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Ephrin-B3 reverse signaling through Grb4 and cytoskeletal regulators mediates axon pruning

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Abstract

Receptor-like roles for ephrin-B proteins are implicated to control axon pathfinding by repulsion, although it is largely unknown how the reverse signals are coupled to downstream intracellular molecules and induce cytoskeletal reorganization at the axon terminal. Here, we show ephrin-B3 (EB3) functions as a repulsive guidance receptor to mediate stereotyped pruning of murine hippocampal mossy fiber (MF) axons during postnatal development. Targeted intracellular point mutants show that axon pruning requires tyrosine phosporylation-dependent reverse signaling and coupling to the SH2/SH3 adaptor protein Grb4 (also known as Nckβ/Nck2). Furthermore, the second SH3 domain of Grb4 is required and sufficient for axon pruning/retraction by mediating interactions with Dock180 and PAK, to bring about guanine nucleotide exchange and signaling downstream of Rac, respectively. These studies reveal a novel pathway that controls axon pruning and elucidate the biochemical mechanism by which ephrin-B reverse signals regulate actin dynamics to bring about the retraction of growth cones.

Keywords

ephrin-B3; reverse signaling; tyrosine phosphorylation; hippocampus; mossy fiber; axon guidance

Axons extending from neurons grow and elongate over long distances toward their target cells under the guidance of various external cues and receptor signaling systems to form functional neural circuits1-3. As they pathfind axon branches often overshoot their intended target and these exuberant branches need to be stereotypically pruned during neural development4, 5. While the spatial and temporal coordination of axon extension and pruning/retraction is of fundamental importance to ensure precise neuronal connectivity as well as attempts to repair damage4-6, the molecular mechanisms *in vivo* that control the outgrowth and pruning/retraction of axons are poorly understood.

An established model of axon pruning in the formation of neural circuits involves hippocampal mossy fiber (MF) axons, which project from granule cell neurons in the dentate gyrus (DG) and target the pyramidal neurons in CA3 area to form the DG-CA3

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neuronal network7. MF axons form two distinct bundles that target CA3 neurons, the supra- and infra-pyramidal bundles (SPB and IPB). Whereas the SPB forms a large extensive track that targets the main dendritic arbors of the CA3 pyramidal neurons, the IPB axons initially grow through the entire length of the CA3 region but then retract and prune their growth cones to join the SPB7-9. Precise regulation of MF axon outgrowth and pruning thus works cooperatively to form the DG-CA3 circuit, which is important for learning and memory and considered as a bottleneck in the processing of sensory information in the brain10.

Here, we have studied the role of ephrin-B3 (EB3) reverse signaling in stereotyped pruning of MF axons by using *in vivo* gene targeting approaches and *in vitro* live cell assays. We show that EB3 tyrosine phosphorylation-dependent reverse signaling is required for the postnatal shortening of IPB axons and that EphB molecules serve as the ligands in CA3 postsynaptic pyramidal neurons to stimulate EB3 reverse signaling into the MF axons. We provide further evidence that implicates the SH2/SH3 adaptor protein Grb4 as a molecular bridge that connects the tyrosine phosphorylated EB3 cytoplasmic tail with the Dock180 guanine nucleotide exchange factor, Rac activation, and the downstream effector PAK to mediate axon retraction and pruning.

RESULTS

Ephrin-B3 reverse signaling prunes MF axons

To investigate the cellular and molecular mechanisms of axon pruning, we characterized the MF phenotype of knockout and knockin mice of various *Eph*/*ephrin* genes, which encode a large family of interacting transmembrane proteins implicated in axon guidance and cell migration11-14. These molecules function upon axon-cell or cell-cell contact to transduce bidirectional signals into both the Eph-expressing and ephrin-expressing cells15-17, and are known to be involved in mediating contact-dependent repulsion events11-14. We first analyzed the expression of *ephrin-B3* (*EB3*) and *Eph* genes in the hippocampal DG-CA3 area at birth (P0), throughout postnatal development (P7–P42), and in adult mice (8–10 weeks old) using X-gal staining and anti-β-gal immunofluorescence (IF) of brain sections from animals carrying the *EB3*^{lacZ}, *EphB1*^{lacZ}, *EphB2*^{lacZ}, and *EphA4*^{lacZ} *lacZ* reporter knockin mutations in conjunction with anti-calbindin IF which labels MF axons (Fig. 1a and Supplementary Figs. 1–4). The *EB3*^{lacZ} and *EphB2*^{lacZ} knockin mice express C-terminal truncated EB3-β-gal and EphB2-β-gal fusion proteins that lack the majority of their respective cytoplasmic segment and traffic normally to the cell surface15, 18, while the *EphB1*^{lacZ} and *EphA4*^{lacZ} knockins are protein-nulls that express unconjugated β-gal that mainly localizes to the cell bodies19, 20. In these knockin mice, β-gal is expressed in the cells that normally express the endogenous Eph/ephrin molecule targeted. While EB3 was not expressed at P0 and only weakly at P7, it became strongly expressed in the DG after P10 and localized to the MF axons, both SPB and IPB tracks (Fig. 1a and Supplementary Fig. 1), which is consistent with previous studies21. EphB1 was localized specifically in the CA3 pyramidal cell layer and subgranular zone of the DG at all stages analyzed, while EphB2 and EphA4 were expressed broadly in both the DG and CA3 regions (Fig. 1a and Supplementary Figs. 2–4). In the CA3 region, the subcellular localization of EphB2 made possible by the EphB2-β-gal fusion protein indicates that this molecule is densely localized

above and below the MF axons (Fig. 1a). Close inspection indicates EphB2 surrounds the both SPB and IPB axon tracts, but does not overlap with the calbindin signal that marks the MF axons themselves. EphB2 was however detected in the dendritic region of the DG (Fig. 1a). EphA4 expression was also obvious in both granule cells in the DG and CA3 pyramidal neurons (Supplementary Fig. 4), again consistent with previous studies22. Together, these data indicate that EB3 is localized within MF axons and that EphB1, EphB2 and EphA4 are expressed in the CA3 target area.

To determine if EB3 has a role in MF axons during development, we examined the SPB and IPB tracks in *EB3*$^{-/-}$ null mice (*EB3*^{neo} in ref. 18) using both anti-calbindin IF and Timm stain in comparison to wild-type littermates (Fig. 1b,c). The absence of EB3 did not cause an obvious change in overall morphology of the hippocampus in adult mice as viewed by staining with an IF dye neurotrace to label nuclei (Fig. 1b). By using anti-calbindin IF in the wild-type mice most MF axons from the DG were seen in the SPB above the CA3 pyramidal cell bodies, while a smaller group of IPB axons traveled underneath the pyramidal cells (**Fig. 1b,** left panels). In wild-type animals, the IPB axons were observed to cross the CA3 cell layer to join the SPB axons shortly after exiting the dentate hilus. In contrast, the IPB axons in *EB3*$^{-/-}$ mutants were observed to extend much farther into the CA3 area underneath the pyramidal neurons (**Fig. 1b,** right panels). The abnormally long IPB in *EB3*$^{-/-}$ mutants was also readily visualized with Timm stain of adult mice (Fig. 1c).

We then examined the postnatal development of MF axons as the IBP is known in wild-type mice to initially extend much farther through the CA3 area prior to being pruned back and reduced to their mature length8, 9. We found that while the IPB length in wild-type animals decreased with age, the IPB in *EB3*$^{-/-}$ null mice did not (Fig. 1d and Supplementary Fig. 5). As the initial outgrowth of the IPB appeared similar between wild-type and mutant neonates, the lack of shortening observed in the absence of EB3 expression as the animal ages indicates an important role for this molecule in the postnatal pruning of these axons.

