The drebrin/EB3 pathway regulates cytoskeletal dynamics to drive neuritogenesis in embryonic cortical neurons

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Abstract
Co-ordinating the dynamic behaviour of actin filaments (F-actin) and microtubules in filopodia is an important underlying process in neuritogenesis, but the molecular pathways involved are ill-defined. The drebrin/end-binding protein 3 (EB3) pathway is a candidate pathway for linking F-actin to microtubules in filopodia. Drebrin binds F-actin and, simultaneously, the microtubule-binding protein EB3 when bound to microtubule plus-ends. We assessed the effect on neuritogenesis of gain- or loss-of-function of proteins in the drebrin/EB3 pathway in rat embryonic cortical neurons in culture. Loss-of-function of drebrin by gene editing or pharmacological inhibition of drebrin binding to F-actin reduced the number of dynamic microtubules in the cell periphery and simultaneously delayed the initiation of neuritogenesis, whereas over-expression of drebrin induced supernumerary neurites. Similarly, loss of EB3 inhibited neuritogenesis, whereas loss of end-binding protein 1 (EB1), a related protein that does not bind to drebrin, did not affect neuritogenesis. Over-expression of EB3, but not EB1, induced supernumerary neurites. We discovered that EB3 is more proximally located at dynamic microtubule plus-ends than EB1 in growth cone filopodia allowing for continuous microtubule elongation as the drebrin/EB3 pathway zippers microtubules to F-actin in filopodia. Finally, we showed that preventing the entry of dynamic microtubules into filopodia using a pharmacological inhibitor of microtubule dynamics is associated with a loss of EB3, but not EB1, from microtubule plus-ends and a concurrent attenuation of neuritogenesis. Collectively, these findings support the idea that neuritogenesis depends on microtubule/F-actin zipping in filopodia orchestrated by the drebrin/EB3 pathway.

KEYWORDS
actin, drebrin, EB3, growth cone, microtubules, neuritogenesis

Abbreviations: +TIP, plus-end tracking; Cdk5, cyclin-dependent kinase 5; CRISPR, clustered regularly interspersed short palindromic repeats; DMSO, dimethyl sulphoxide; EB1/EB3, end-binding protein 1/3; Ex, exon; GFP/RFP/YFP, green, red or yellow fluorescent protein; gRNA, guide RNA; mAb, pAb, monoclonal or polyclonal antibody; RRID, Research Resource Identifier; RT, room temperature.

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INTRODUCTION

Neuritogenesis is the formation of neurites by developing neurons (Flyn, 2013). The process begins with the emergence of a growth cone at the cell surface and as the growth cone moves away from the cell body, it forms a neurite. Neurites differentiate into axons and dendrites. Neuritogenesis is an essential step in neuronal development because unless growth cones emerge at an appropriate point in time and space, a properly connected nervous system will not develop. The molecular mechanisms determining how and where a growth cone will form are not well known (Flynn, 2013).

Developing neurons have a circumferential lamellipodium containing actin filaments (F-actin), organized into dendritic networks and actin arcs, and filopodia with bundled F-actin (Smith, 1994a, 1994b; Worth et al., 2013). Microtubules are mainly in circumferential bundles but occasionally extend individually into the lamellipodium. Segmentation of the lamellipodium is associated with growth cone emergence (Dehmelt et al., 2003), which is preceded by an accumulation of F-actin at the site of subsequent growth cone formation (Zhang et al., 2016). Filopodia predict (Smith, 1994a, 1994b) and are necessary for (Dent et al., 2007) growth cone emergence. The microtubules that occasionally extend into the lamellipodium from the circumferential bundle may invade filopodia and there is a correlation between microtubule invasion and the emergence of a growth cone (Flynn et al., 2012; Smith, 1994a). Compounds that inhibit microtubule or F-actin dynamics block neuritogenesis (Dent et al., 2007; Flynn et al., 2012). Neuritogenesis, therefore, depends on orchestrating the dynamic behaviour of microtubules and F-actin in filopodia (Flynn, Gordon-Weeks, 2017).

