. Representative images of neuronal morphology are shown. ***B***, Quantification of total average dendritic length from *δ-catenin*+/+ and *δ-catenin*−/− hippocampal neurons expressing vector or erbin shRNA (*δ-catenin*+/+ + vector, 2186.32 μm ± 193.05 μm; *δ-catenin*+/+ + shRNA, 987.88 μm ± 158.25 μm; $p < 0.0005$; *δ-catenin*−/− + vector, 982.29 μm ± 91.56; *δ-catenin*−/− + shRNA, 804.40 μm ± 98.31; $p = 0.2$; $n > 10$ neurons). ***$p < 0.0005$. ***C***, Rat hippocampal neurons were transfected at DIV 11 with the indicated constructs and fixed at DIV 17. Representative images of neuronal morphology are shown. ***D***, Quantification of total average dendritic length from neurons expressing the indicated constructs (vector, 1669.14 ± 104.56 μm; erbin shRNA, 549.83 ± 60.71 μm; δ-catenin shRNA, 707.67 ± 58.17 μm; $p <$ 0.00001 for vector vs erbin shRNA and vector vs δ-catenin shRNA; $n > 22$ neurons). ***$p < 0.0001$. NS, Not significant. Scale bars, 20 μm. Error bars indicate SEM.

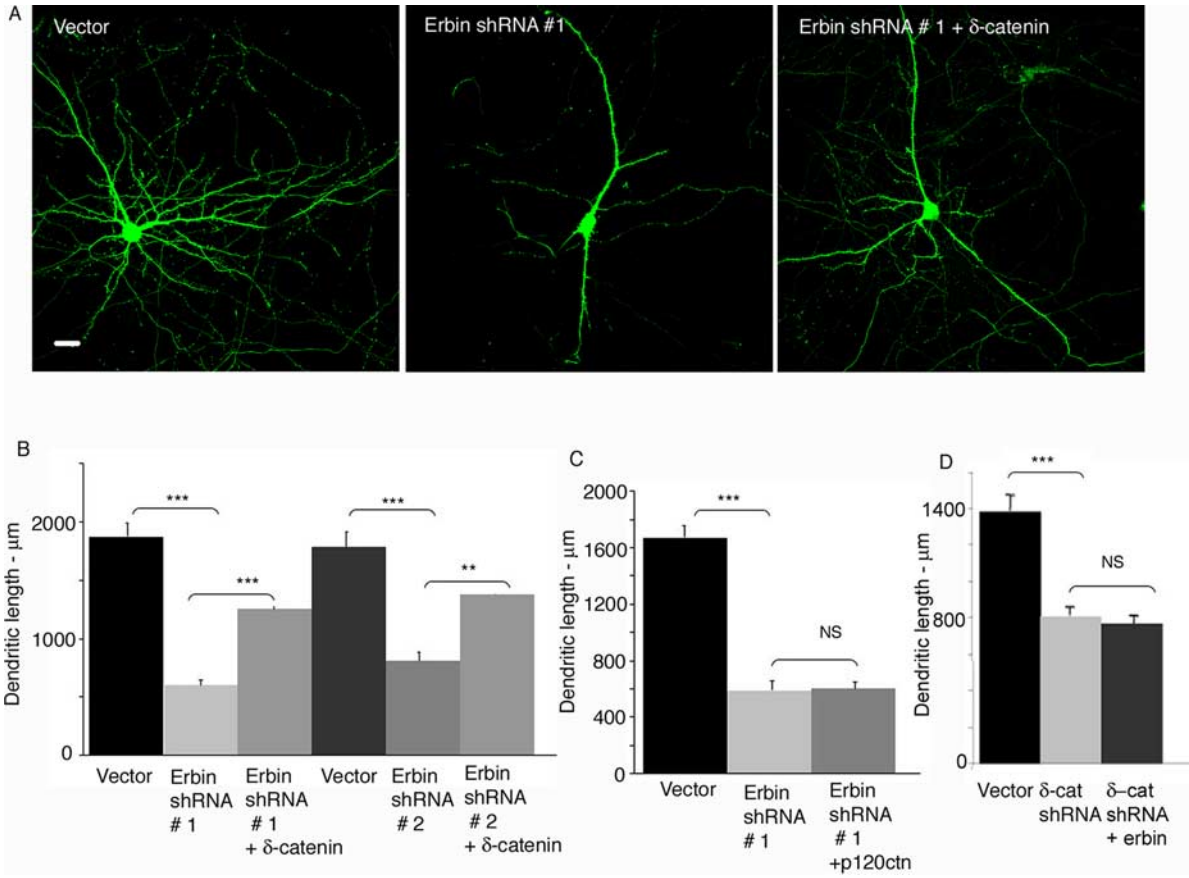

Figure 6. Erbin is upstream of δ-catenin in regulating dendritic morphogenesis. ***A***, Rat hippocampal neurons were transfected at DIV 11 with vector, erbin shRNA 1, or erbin shRNA 1 plus EGFP δ-catenin and fixed at DIV 17. Representative images show dendritic morphology. Scale bar, 20 μm. ***B***, Quantification of dendritic length from neurons transfected at DIV 11 with the indicated constructs and fixed at DIV 17 (vector, 1871.7 ± 119.56 μm; shRNA 1, 590.26 ± 39.67 μm; shRNA 1 + δ-catenin, 1252.68 ± 97.59 μm; $p < 0.0001$ for vector vs shRNA 1; $p < 0.0001$ for shRNA 1 vs shRNA 1 + δ-catenin; vector, 1780.54 ± 127.96 μm; shRNA 2, 806.37 ± 69.07 μm; shRNA 2 + δ-catenin, 1397.63 ± 142.13 μm; $p < 0.0001$ for vector vs shRNA 2; $p < 0.001$ for shRNA 2 vs shRNA 2 + δ-catenin). ***C***, Quantification of total average dendritic length from neurons transfected with vector 1, erbin shRNA 1, or erbin shRNA 1 and p120ctn (vector, 1666.96 ± 87.86 μm; shRNA 1, 586.72 ± 69.82 μm; shRNA 1 + p120ctn, 600.29 ± 44.47 μm; $p < 0.0001$ for vector vs shRNA 1; $p = 0.87$ for shRNA 1 vs shRNA 1 + p120ctn; $n > 18$ neurons each). ***D***, Quantification of total dendritic length in neurons expressing vector, δ-catenin (δ-cat) shRNA, or δ-catenin shRNA plus full-length erbin ($n > 20$ neurons each). $^{**}p < 0.001$; $^{***}p < 0.0001$. NS, Not significant. Error bars indicate SEM.