We next asked if the cytoplasmic domain of EB3 is required for IPB axon pruning. As described previously18, the *EB3*^{lacZ} mutation expresses a truncated EB3-β-gal fusion protein that retains the extracellular and transmembrane domains to provide ligand-like activities to initiate forward signaling, but lacks the conserved cytoplasmic segment and thus cannot transduce reverse signals requiring tyrosine phosphorylation/SH2 and C-terminal/PDZ protein-protein interactions. As visualized using anti-calbindin IF and Timm stain (Supplementary Fig. 6), the *EB3*$^{lacZ/lacZ}$ mutants showed a longer IPB that was similar to the *EB3*$^{-/-}$ mice. Quantitation of IPB length in adults showed that both *EB3*$^{-/-}$ and *EB3*$^{lacZ/lacZ}$ genotypes presented with a significantly longer IPB than that of wild-type controls throughout the rostral-caudal range of the hippocampus, but no significant difference was observed between the two mutants (Fig. 1e). This data suggests that the functional role of EB3 in IPB shortening is due to its ability to act like a receptor and transduce reverse signals.

The high levels of expression of many EphB receptors and EphA4 in the CA3 region (Fig. 1a and Supplementary Figs. 2–4) raise the possibility that these molecules may act as ligands to bind EB3 and activate reverse signaling in MF axons. We therefore analyzed the

length of the IPB in adult mice single homozygous for *EphB1*, *EphB2*, *EphB3*, or *EphA4* protein-null mutations using anti-calbindin IF to determine which, if any, is the biologically significant partner. This analysis revealed defective IPB shortening in the *EphB1*$^{-/-}$, *EphB2*$^{-/-}$, and *EphB3*$^{-/-}$ mutants, but not in the *EphA4*$^{-/-}$ mutants (Fig. 2). However, the SPB axons in *EphA4*$^{-/-}$ mutants appeared much thinner and less robust than that of the wild-type mice (or the other mutants analyzed here), suggesting a different role for EphA4 in MF targeting that does not involve EB3. To further examine whether the EphB molecules act as ligands coordinately, we tested double and triple knockout mice lacking EphB1/2 or lacking EphB1/2/3 respectively. The data showed that *EphB1/2* and *EphB1/2/3* compound knockout mice exhibited more severe defects in IPB shortening compared with the single *EphB* mutant mice (Fig. 2). These data indicate that multiple EphB receptors are involved in pruning of IPB axons *in vivo*.

Axon pruning requires ephrin-B3 tyrosine phosphorylation

We then asked how EB3 reverse signaling may bring about the pruning of MF axons. A major component of reverse signaling is thought to involve tyrosine phosphorylation of the ephrin-B cytoplasmic domain16, 17 and subsequent coupling to downstream SH2 domain-containing proteins, such as Grb4 (also known as Nck2 or Nckβ)23. To determine if tyrosine phosphorylation-dependent reverse signaling is important, we engineered two new mutations in the mouse germline that specifically targets the highly conserved tyrosine (Y) codons and replaces them with phenylalanine (F) (Fig. 3a and Supplementary Fig. 7a). These mutations, termed *EB3*^{3F} and *EB3*^{5F}, modify the last (fifth) exon by replacing the tyrosine codons at positions 311, 318, and 323 (*EB3*^{3F}), or 311, 318, 323, 337, and 338 (*EB3*^{5F}) in the cytoplasmic domain with phenylalanines (Fig. 3a). As a peptide encompassing residues 311, 318, and 323 is known to contain the Grb4 SH2 domain binding site23, both mutant proteins will be unable to associate with Grb4 (see below).

As expected, the *EB3*$^{3F/3F}$ and *EB3*$^{5F/5F}$ homozygotes were found to be viable at expected Mendelian ratios and the adults appeared healthy, fertile and long lived as previously shown for the *EB3*$^{-/-}$ and *EB3*$^{lacZ/lacZ}$ homozygotes18. In order to confirm expression of the EB3 mutant proteins and their lack of tyrosine phosphorylation, we first cultured primary hippocampal granule cell neurons from 2 week old *EB3*$^{+/+}$ and *EB3*$^{5F/5F}$ mice for 6 days and then exposed the neurons to soluble preclustered EphB1-Fc extracellular domains or unconjugated Fc as the control. Exposure to EphB1-Fc led to clustering and obvious tyrosine phosphorylation of EB3 within calbindin-positive neurons from wild-type cultures (Supplementary Fig. 7b, upper panels). However, while cultures from *EB3*$^{5F/5F}$ homozygotes showed clear expression and clustering of the EB3-5F protein in calbindin-positive neurons and outgrowing axons in response to the applied EphB1-Fc reagent, there was no signal for phospho-ephrin (Supplementary Fig. 7b, lower panels). Therefore, while the *EB3*^{5F} mutation does lead to normal expression of the EB3-5F protein on the surface of calbindin-positive granule cell neurons, it is not able to become tyrosine phosphorylated after being clustered by application of EphB1-Fc reagent. We then visualized the MF axons of adult *EB3*$^{3F/3F}$ and *EB3*$^{5F/5F}$ mutants with anti-calbindin IF (Fig. 3b,c) and Timm stain (data not shown) to determine the role of EB3 phosphorylation in pruning of the IPB axons. We found that the *EB3*$^{3F/3F}$ and *EB3*$^{5F/5F}$ mice showed a similar long IPB comparable to

that of the *EB3*$^{-/-}$ or *EB3*$^{lacZ/lacZ}$ homozygotes. This data indicates an essential role for tyrosine phosphorylation of the EB3 cytoplasmic tail in the pruning of MF axons.

MF axon pruning was further verified by *in vitro* co-culture of primary hippocampal dentate granule cell neurons with Cos-1 cells which express endogenous EphB2 protein24. Following 6 days culture of granule cell neurons from *EB3* mutant and wild-type mice to allow growth of neurites, Cos-1 cells were plated on the layer of hippocampal neurons to stimulate ephrin-B reverse signaling. After 1–48 hr incubation, the co-cultures were fixed and treated for IF with antibodies to detect EB3 and Tau, a specific axon microtubule associated protein, to identify axons. The data showed that after 8–48 h incubation wild-type dentate granule cell neurons had only short neurites (from 65.8 ± 5.4 to 117.7 ± 9.5 μm) in contrast to the *EB3*$^{-/-}$ group which exhibited longer neurites (from 165.6 ± 11.1 to 193.8 ± 18.6 μm) (Fig. 4a-c, and Supplementary Fig. 8). Similarly, at the 48 h time point, neurons from *EB3*$^{3F/3F}$ or *EB3*$^{5F/5F}$ homozygotes, in which EB3 phosphorylation was eliminated (Fig. 4d), exhibited longer neurites (155.8 ± 11.5 μm for *EB3*$^{3F/3F}$ and 146.4 ± 13.1 μm for *EB3*$^{5F/5F}$) as the *EB3*$^{-/-}$ group (Fig. 4e,f). In these assays, greater than 70% of the neurites from the various *EB3* mutant cells were longer than 100 μm, while only ∼20% of the neurites from the wild-type group were longer than 100 μm after 48 h Cos-1 co-culture (Fig. 4e). We also compared the pruning of calbindin-positive and calbindin-negative neurons from wild-type mice in this co-culture system. The data showed calbindin-positive neurons exhibited shorter neurites compared to the calbindin-negative neurons (Fig. 4g,h), indicating it is the dentate granule cells that become pruned in response to Cos-1 co-culture. To further determine the role of EB3 reverse signaling on neurite retraction, we transfected various EB3 expression vectors with a membrane-anchored farnesylated enhanced green fluorescent protein (f-EGFP) reporter into NG108 neuronal cells and performed time-lapse imaging of differentiated neurites following exposure to pre-clustered EphB1-Fc to stimulate reverse signaling. These experiments demonstrated that EB3 reverse signaling induced neurite retraction and that this requires intact tyrosine residues in its cytoplasmic tail (Supplementary Fig. 9). Together, these results indicate that stereotyped pruning of granule cell MF axons can be induced by EphB molecules acting as ligands to stimulate EB3 tyrosine phosphorylation-dependent reverse signaling.