What are the molecular pathways that co-ordinate the dynamics of microtubules and F-actin that drives neuritogenesis? We have shown that an interaction between the F-actin-binding protein drebrin and the microtubule plus-end-tracking (+TIP) end-binding protein 3 (EB3) cross-links dynamic microtubules to F-actin (Gerald et al., 2008; Worth et al., 2013). Drebrin, bound to F-actin in filopodia, interacts with EB3 located at the plus-ends of dynamic microtubules entering filopodia (Geraldo et al., 2008; Worth et al., 2013). We have demonstrated that a construct containing the drebrin-binding domain of EB3 (EB3 M) inhibits neuritogenesis and neuronal migration, whereas a corresponding construct (EB1 M), of an EB family member that does not bind to drebrin (EB1), has no effect (Gerald et al., 2008; Trivedi et al., 2017; Zhao et al., 2017). Drebrin has one F-actin-binding site in a coiled-coil domain and a second in an adjacent helical domain and can exist in a closed conformation, in which the F-actin-binding site in the coiled-coil domain is occluded, and an open conformation in which both F-actin-binding domains are exposed (Worth et al., 2013). In the closed conformation, drebrin binds single actin filaments, whereas in the open conformation, it binds to F-actin bundles. Phosphorylation of drebrin at S142 by cyclin-dependent kinase 5 (Cdk5) switches drebrin from a single F-actin filament-binding protein to one that can bind to F-actin bundles (Worth et al., 2013). We have proposed that Cdk5 phosphorylation of drebrin might target it to F-actin bundles in filopodia (Gordon-Weeks, 2017). Consistent with this proposal, pS142-drebrin accumulates in filopodia in stage I neurons along with dynamic microtubules (Worth et al., 2013). Crucially, EB3 binds preferentially to pS142-drebrin and therefore to the open conformation of drebrin (Worth et al., 2013).

Consistent with a role for the drebrin/EB3 pathway in microtubule/F-actin coupling underlying neuritogenesis, we show here that gain- or loss-of-function of drebrin or EB3 enhances or delays neuritogenesis, respectively, in embryonic cortical neurons in culture. Furthermore, we provide evidence that S142-drebrin phosphorylation regulates the drebrin/EB3 pathway and, additionally, we found direct evidence that microtubule insertion into filopodia is a necessary step in neuritogenesis. Based on these findings and the discovery of a differential distribution of EB1 and EB3 at microtubule plus-ends, we built a model in which the drebrin/EB3 pathway produces a zipping of dynamic microtubules to F-actin in filopodia that underlies neuritogenesis.

MATERIALS AND METHODS

2.1 Materials

Antibodies used for immunoblotting and immunofluorescence are listed in Table 1. Alexa-conjugated phalloidin was from Molecular Probes/Life Technologies. Eribulin mesylate, a microtubule-targeting drug, was from Eisai Ltd. BTP2 (YM-58483), an inhibitor of drebrin binding to F-actin, was from Cambridge Bioscience (Cat# CAY13246).

2.2 Plasmids

CRISPRgRNA sequences were designed using the MIT CRISPR designer (http://crispr.mit.edu/). Four individual gRNA sequences were used to target rat(Db1: one in Exon 1 (Ex1; GCTGCTGGCCGCGTACGAGG), two in Exon 2 (Ex2.1: GTCTCTGACTTACGAAGAAGA and Ex2.2: GACCTCAAGCTTGCACTATC) and one in Exon 3 (Ex3: AGAAACCGTACATCCTTTTC). Four guides were also designed for rat(Mapre1: one in Ex2 (Ex2; GTGCATGACATCGATCG), one in Exon 3 (Ex3; GTGCTGATCTGCTGATCTG) and two in Exon 4 (Ex4;1: GATCCTGCTGGCCGACAAA, Ex4;2: GTTCTGTATCTGGCCTACT) and Mapre3: two in Exon 3 (Ex3.1 GTCGCACTATGCTGCTGCT, Ex3;2: GTGCTGATCTGTATCGTAAATG) and two in Exon 4 (Ex4.1: GTATGCTGATCTCGTCTG) and Ex4.2: GCAAGCGCTTCTCAAGAGA). CRISPR oligos were synthesized by Sigma and cloned into the pSpCas9(2B)-2A-GFP (PX458) vector, a gift from Feng Zhang (Ran et al., 2013) (Addgene, plasmid #48138) at the Bbs1 restriction site. DNA from positive clones was sequenced.
A eukaryotic expression plasmid containing C-terminal Myc and His-tagged human drebrin (DBN1, NM_004395.3) was obtained from GeneCopoeia (EX-Z7530-M10) (Geraldo et al., 2008). Drebrin-YFP, S142A drebrin-YFP, S142D drebrin-YFP, EB1-GFP and EB3-mCherry have been described (Geraldo et al., 2008) and the drebrin constructs are available at Addgene (#40359, #58335, #58336).