not including the PDZ interaction motif (Martinez et al., 2003; Kim et al., 2008), is necessary for the ability of δ-catenin to induce dendritic branching. Our results are consistent with a model in which erbin interacts with the C-terminal PDZ interaction motif of δ-catenin to localize this protein. δ-Catenin, in turn, functions to promote arbor growth through other interactions within the C-terminal region.

Loss of δ-catenin does not affect the levels of active Rac1 and RhoA

Previous studies have suggested a number of candidate proteins that might affect the ability of δ-catenin to regulate branching. It has been suggested that overexpression of δ-catenin inhibits RhoA via p190RhoGEF (Martinez et al., 2003; Kim et al., 2007) Furthermore, our lab has demonstrated previously that loss of p120ctn, a member of the same family as δ-catenin, results in a significant increase in the levels of active RhoA with a concomitant decrease in active Rac1 (Elia et al., 2006). Hence, we chose to examine whether loss of δ-catenin leads to alterations in the levels of active Rac1 and RhoA. Contrary to our expectations, hippocampal neurons from δ-catenin mice examined at DIV 17 did not show any significant alterations in the levels of active Rac1 and RhoA compared with heterozygous littermate controls (active RhoA percentage of control: control, 100 ± 7.16; mutant, 103.44 ± 5.54; $p = 0.71$; active Rac1 percentage of control: control, 100 ± 4.96; mutant, 94.4 ± 14.05; $p = 0.72$; number of animals for RhoA: control, $n = 9$; mutant, $n = 6$; Rac1: control, $n = 8$; mutant, $n = 5$). Heterozygous mice did not have any changes in dendritic length, as examined by the total dendritic length, in compared with δ-*catenin*+/+ neurons (data not shown). These results suggest that δ-catenin can regulate dendritic morphogenesis via an Rac1/RhoA-independent pathway. Furthermore, together with previous results from our lab (Elia et al., 2006), these results suggest that δ-catenin and p120ctn may regulate dendritic morphogenesis via different mechanisms.