Grb4 transduces ephrin-B3 reverse signals in axon pruning

Among the downstream molecules that may mediate ephrin-B reverse signaling, the SH2/SH3 adaptor protein Grb4 is known to bind ephrins in a tyrosine phosphorylation-dependent fashion23. While Grb4 binds phosphorylated ephrin-B molecules with its single SH2 domain, its three N-terminal SH3 domains can associate with several PXXP-containing proteins which may help bridge ephrins with downstream regulators of the cytoskeleton12. Since Grb4 and EB3 become co-expressed endogenously in mature calbindin-positive granule cell neurons (Fig. 5a and Supplementary Fig. 10), we tested the role of Grb4 in axon pruning by overexpressing in 4 day cultured primary hippocampal neurons a DsRed-conjugated Grb4 wild-type fusion protein (Grb4-DsRed) or a dominant-negative construct in which the three SH3 domains were inactivated by point mutations (Grb4-SH3Mut-DsRed). Two days after transfection, the cells were co-cultured with Cos-1 cells expressing EphB2 as the ligand to stimulate reverse signaling and live neurons expressing DsRed were visualized.

The data showed that the average length of neurites from Grb4-DsRed expressing live neurons was dramatically decreased from 181.9 ± 18.1 to 20.1 ± 5.6 μm after co-culturing with Cos-1 cells ($P < 0.0001$), while the length of neurites in Grb4-SH3Mut-DsRed positive live neurons were not significantly decreased (from 210.8 ± 40.9 to 151.2 ± 29.1 μm) and remained much longer than that of co-cultured Grb4-DsRed neurons ($P < 0.0001$) (Fig. 5b,c). This suggests an essential role of Grb4 SH3 domains in axon retraction and pruning in response to exposure to EphB2-expressing Cos-1 cells. To further determine the sufficiency of Grb4 SH3 domains in ephrin-B signaling, we generated EB3-Grb4-SH3 chimeric fusion proteins, in which the EB3 intracellular sequence encompassing the first three tyrosine phosphorylation sites (residues 311, 318, and 323) was deleted and replaced with the Grb4 SH3 domains. Both the wild type Grb4 SH3 domains (EB3-Grb4-SH3) and the triple SH3 point mutant (EB3-Grb4-SH3Mut) (see cartoon in Fig. 5g) were subcloned into an expression vector that also expressed the membrane-anchored f-EGFP reporter. The chimeric fusion protein expression vectors were transfected into 4 day cultured primary hippocampal neurons from *EB3*$^{-/-}$ mice and scored two days later in the absence of Cos-1 cells. We found that *EB3*$^{-/-}$ neurons expressing EB3-Grb4-SH3, which is indicated by f-EGFP expression in live neurons (**Fig. 5d,** upper panels) and also by anti-EB3/anti-Grb4 IF in fixed cultures (**Fig. 5d**, middle panels), exhibited significantly shorter neurites compared to the neurons in the same cultures that did not become transfected or in mock-transfected neurons (Fig. 5d,e). Expression of the EB3-Grb4-SH3Mut chimeric protein did not lead to a shortening of neurites as they were significantly longer than the EB3-Grb4-SH3 transfected neurons ($P < 0.0001$). Furthermore, co-culture with Cos-1 cells did not dramatically alter the response of the transfected neurons in either group (**Fig. 5d,** lower panels). Together, this data indicates that the EB3-Grb4-SH3 chimeric fusion protein provides for a gain-of-function of EB3 to induce axon shortening in the absence of EphB exposure while the EB3-Grb4-SH3Mut acts as loss-of-function, and suggests that Grb4 is critical for the transduction of ephrin-B reverse signals involved in axon retraction/pruning.

The effects of EB3-Grb4-SH3 and EB3-Grb4-SH3Mut were also reproducible in differentiated NG108 neuronal cells. EB3-Grb4-SH3 expressing NG108 cells exhibited a gain-of-function phenotype as exemplified by either short neurites or a rounded up morphology, while cells expressing EB3-Grb4-SH3Mut showed long neurites (Fig. 5f). We then used this NG108 cell system to screen the functional roles of each Grb4 SH3 domain by generating a series of EB3-Grb4-SH3 point mutants that inactivated the individual SH3-1, SH3-2, or SH3-3 domain or various combinations of the SH3 domains (SH3-1/SH3-2; SH3-1/SH3-3; SH3-2/SH3-3). The data showed that disruption of SH3-2 resulted in cells with long neurites while disruption of SH3-1 and/or SH3-3 had minimal effect on the neurites (Fig. 5g). This indicates that the second SH3 domain of Grb4 is essential and sufficient for mediating EB3 reverse signals involved in the retraction/pruning of axons.

Grb4 couples ephrin-B3 to PAK, Dock180, and Rac/Cdc42

Grb4 has been demonstrated to bind via its three SH3 domains to numerous PXXP-containing proteins that are involved in regulating cytoskeletal dynamics12, 23. Among the candidate downstream proteins, Pak1 was found to bind with the second SH3 domain and is known to regulate actin remodeling as a downstream effector of the small GTPases Rac and

Cdc42. We expressed a dominant-negative fusion protein Pak1DN-DsRed in neurons to examine whether PAK is involved in the ephrin-B3-Grb4 pathway. The Pak1DN-DsRed consists of the Pak1 autoinhibitory domain, which binds to the catalytic domain of all three PAKs to block their autophosphorylation and consequently their catalytic activity, leading to inhibition of actin remodeling25. Data obtained from live differentiated NG108 cells showed that cells co-transfected with the gain-of-function EB3-Grb4-SH3 construct and Pak1DN-DsRed have much longer neurites than the DsRed mock control cells expressing only EB3-Grb4-SH3 ($P < 0.0001$, Fig. 6a,b). This indicates that interfering with PAK activation blocks the ability of the EB3-Grb4 gain-of-function from propagating reverse signals involved in axon retraction/pruning. The inhibitory role of Pak1DN-DsRed in neurite retraction of differentiated NG108 cells had minimal effect in cells co-transfected with EB3-Grb4-SH3Mut, which already presents with long neurites (Fig. 6a,b). The effect of Pak1DN-DsRed expression was also reproduced in live wild-type primary hippocampal neurons co-cultured with Cos-1 cells which presented much longer axons than the DsRed mock control neurons ($P < 0.001$) (Fig. 6c,d). These results indicate that PAK acts as a key downstream component of ephrin-B3-Grb4 reverse signaling to mediate axon retraction and pruning.