2.3 Embryonic cortical cultures and transfections

This study was not pre-registered. All animals used for cell isolation were treated according to the ethical and legal requirements of King’s College London under project licence (70/7886) from the U.K. Home Office. Pregnant Wistar rats (Harlan UK Ltd) were singly housed with food and water ad libitum. Rats were killed by CO₂ inhalation and
2.4 Gel electrophoresis and immunoblotting

Cultured cells were washed in PBS and lysed in hot Laemmli sample buffer. Gels were transferred onto polyvinylidene difluoride membranes (ThermoFisher) and blocked in 5% non-fat milk solids (Marvel dried milk powder)/Tris-buffered saline Tween 20. Primary antibodies were incubated for 1 h at room temperature (20°C) or overnight at 4°C and secondary antibodies for 1 h at room temperature. Blots were developed in 100 µl of nucleofection solution (Lonza, Cat# VPG-1003) containing 2–3 µg of DNA, transferred to a cuvette and transfected using an Amx Nucleofector and program O-03. Transfected neurons (2 × 10⁶) were plated into six-well plates coated with poly-L-lysine (20 µg/ml, Sigma, Cat# P4832) and cultured in Neurobasal medium (Gibco BRL, Cat# LS21103049) supplemented with 2% (v/v) B27 supplement (Thermo Fisher Scientific, Cat# A3582801), GlutaMAX (2 mM, Sigma, Cat# G7513), penicillin (100 I.U./ml), streptomycin (100 I.U./ml, Sigma Cat# P0781) at 37°C in 5% CO₂ in humidified air for 3 days for over-expression studies and 5 days for knockdown studies. Cultures were then washed with PBS and cells detached using trypsin-EDTA (0.25% in HBSS, Gibco BRL, Cat# 14180046) at 37°C for 15 min and then trypsin inhibitor (Sigma, Cat# T6522) in PBS added. Detached cells were pelleted by centrifugation at 750 g for 10 min and the supernatant removed. Pelleted cells were re-suspended in Neurobasal medium (Gibco BRL, Cat# LS21103049) as above and plated into four-well plates containing 14-mm glass coverslips, for immunolabelling, each coated with poly-L-lysine (100 µg/ml, Sigma, Cat# P4832). Primary neuronal cultures were passaged (re-plated) no more than once.

BTP2 and eribulin were dissolved in dimethyl sulphoxide (DMSO) and stock solutions stored at −80°C. BTP2 or DMSO were added to cortical cultures for 3 h. Neurons were then re-plated to initiate neuritogenesis and cultured in fresh BTP2 or DMSO for a further 3 h. For washout experiments, cultures were treated overnight with BTP2 (10 µM) or DMSO, washed in PBS and then placed in fresh medium with DMSO or BTP2 for 5 h. Eribulin or DMSO was added to dissociated neurons in suspension for 5 min at 37°C before plating and culturing for 20 min to 3 h.

2.6 Neuritogenesis assay

To quantify neuritogenesis, neurons were imaged with a 20x/0.5 Plan Neo objective. Neurons were identified by pIII tubulin labelling and transfected neurons (100) from each of three independent culture preparations were scored for the absence (Stage I) or presence (Stage II) of a neurite, defined as a process of length equal to or greater than one cell diameter. Image files were manipulated using ZEN 2012 SP2 (blue edition) Carl Zeiss software.

2.7 Statistical analysis

Data were analysed using Prism 8 software (GraphPad Software https://www.graphpad.com/) and are expressed as mean ± SEM.
Data were collected from three or more independent culture preparations. Wherever possible analysis of images was done blinded. No test for outliers was conducted. No sample calculation was performed. No exclusion criteria were included. No randomization was performed when selecting cultures. Data sets were assessed for Normality using a Shapiro–Wilk test in Prism 8 software. The Student’s t test or Anova followed by a post hoc test was used for statistical analysis whichever was appropriate. Differences between values in all statistical tests were considered significant if \( p < 0.05 \).