Knockdown of erbin or perturbation of the interaction between erbin and δ-catenin leads to altered localization of δ-catenin

How does the loss of erbin impair the ability of δ-catenin to regulate dendritic morphogenesis? To examine the impact of loss of erbin on δ-catenin, we took advantage of the ability to express EGFP-tagged δ-catenin in neurons. EGFP-δ-catenin showed a somewhat diffuse distribution including concentration associated with dendrites and spines (supplemental Fig. 4*A*, available at www.jneurosci.org as supplemental material) and some diffuse

fluorescence in axons. The dendritic staining partially colocalizes with the presynaptic marker synaptophysin, similar to the distribution of endogenous δ-catenin (supplemental Fig. 4*B*, available at www.jneurosci.org as supplemental material).

We examined the effect of expressing the erbin shRNA or the C terminus of erbin on the localization of EGFP-δ-catenin. Expression of EGFP-δ-catenin with vector only resulted in a pattern similar to that observed with EGFP-δ-catenin alone, whereas coexpression of shRNA 1 to erbin resulted in a more punctate pattern of expression of EGFP δ-catenin (Fig. 8*A*,*B*). These punctate structures were observed in the cell bodies, dendrites and axons, but more prominently in dendrites and cell bodies. Similarly, the pattern of distribution of EGFP-δ-catenin was altered by coexpression of the C terminus of erbin (Fig. 8*C*,*D*) or coexpression of the PDZ domain of erbin (Fig. 8*E*,*F*).

Consistent with the role of the erbin–δ-catenin PDZ interaction in localizing δ-catenin, EGFP-δ-catenin-δ-PDZ, a protein lacking the last four PDZ-binding amino acid motif, did not show any appreciable change in distribution when coexpressed with the C terminus of erbin (EGFP-δ-catenin-δ PDZ: diffused distribution, $n = 14$; punctate distribution, $n = 32$; EGFP-δ-catenin plus C terminus of erbin: diffused distribution, $n = 10$; punctate distribution, $n = 23$). The identity of organelles defined by these puncta is under investigation, but our preliminary data indicate that they do not colocalize with the golgi marker GM130, early endosome marker EEA1, or synaptophysin. These results indicate that knockdown of erbin or perturbation of the interaction between δ-catenin and erbin results in altered localization of δ-catenin.