Dock180, a guanine nucleotide exchange factor for the small Rho family GTPase Rac (ref. 26), is another candidate molecule found to bind with the second and third Grb4 SH3 domains27. It is also co-expressed with EB3 in calbindin-positive hippocampal granule cell neurons (Fig. 6e). We tested the role of Dock180 in axon pruning by overexpressing in primary hippocampal neurons a Flag-tagged Dock180 wild-type protein (Flag-Dock180-WT) or a dominant negative mutant (Flag-Dock180-ISP) in which three contiguous residues at positions 1487–1489, Ile-Ser-Pro, were mutated into Ala-Ala-Ala (ref. 26), and then co-culturing with Cos-1 cells to stimulate reverse signaling. The data showed that the length of neurites from Flag-Dock180-WT expressing neurons detected by anti-Flag antibody was decreased on average from 155.8 ± 27.6 to 27.1 ± 4.8 μm after co-culturing with Cos-1 cells ($P < 0.0001$), while the length of neurites in Flag-Dock180-ISP expressing live neurons were not significantly decreased ($P < 0.0001$) (Fig. 6f,g). This indicates that Dock180 is required for axon retraction/pruning following activation of EB3 reverse signaling. We further tested for a physical interaction between EB3, Grb4, and Dock180. NG108 cells stably expressing either Flag-tagged EB3 or EB3-3F proteins were exposed to pre-clustered EphB2-Fc to stimulate reverse signaling and protein-protein associations between EB3, Grb4, and Dock180 were analyzed by co-immunoprecipitation. This showed that wild-type EB3 formed a stable complex with endogenous Grb4 and Dock180 proteins in response to EphB2-Fc exposure, while the EB3-3F mutant did not (Fig. 6h). These results demonstrate that Grb4 and Dock180 are recruited to tyrosine phosphorylated EB3 following EphB stimulation of reverse signaling.

To determine if Rac is required for axon pruning downstream of EB3, we overexpressed an mCherry-conjugated Rac1 wild-type fusion protein (mCherry-Rac1-WT) or a dominant-negative construct (mCherry-Rac1-N17) in 4 day cultured primary hippocampal neurons and then co-cultured with Cos-1 cells to activate reverse signaling. Co-culturing mCherry-Rac1-WT expressing live neurons with Cos-1 cells resulted in a significant reduction in the length of neurites from 181.2 ± 22.2 to 36.3 ± 5.6 μm ($P < 0.0001$), while the neurites from mCherry-Rac1-N17 expressing neurons remained much longer (95.2 ± 7.1, $P < 0.0001$)

(Fig. 7a,b). This indicates that Rac1 is involved in EB3 reverse signaling mediated axon retraction and pruning. Finally, to further determine whether the activity of Rho family GTPases are regulated in response to EB3 reverse signaling, we measured the levels of GTP-bound Rho, Rac, and Cdc42 in NG108 cells which stably express EB3-WT in the presence EphB2-Fc stimulation for up to 60 min. We found that EphB2-Fc exposure induced a biphasic GTP loading of both Rac and Cdc42, with an initial peak observed at 4–8 min stimulation followed by a second peak noted after 32 min (Fig. 7c). Levels of Rho-GTP were not observed to change in response to EphB2-Fc stimulation of EB3 reverse signaling (data not shown). We also compared the levels of GTP-bound Rac and Cdc42 in NG108 cells which stably express either EB3-WT or EB3-3F in the presence or absence of EphB2-Fc stimulation. The data showed that EphB2-Fc exposure induced GTP loading of Rac and Cdc42 in cells expressing EB3-WT but not in cells expressing EB3-3F (Fig. 7d). Together, this data demonstrates that stimulation of EB3 tyrosine phosphorylation-dependent reverse signaling leads to GTP loading of Rac and Cdc42.

DISCUSSION

The transmembrane ephrin-B proteins, ligands to Eph receptor tyrosine kinases, are capable of transducing reverse signals into their own cell to give rise to an autonomous cellular response15-17. While receptor-like roles for ephrin-B proteins have been implicated to control numerous cell-cell interactions such as those during axon pathfinding and directed cell migrations, it is largely unknown how the reverse signals are interpreted by the cell and coupled with downstream intracellular molecules. By generating and analyzing gene targeted point mutant mice, our study provides evidence that EB3 transduces tyrosine phosphorylation-dependent reverse signals into hippocampal MF axons to control stereotyped pruning of exuberant IPB fibers. Our results are in contrast to the well documented ligand-like roles for EB3 in the spinal cord as a midline-expressed repellant that is crucial for the wiring of corticospinal tract and central pattern generator axons important in motor control and locomotion18, 28, 29. Our present analysis further reveals a novel biochemical pathway required for axon pruning in which EB3 binds the cytoplasmic SH2/SH3 adaptor protein Grb4 which functions as a molecular bridge to connect the activated tyrosine-phosphorylated ephrin-B intracellular domain to the guanine nucleotide exchange factor Dock180, Rac/Cdc42 activation, and downstream PAK to induce axon pruning (Supplementary Fig. 11). This study thus provides a direct physical link between ephrin-B reverse signaling and important cytoskeletal regulatory proteins in axon pruning.

Reverse signaling mediated by ephrin-B proteins acting as receptors has been implicated in axon pathfinding15, 30, 31, synapse formation32-34, and plasticity34-38. However, opposed to the large body of evidence linking Eph forward signaling to axon guidance and synaptic functions, progress in understanding how and in what context ephrin reverse signals are utilized to control neuronal connectivity has been relatively slow. It is noteworthy, though, that many other receptor-like roles for the three ephrin-B molecules have been provided by studies outside the axon guidance and synaptogenesis fields14.

Mechanistically, upon interacting with cognate Eph extracellular domains, ephrin-B proteins become clustered and tyrosine residues in the highly conserved cytoplasmic domain become

phosphorylated by Src family kinases16, 17, 23, 39, leading to formation of a protein-protein interaction with the cytoplasmic SH2/SH3 domain adaptor protein Grb4 (ref. 23). Cell-based studies indicate that the SH2 interactions of Grb4 with tyrosine phosphorylated ephrin-B molecules ultimately leads to the transduction of signals into the cell that bring about the loss of stress fibers and disassembly of focal adhesions23. These effects on the actin cytoskeleton are likely due to the ability of Grb4 to also associate via its three SH3 domains with a large set of other PXXP motif containing proteins implicated in cytoskeletal dynamics12, 23, and with the G protein-coupled receptor kinase-interacting protein-1 (GIT-1) (ref. 40). Our data here provides evidence that Grb4 bridges EB3 to Dock180 and PAK, leading to activation of the Rac and Cdc42 GTPases, and downstream signaling which ultimately results in axon retraction/pruning. To the best of our knowledge, this is first identified *in vivo* reverse signaling pathway that clarifies how ephrin-B proteins communicate with downstream proteins to regulate cytoskeletal dynamics and control stereotyped axon pruning.

Our present results are contrary to the well-established role for Rac as an attractive force to drive axon elongation and outgrowth41, 42. However, there are reports that link the Robo and Plexin/Neuropilin axon guidance receptor systems to Rac activation and growth cone collapse/repulsion43-46. Indeed, recent evidence shows that clathrin mediated endocytosis is required for the activation of Rac induced by motogenic stimuli47. We suggest that the role of activated Rac in axon pruning may be to mediate the spatially restricted endocytosis of repulsive axon guidance receptors and associated plasma membranes at the axon terminal to help bring about the collapse of growth cones and retraction of the distal segment of the axon48-50. Interestingly, we find that *EB3*$^{-/-}$ null hippocampal neurons still respond to semaphorin exposure by pruning their neurites *in vitro* (Supplementary Fig. 12) and that Sema3A stimulated pruning does not appear to be mediated by Grb4 (Supplementary Fig. 13). This suggests that both Sema-Npn/Plex and EphB-EB3 systems are required independently to mediate the pruning of MF axons. Taken together, our study identifies a molecular mechanism by which ephrin-B tyrosine phosphorylation-dependent reverse signaling brings about the pruning of exuberant axon outgrowths. Given the widespread role of axon pruning in neural development and plasticity, as well as potential implications for neurological diseases and injury4-6, it will be important to determine if ephrin-B reverse signaling is a general mechanism utilized to trim excessive neuronal processes throughout the nervous system.