3 | RESULTS

3.1 | Loss of function of drebrin delays neuritogenesis in embryonic rat cortical neurons

To test whether drebrin has a role in neuritogenesis, we used clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) gene editing to remove drebrin from primary embryonic rat cortical neurons. We screened four individual guide RNA (gRNA) sequences for drebrin, targeting exons (Ex): 1 (Ex1), 2 (Ex2.1 and Ex2.2) and 3 (Ex3) (see Materials and Methods) with the aim to introduce indel mutations. Following transfection with CRISPR plasmids, neurons were cultured for 5 days before re-plating to allow time for loss of drebrin protein and at the same time follow neuritogenesis. We used quantitative immunoblotting to assess the effects of each plasmid on drebrin protein levels. This showed that drebrin gRNA sequences targeting Ex1 and Ex2.1 reproducibly decreased drebrin protein levels, whereas gRNA sequences targeting Ex2.2 and Ex3 did not (Figure 1a, b). Cultures were fixed and immunolabelled with antibodies to drebrin, βII/III tubulin, to identify neurons, and green fluorescent protein (GFP), to identify transfected cells and scored for the absence (Stage I) or presence (Stage II) of a neurite. In control cultures transfected with the empty CRISPR-Cas9 plasmid that were fixed 3 h after re-plating, most Ex1- or Ex2.1- cells had drebrin, as judged by immunofluorescence (Figure 1c). Most of these neurons were at Stage I, whereas most GFP−/drebrin+ cells expressed drebrin (not shown). In cultures that were transfected with gRNA sequences targeting Ex1 or Ex2.1, we found βII/III tubulin+/GFP+ cells that had no drebrin as judged by immunofluorescence (Figure 1c). Most of these neurons were at Stage I, whereas most GFP−/βII/III tubulin+ cells that had drebrin were at Stage II (Figure 1d). In contrast, in cultures that were transfected with gRNA sequences targeting Ex3, all βII/III tubulin+/GFP+ cells had drebrin, as judged by immunofluorescence (Figure S1), and the majority were at Stage II (Figure 1d). In cultures that were fixed 24 h after re-plating, most Ex1- or Ex2.1-transfected βII/III tubulin+/GFP+/drebrin− cells had entered Stage II/III (Figure 1e, f). These results show that loss of drebrin from embryonic rat cortical neurons results in a delay in the initiation of neuritogenesis. To determine whether neuritogenesis delay is specific to drebrin loss and not an off-target effect, we attempted to rescue the phenotype by simultaneously transfecting neurons with drebrin gRNA sequences targeting Ex1 or Ex2.1 and a plasmid containing His-tagged human drebrin that is not recognized by these rat drebrin targeting gRNA sequences. Neurons that had been co-transfected were identified with antibodies to GFP and His-tag and after re-plating for 3 h many of these neurons had undergone neuritogenesis similar to control transfected neurons, suggesting that neuritogenesis delay is directly due to drebrin loss (Figure 1g, h).

Loss of drebrin did not obviously affect the overall organization of the cytoskeleton in stage I neurons (Figure 1i). Lamellipodia appeared normal with actin arcs and F-actin bundles in filopodia (Figure 1i). However, the frequency with which dynamic microtubules entered lamellipodia was greatly reduced after drebrin loss (Figure 1i). In control cultures, 6.47 ± 1.15 (mean ± SEM) microtubules per unit length (10 µm) of lamellipodium were found on average, whereas in neurons lacking drebrin, 2.17 ± 0.46 (mean ± SEM) microtubules per unit length of lamellipodium were found (Data from 30 neurons from three independent culture preparations for each experimental condition. Student’s t test, \( p < 0.05, t = 3.47 \)).

3.2 | BTP2, a small molecule inhibitor of drebrin binding to F-actin, inhibits neuritogenesis

To independently confirm a role for drebrin in neuritogenesis, we treated cortical cultures with BTP2, a small molecule that inhibits binding of drebrin to F-actin (Dart et al., 2017; Mancini et al., 2011; Mercer et al., 2010; Shan et al., 2021; Sonego et al., 2015). BTP2 produced a dose-dependent inhibition of neuritogenesis in cortical neurons (Figure 2a, b). Neuritogenesis inhibition was first evident at a BTP2 concentration of 2 µM, and at 10 µM only 25% of neurons produced neurites (Figure 2b). The inhibitory effect of BTP2 (10 µM) on neuritogenesis was reversed on washout of the drug (Figure 2c). Binding of BTP2 to drebrin requires two adjacent lysines: K270 and K271 in human drebrin (Mercer et al., 2010). Mutation of these lysines to methionine abrogates BTP2 binding but does not appear to affect the F-actin binding of drebrin (Mercer et al., 2010). We used the K270 M, K271 M drebrin mutant to directly test the role of drebrin in the inhibition of neuritogenesis produced by BTP2. We transfected cortical neurons with GFP, wild-type drebrin-GFP or K270 M, K271 M drebrin-GFP and compared the effects of BTP2 treatment on neuritogenesis (Figure 2d). The K270 M, K271 M drebrin-GFP construct rescued the BTP2 inhibitory effect on cortical neuritogenesis (Figure 2e, f), showing that the action of BTP2 on neuritogenesis is mediated by inhibiting binding of drebrin to F-actin and not because of an off-target effect.