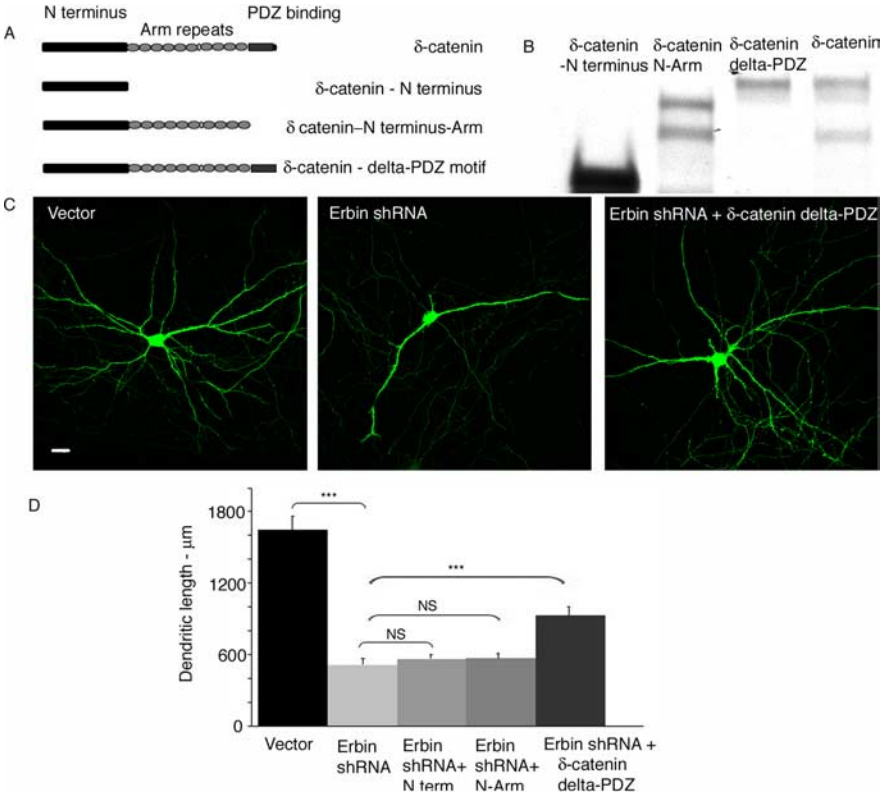

Figure 7. The C-terminal region of δ-catenin is necessary for δ-catenin to rescue the loss of function of the erbin phenotype. ***A***, Schematic of constructs of δ-catenin. ***B***, Immunoblots from tsa201 cells expressing the constructs in ***A*** with anti-δ-catenin. ***C***, Rat hippocampal neurons in culture were transfected with the indicated constructs at DIV 11 and fixed at DIV 17. Representative images show dendritic morphology. ***D***, Quantification of total average dendritic length from neurons expressing the indicated constructs (vector, 1644.93 ± 116.91 μm; shRNA, 510.51 ± 50.56 μm; shRNA + N-term, 560 ± 37.86; shRNA + N-Arm, 563.35 ± 45.33 μm; shRNA + Δ-PDZ, 932.52 ± 76.30 μm; *p* values vs shRNA: vector, <0.0001; shRNA + N-term, 0.43; shRNA + N-Arm, 0.44; shRNA + Δ-PDZ, <0.0001; $n > 19$ neurons). ***$p < 0.0001$. Scale bar, 20 μm. Error bars indicate SEM.

Discussion

Motivated by the known critical roles of LAP proteins in maintenance of apical-basolateral polarity in epithelial cells (Bilder and Perrimon, 2000), which is analogous to axon-dendrite polarity in neurons (Jareb and Banker, 1998; Parrish et al., 2007), we examined the function of one member of this family, erbin, which is highly expressed in the nervous system. Our results indicate that erbin functions to promote dendrite growth and branching in hippocampal neurons. Our data also indicate that erbin functions, at least in part, through controlling the localization and possibly activity of δ-catenin, a cadherin-associated protein known to regulate organization of the F-actin cytoskeleton through control of the Rho family of GTPases and cortactin activities (Martinez et al., 2003).

The maintenance of proteins in an appropriate cellular localization is often critical for their function (Shi et al., 2003; Zhang et al., 2003; Hoogenraad et al., 2005). A number of studies have indicated that LAP proteins, including scribble, erbin, and densin-180, are tethered to the membrane through their leucine-rich repeat motifs (Legouis et al., 2003; Navarro et al., 2005; Quitsch et al., 2005). Erbin is further concentrated in neurons at the postsynaptic density (Huang et al., 2001). We propose a model in which membrane-localized erbin concentrates δ-catenin at sites optimal for the ability of δ-catenin to regulate the cytoskeleton and hence dendrite morphogenesis (supplemental Fig. 5, available at www.jneurosci.org as supplemental material).