METHODS

Mice and sample preparation

EB3^{-}, *EB3*^{lacZ}, *EphB1*$^{-}$, *EphB2*$^{-}$, *EphB2*^{lacZ}, *EphB3*$^{-}$, and *EphA4*$^{-}$ knockout and knockin mutant mice and genotyping methods have been described15, 18-20. Consecutive backcrosses to the CD1 strain were performed to move the mutations to CD1 background. Mice were anesthetized (ketamine, 450 mg/kg), perfused transcardially with 0.1 M PBS followed by 4% paraformaldehyde in phosphate buffer. The brains were then removed, postfixed and sectioned at 50 μM using a vibratome. All experiments involving mice were carried out in accordance with the National Institutes of Health *Guide for the Care and Use*

of Animals under an Institutional Animal Care and Use Committee (IACUC) approved protocol and Association for Assessment and Accreditation of Laboratory Animal Care approved facility at UT Southwestern Medical Center.

Immuofluorescence and X-gal staining of mouse brains

Vibratome sections were blocked in 10% normal donkey serum, 0.3% triton-100X in PBS overnight at 4 °C. Primary antibodies were as follows: mouse anti-Calbindin 28K (1:3000, Swant), goat anti-ephrin-B3 (1:1000, R&D Systems), goat anti-EphB2 (1:200, R&D Systems), and rabbit anti-β-gal (1:1000, MP Biomedicals). Fluorescent-conjugated secondary antibodies (1:200, Jackson Immunoresearch) or green fluorescence dye NeuroTrace 500/525 (Molecular Probes) were used for visualization. To detect the β-gal expression by X-gal stain, mouse brain sections were processed as described15.

Generation of *EB3* tyrosine to phenylalanine mutant mice

Tyrosine to phenylalanine point mutant mice that target the intracellular domain of EB3 were generated through two-step gene knock-in strategy. Additional details are provided in the Supplementary Methods.

Primary neuron culture and immunocytochemistry

Dentate granule cell neurons were dissociated from micro-dissected DGs of 2 week old mice. For transfections, postnatal day 1 (P1) mice were used and whole hippocampi were dissected. Primary neurons or differentiated NG-108 neuronal cells were used for live cell analysis. Additional details are provided in the Supplementary Methods.

Statistical analysis

The results are presented as mean ± s.e.m. Statistical differences were determined by Student's t test for two-group comparisons or ANOVA followed by Tukey test for multiple comparisons among more than two groups.

Cell stimulations and biochemistry

NG108 cells stably expressing EB3 and various mutants were used to assay for protein-protein interactions between EB3, Grb4 and Dock180, and to examine the activity of Rac1 and Cdc42. Additional details are provided in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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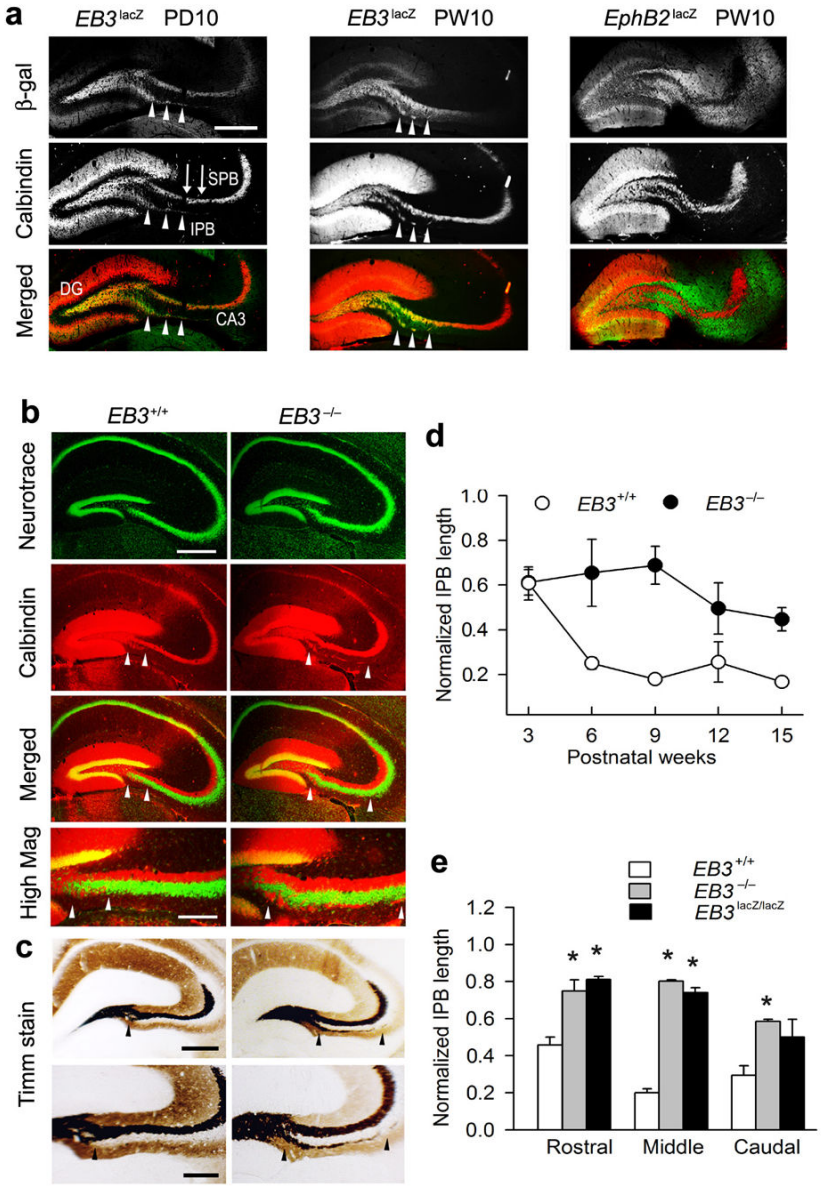

Figure 1.

Defective hippocampal MF pruning in *EB3* mutants. **a**, Confocal IF detects the EB3-β-gal fusion protein in calbinding-positive SPB (arrows) and IPB (arrowheads) MF axons extending from the DG towards the CA3 area in postnatal day 10 (PD10) and 10 week old adult (PW10) mice. The EphB2-β-gal fusion protein is expressed broadly above and underneath the MF bundles, but is not localized with calbindin in the MF axons. **b** and **c**, Anti-calbindin IF (**b**) and Timm stain (**c**) shows that IPB axons in 8–10 week old adult $EB3^{-/-}$ mice are much longer than in WT littermates (distance between arrowheads). **d**, Quantification of the ratio of IPB length/length from hilus to curvature of CA3 area in $EB3^{-/-}$ and WT littermates during postnatal development (n = 3–4 per group). **e**, Quantification of IPB length in $EB3^{-/-}$, $EB3^{lacZ/lacZ}$, and WT adult mice (n = 8–9 per

group). Mean ± s.e.m. Scale bars: 300 μm in **a**, **b** and **c** (upper panels); 150 μm in **b** and **c** (bottom panels).

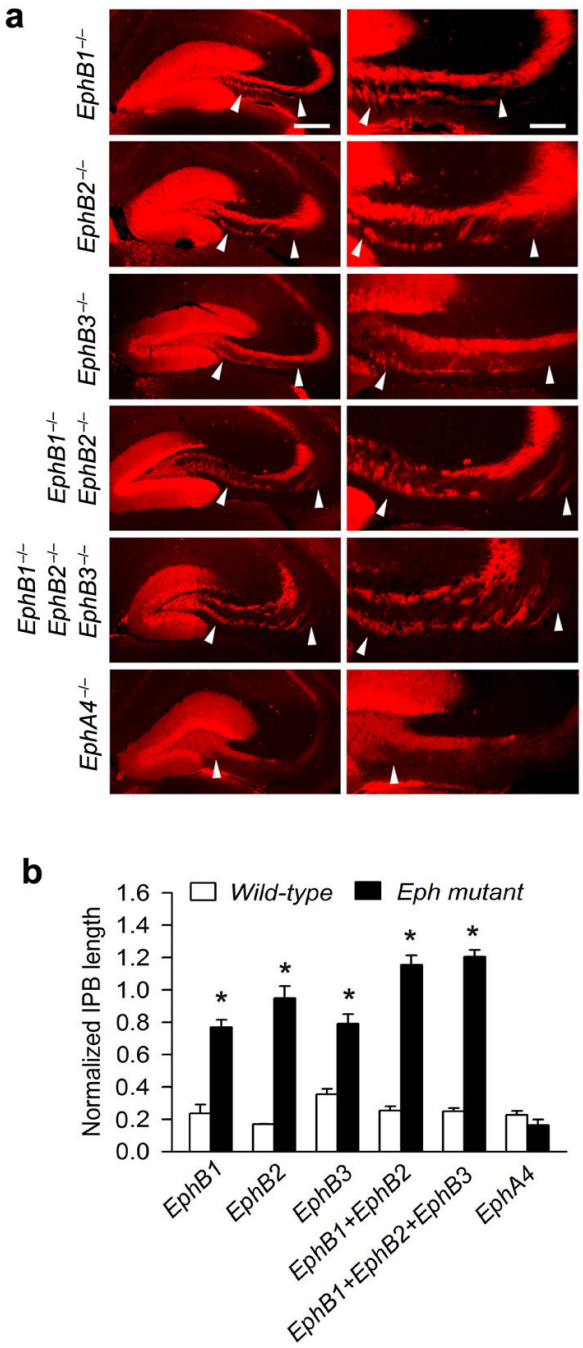

Figure 2.

Defective hippocampal MF pruning in *EphB* mutants. **a**, Anti-calbindin IF shows long IPB axons in indicated *EphB* single and compound mutants at 8–10 weeks. **b**, Quantification of IPB length in $EphB1^{-/-}$ (n = 6), $EphB2^{-/-}$ (n = 5), $EphB3^{-/-}$ (n = 4), $EphB1^{-/-}$; $EphB2^{-/-}$ (n = 6), $EphB1^{-/-}$; $EphB2^{-/-}$; $EphB3^{-/-}$ (n = 6), and $EphA4^{-/-}$ (n = 6) mutants (* $P < 0.001$). Mean ± s.e.m. Scale bars: 300 μm in **a** (left panels); 150 μm in **a** (right panels).

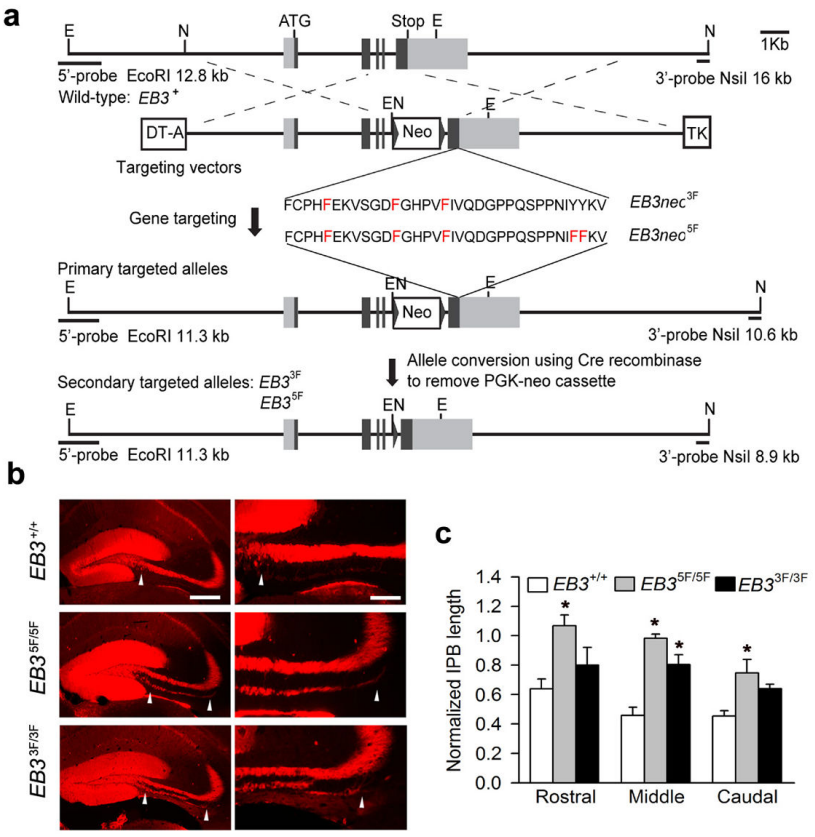

Figure 3.

Mossy fiber pruning requires tyrosine phosphorylation of the EB3 cytoplasmic tail. **a**, Generation of *EB3* tyrosine (Y) to phenylalanine (F) point mutations. Exons are shown as filled boxes (coding segments are dark and non-coding segments are gray), introns and 5′ and 3′ nontranscribed regions as lines, and EcoRI (E) and NsiI (N) restriction sites are indicated. The last (fifth) exon encoding EB3 amino acids 205–340 including the cytoplasmic domain was engineered to generate 3F or 5F mutations (red) following homologous recombination in ES cells and Cre-mediated excision of the *loxP*-flanked *PGK-neo* cassette after germline transmission of the initial targeted *EB3neo* insertions. The location of 5′ and 3′ external probes used to confirm the various recombination events by Southern blot are shown with the expected sizes indicated (see Supplementary Fig. 7). **b**, The IPB axons in both $EB3^{3F/3F}$ and $EB3^{5F/5F}$ mutants are longer than in WT mice. **c**, Quantification of IPB length in 8–10 week old $EB3^{3F/3F}$, $EB3^{5F/5F}$, and WT mice (n = 9 per group, * $P < 0.01$). Mean ± s.e.m. Scale bars: 300 μm in **b** (left panels) and 150 μm in **b** (right panels).

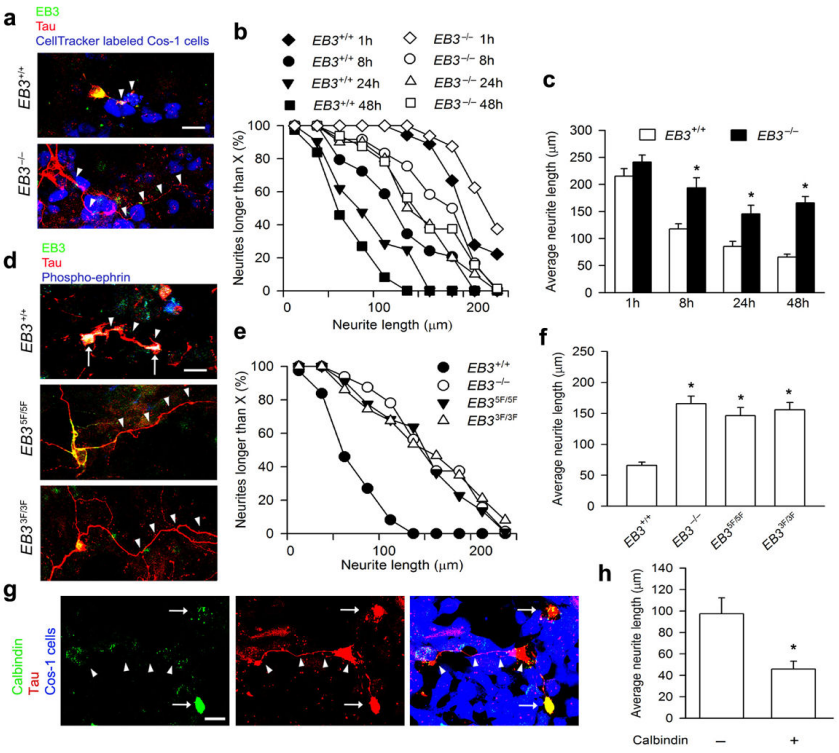

Figure 4.