It is not entirely clear how the loss of erbin perturbs the localization of δ-catenin. δ-Catenin has a diffuse distribution in neurons with some localization to synaptic sites. Erbin has been proposed to be localized to synaptic sites. Given this distribution, it is likely that only a proportion of δ-catenin is associated with erbin. This is consistent with the ability of δ-catenin to bind to other PDZ domain-containing proteins, including densin-180 (Izawa et al., 2002; Quitsch et al., 2005), papin (Deguchi et al., 2000), and S-SCAM (synaptic scaffolding molecule) (Ide et al., 1999). We propose that the absence of erbin promotes the association of δ-catenin with other proteins in a different subcellular location and thereby impairs the ability of δ-catenin to promote dendritic morphogenesis.

LAP family proteins in neuronal morphogenesis

The functional roles of LAP family of proteins have been predominantly explored in non-neuronal cell types and perhaps the most well studied member of the family is scribble. In *Drosophila*, scribble is required for maintaining apical-basolateral polarity and synaptic architecture and plasticity (Roche et al., 2002). In mammalian cells, scribble regulates planar cell polarity (Montcouquiol et al., 2003; Phillips et al., 2007), receptor trafficking (Lahuna et al., 2005), localization of adenomatous polyposis coli

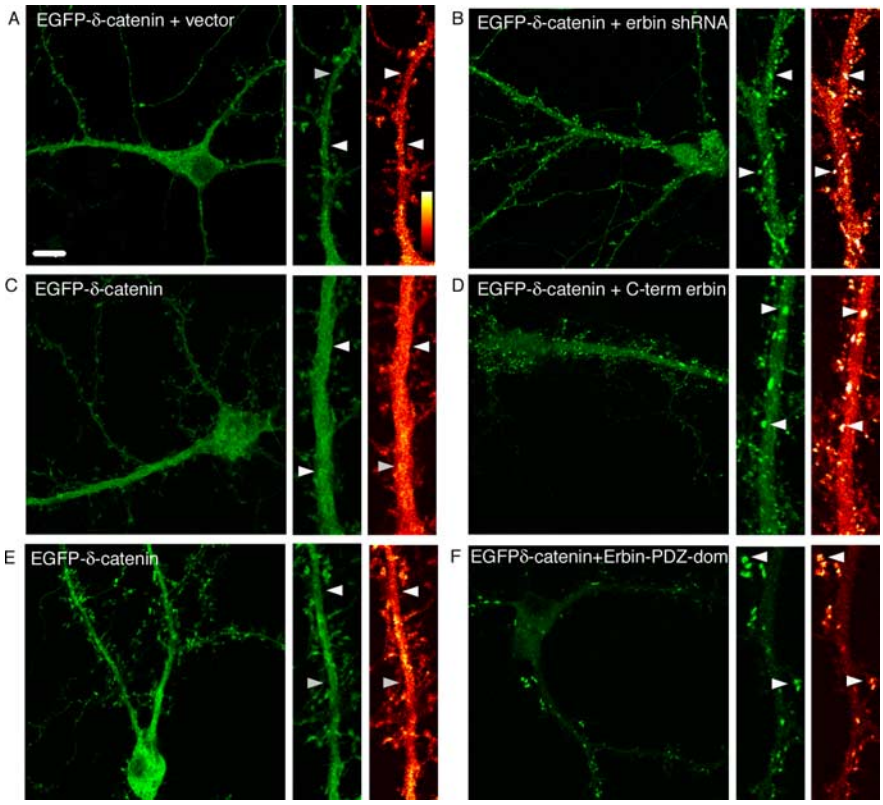

Figure 8. Erbin knockdown or overexpression of the C terminal or PDZ domain of erbin leads to altered localization of δ-catenin. ***A–F***, Altered localization (arrowheads) of EGFP-δ-catenin in hippocampal neurons that express EGFP-δ-catenin and vector (***A***), EGFP-δ-catenin and erbin shRNA (***B***), EGFP-δ-catenin (***C***, ***E***), EGFP-δ-catenin and the C terminus of erbin (***D***), and EGFP δ-catenin and the PDZ domain of erbin (***F***). Images are pseudocolored for fluorescence intensity (EGFP-δ-catenin + vector: diffuse distribution, $n = 12$; punctate distribution, $n = 4$; EGFP-δ-catenin + erbin shRNA 1: diffuse distribution, $n = 1$; punctate distribution, $n = 17$; EGFP-δ-catenin: diffuse distribution, $n = 19$; punctate distribution, $n = 3$; EGFP-δ-catenin + C terminus of erbin: diffuse distribution, $n = 0$; punctate distribution, $n = 28$; EGFP-δ-catenin: diffuse distribution, $n = 18$; punctate distribution, $n = 2$; EGFP-δ-catenin + PDZ domain (dom) of erbin: diffuse distribution, $n = 0$; punctate distribution, $n = 8$). Scale bar, 10 μm.

to the basolateral membrane (Takizawa et al., 2006), and exocytosis (Audebert et al., 2004), but there is no clear evidence for a role in regulating apical–basolateral polarity (Murdoch et al., 2003). Both scribble (Audebert et al., 2004; Dow et al., 2007) and densin-180 (Walikonis et al., 2001) have been suggested to serve as scaffolding proteins. The functional roles of LAP proteins in mammalian neurons have not been extensively investigated. A mouse mutant of *scribble*, *circletail*, exhibits severe neural tube closure deficits (Murdoch et al., 2003). Consistent with our results, overexpression of densin-180, another LAP protein that interacts with δ-catenin, promotes dendritic branching in hippocampal neurons (Quitsch et al., 2005). Because overexpression of shank inhibits densin-180-induced dendritic branching and the association of densin-180 with δ-catenin, it has been suggested that densin regulates dendritic branching through δ-catenin.