Axons pruning of hippocampal granule cell neurons induced by Cos-1 co-culture *in vitro* requires EB3 tyrosine phosphorylation. **a**, Primary dentate granule cell axons (arrowheads) are pruned in WT but not $EB3^{-/-}$ neurons after co-culturing with Cos-1 cells which express endogenous EphB2 and are labeled with CellTracker (blue). **b** and **c**, Comparison of neurite lengths following time course of co-culturing primary granule cells from WT and $EB3^{-/-}$ dentates with Cos-1 cells expressing endogenous EphB2 (n = 21–37 neurons per group, * P < 0.001). **d**, EB3 is tyrosine phosphorylated in primary dentate granule cell neurons from WT but not $EB3^{3F/3F}$ or $EB3^{5F/5F}$ mutant neurons after co-culturing with unlabeled Cos-1 cells. Arrows indicate co-localization of EB3, phosphorylated EB, and tau in the growth cone and cell body of a WT neuron (white). No EB phosphorylation is detected in the $EB3^{3F/3F}$ or $EB3^{5F/5F}$ mutant neurons and the axons did not retract (arrowheads). **e** and **f**, Comparison of neurite lengths from WT and indicated *EB3* mutants after 48 h co-culture with Cos-1 cells (n = 22–43 per group, * P < 0.001). **g** and **h**, Comparison of neurite lengths of calbindin-negative cells (n = 21) and caldindin-positive cells (n=31) from *EB3* wild-type mice after 48 h co-culture with Cos-1 cells (* P < 0.01). Mean ± s.e.m. Scale bars, 20 μm.

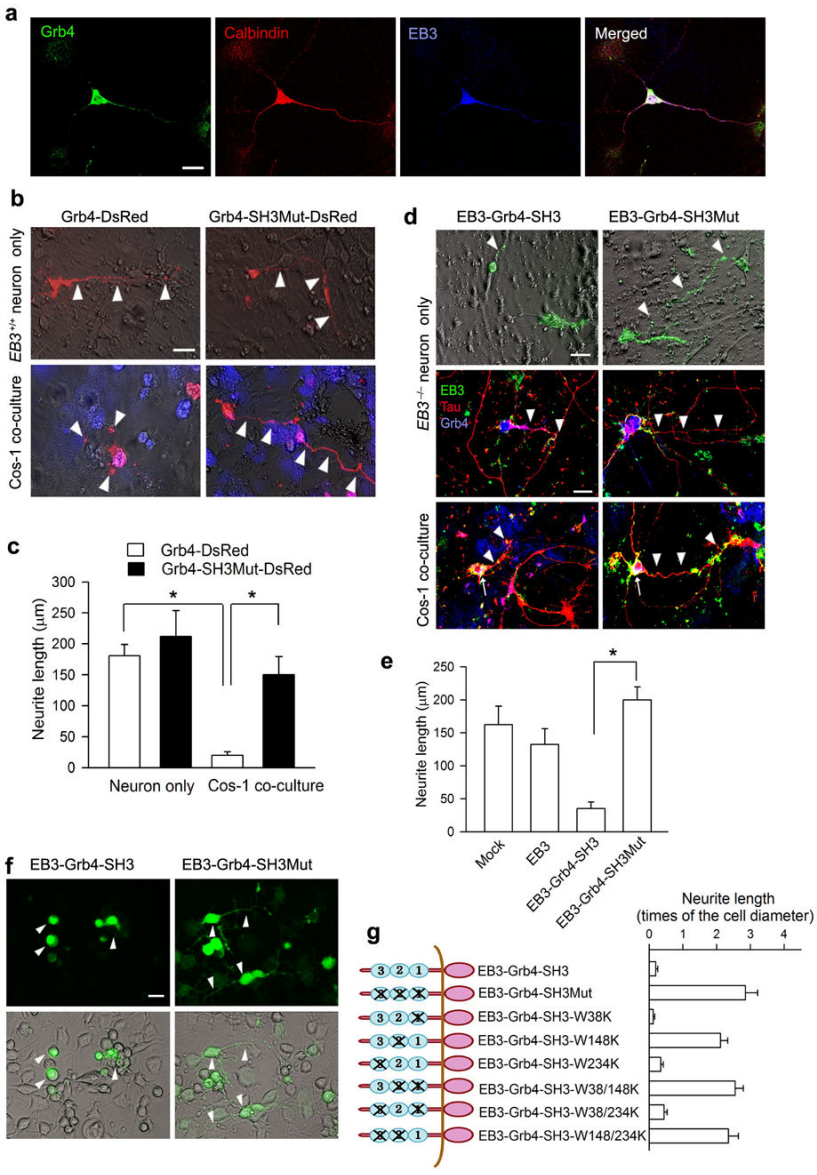

Figure 5.

Grb4 is a key mediator to transduce EB3 reverse signals involved in axon pruning. **a,** Co-expression of Grb4 and EB3 in hippocampal granule cell neurons. **b** and **c**, Expression of dominant-negative Grb4-SH3Mut-DsRed (W38K, W148K and W234K) but not WT Grb4-DsRed (red fluorescence) in hippocampal neurons blocks axon pruning in live cells initiated by co-culture with CellTracker labeled Cos-1 cells (blue fluorescence) for 48 h (n = 11–16 neurons per group, * $P < 0.0001$). **d**, Expression of an EB3-Grb4-SH3 chimeric fusion protein in *EB3*$^{-/-}$ hippocampal neurons leads to axon shortening without addition of Cos-1 cells in live transfected neurons labeled with f-EGFP (upper panels) or in fixed cultures labeled with the indicated antibodies (lower panels). The EB3-Grb4-SH3Mut has no effect on axon shortening in *EB3*$^{-/-}$ neurons either with or without co-culture with Cos-1 cells. Arrowheads indicate axons and arrows indicate neuronal cell bodies. **e**, Quantification of neurite length in transfected *EB3*$^{-/-}$ neurons in the absence of Cos-1 co-culture (n = 8–10

neurons per group, * $P < 0.0001$). **f**, Expression of the EB3-Grb4-SH3 chimeric fusion protein in live differentiated NG108 cells is sufficient to mediate neurite shortening and rounding as indicated by co-expressed f-EGFP. **g**, Various EB3-Grb4-SH3 chimeric fusion proteins with indicated SH3 domain point mutations were scored for neurite length in live transfected NG108 cells. Quantification indicates the second SH3 domain is essential and sufficient to induce neurite shortening (n = 70–104 transfected cells per group). Mean ± s.e.m. Scale bars: 20 μm.