Our studies on erbin indicate that in hippocampal neurons it serves as a scaffold that is necessary for maintaining the appropriate localization within dendrites of δ-catenin and probably additional proteins. Loss of erbin does not disrupt the established polarity of neurons as assessed by the presence of axons and dendrites, but does impair growth and branching of dendritic processes. Our results indicate that the function of erbin, and potentially additional LAP proteins, is to localize proteins involved in dendrite growth and branching.

δ-Catenin/p120ctn family in dendritic morphogenesis

Two members of the p120catenin family, δ-catenin (the present study) and p120ctn (Elia et al., 2006), function to promote dendrite growth and branching. Unlike δ-catenin, P120ctn does not have a PDZ-binding motif and does not interact with erbin (Laura et al., 2002). Our data suggest that in contrast to p120ctn (Elia et al., 2006), δ-catenin may not entirely regulate dendritic branching through global regulation of active Rac1 and RhoA, because the active levels of these proteins are not significantly altered in neurons from mice that lack δ-catenin, although these neurons have compromised dendritic branching.

Our studies also indicate that although δ-catenin and p120ctn are members of the same family of proteins, their functional roles may be different and they are regulated by different upstream regulators and may also have specific downstream effector mechanisms that are not entirely functionally redundant.

In addition to erbin-mediated localization of δ-catenin via its C-terminal PDZ binding motif, cadherins also localize δ-catenin to the plasmalemma through an interaction between the cadherin cytoplasmic domain and the armadillo repeats in δ-catenin. Although cadherins are present in hippocampal neurons, the presence of erbin promotes the ability of δ-catenin to promote dendrite growth and branching. This indicates that membrane anchoring of δ-catenin per se in the absence of erbin is not sufficient for δ-catenin to be fully active in promoting dendrite growth. One possibility is that erbin localizes δ-catenin to membrane regions different from those at which it is concentrated by cadherin binding. Another is that erbin binding to δ-catenin promotes effector functions of δ-catenin, such as activation of cortactin, which stimulate dendrite growth. There are many examples where interactions of catenins with other proteins regulate their stability and effector functions (Gottardi and Gumbiner, 2004a,b)

In summary, our data reveals a novel functional role for the LAP protein erbin in dendritic branching in hippocampal neurons and provides a mechanism for this functional role. In the future, it will be interesting to examine the regulation and dynamics of erbin–δ-catenin interactions and assess if these are regulated in a temporal manner by development and activity.

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