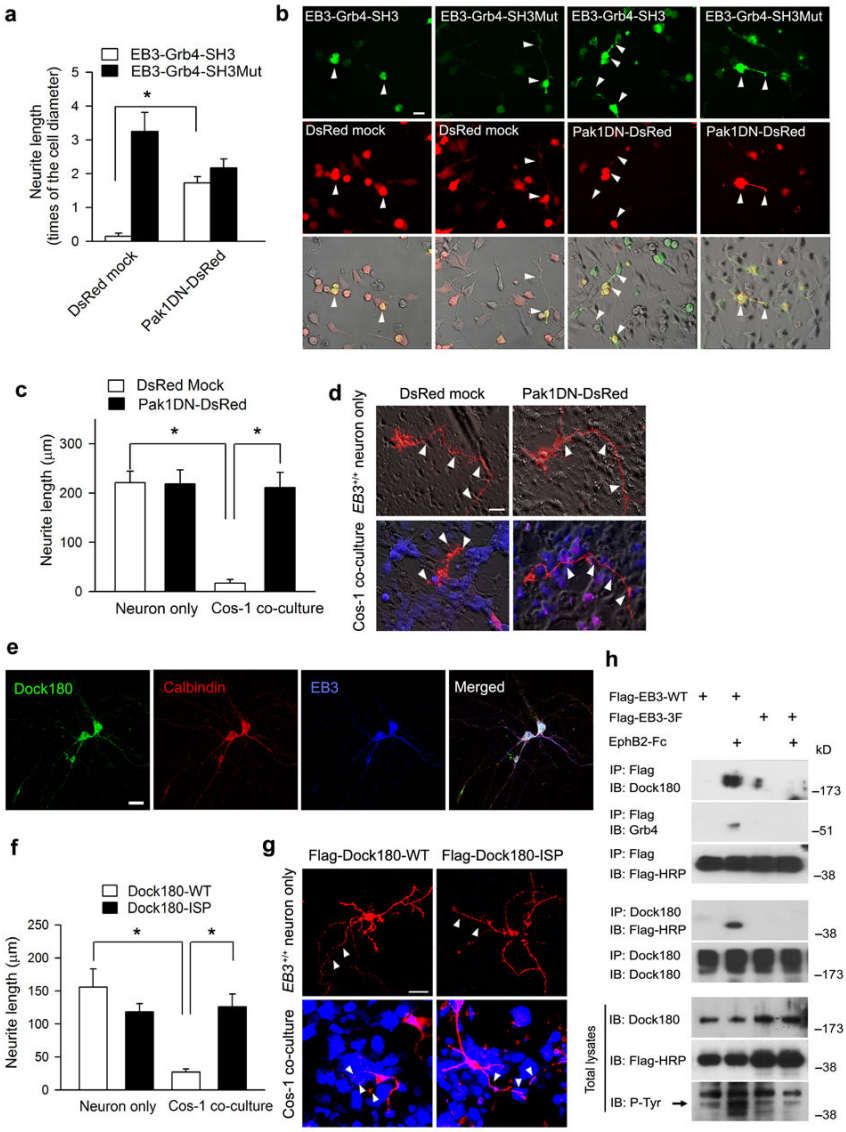

Figure 6.

EB3 reverse signaling requires PAK and Dock180. **a** and **b,** Expression of dominant-negative Pak1DN-DsRed (indicated by red fluorescence) blocks neurite shortening induced by EB3-Grb4-SH3 (indicated by green f-EGFP fluorescence) in live NG108 cells (n = 45–89 transfected cells per group, *P < 0.001). **c** and **d**, Expression of Pak1DN-DsRed (red fluorescence) blocks axon pruning initiated by co-culture of primary hippocampal neurons with CellTracker labeled Cos-1 cells (blue) for 48 h in live cell assays (n = 11–13 transfected neurons per group, * P < 0.0001). **e,** Co-expression of Dock180 and EB3 in hippocampal granule cell neurons. **f** and **g**, Expression of dominant-negative Dock180-ISP (indicated by red immunofluorescence with anti-Flag) blocks axon pruning initiated by co-culture of primary hippocampal neurons with CellTracker labeled Cos-1 cells (blue) for 48 h (n = 10–14 transfected neurons per group, *P < 0.0001). Mean ± s.e.m. Scale bars: 20 μm. **h**, Grb4 and Dock180 co-immunoprecipitate with EB3-WT stably expressed in NG108 cells, but not with EB3-3F following 30 min EphB2-Fc stimulation of reverse signaling.

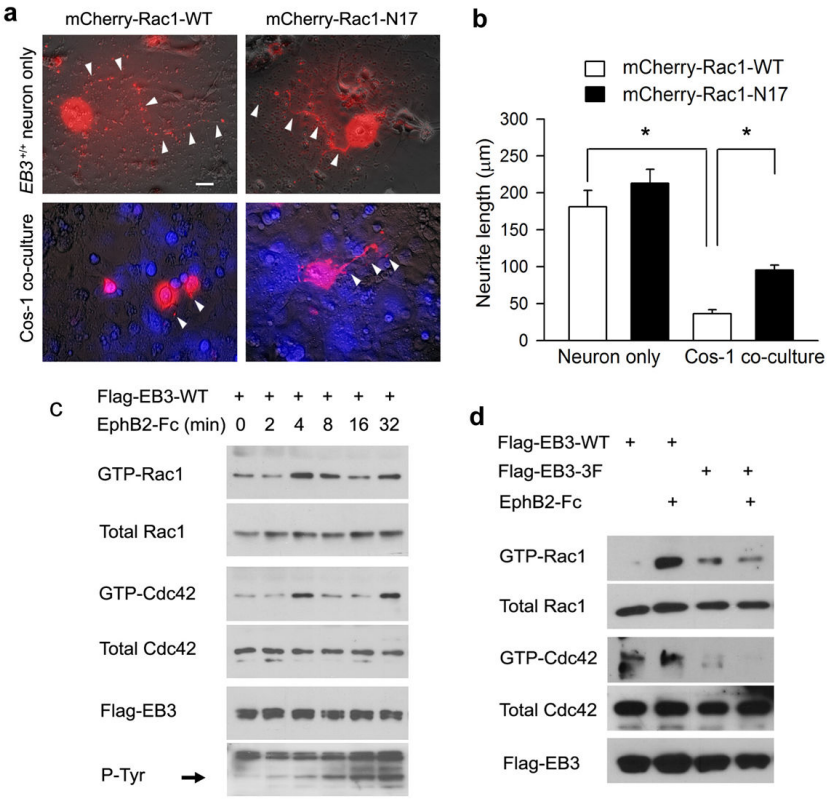

Figure 7.

Activation of EB3 reverse signaling leads to increased Rac and Cdc42 GTP levels. **a** and **b**, Expression of a dominant-negative Rac1, mCherry-Rac1-N17, blocks axon pruning initiated by co-culture of primary hippocampal neurons with CellTracker labeled Cos-1 cells (blue) for 48 h in live cell assays (n = 13–27 transfected neurons per group, *P < 0.0001). Mean ± s.e.m. Scale bars: 20 μm. **c,** GTP-bound Rac1 and Cdc42 were precipitated by GST-PDB fusion protein pull downs from EB3-WT expressing NG108 cells following a time course of EphB2-Fc stimulation of reverse signaling. The immunoblots show a biphasic activation of Rac1 and Cdc42 in response to EphB2-Fc. Arrow indicates increased EB3 tyrosine phosphorylation. **d,** GTP-bound Rac1 and Cdc42 were precipitated by GST-PDB fusion protein pull downs from EB3-WT and EB3-3F expressing NG108 cells following 30 min EphB2-Fc stimulation of reverse signaling. The immunoblots show that Rac1 and Cdc42 are activated by EphB2-Fc stimulation in EB3-WT cells but not in EB3-3F cells.