In stage I embryonic cortical neurons, drebrin mainly localizes to F-actin arcs and occasionally to the proximal region of filopodia, co-localizing with F-actin (Figure 2g, Worth et al., 2013). BTP2 (5 µM) produced a dramatic reduction in the amount of F-actin-bound drebrin in F-actin arcs without changing the levels of F-actin or the organization of F-actin arcs (Figure 2g, h).
3.3 | Gain of function of drebrin in rat cortical neurons induces supernumerary neurites

Previous work has shown that S142 phosphorylated drebrin accumulates at the base of filopodia in nascent growth cones during neuritogenesis (Worth et al., 2013). To investigate the role of drebrin and the impact of phosphorylation at S142 (Worth et al., 2013) on neuritogenesis, we transfected neurons with yellow fluorescent protein (YFP), drebrin-YFP, the pS142 phospho-dead mutant S142A drebrin-YFP or the pS142 phospho-mimetic mutant S142D drebrin-YFP. Embryonic cortical neurons expressing YFP reached Stage II in culture mainly with <5 neurites and often with large growth cones terminating neurite ends (Figure 3a, b). In contrast, expression of drebrin-YFP in embryonic neurons stimulated neuritogenesis, as noted previously (Biou et al., 2008; Shirao et al., 1994; Worth et al., 2013), and induced supernumerary (>5) neurites often terminating without a growth cone (Figure 3a). Expression of the pS142 phospho-dead mutant S142A drebrin-YFP was less effective than drebrin-YFP in inducing supernumerary neurites, whereas the pS142 phospho-mimetic mutant S142D drebrin-YFP was more effective (Figure 3b). Drebrin-YFP expressed in supernumerary neurites was highly phosphorylated at S142, as indicated by labelling with pS142 drebrin-specific antibodies and contained bundles of F-actin and dynamic microtubules, confirming neurite identity (Figure 3c, d). As expected, pS142 drebrin antibodies failed to recognize S142A drebrin-YFP but did cross-react slightly with S142D drebrin-YFP (Figure 3c). Neuronal expression of drebrin-YFP and S142D drebrin-YFP, but not S142A drebrin-YFP, increased the level of F-actin, although this only reached statistical significance for S142D drebrin-YFP (Figure 3e). Together, these findings show that drebrin-driven neuritogenesis is regulated by S142 phosphorylation and is consistent with our previous report that EB3 preferentially binds to pS142-drebrin (Worth et al., 2013).

3.4 | the +TIP proteins EB1 and EB3 are differentially distributed at the plus-ends of dynamic microtubules in embryonic cortical neurons

Drebrin binds to the +TIP protein EB3, but not to EB1, when EB3 is bound to dynamic microtubules (Geraldo et al., 2008; Worth et al., 2013). We recently discovered that EB1 and EB3 are differentially distributed at the plus-ends of microtubules in human prostate cancer cell lines (Dart et al., 2017). Embryonic neurons also express EB1 and EB3 and so we investigated whether the EB proteins in these cells are similarly differentially distributed. We labelled embryonic rat cortical neurons with antibodies against EB1 and EB3 and tyrosinated α-tubulin to label dynamic microtubules (Kilmartin et al., 1982). In cultured embryonic neurons undergoing neuritogenesis,
Dynamic microtubules frequently enter the lamellipodium and occasionally run alongside F-actin bundles in filopodia. In stage I embryonic cortical neurons labelled with antibodies against EB1 and EB3, microtubule plus-ends in the lamellipodium labelled for both EB1 and EB3. EB1 was located closer to the microtubule plus-end than EB3 and occupied a slightly shorter length along the microtubule (Figure 4a, b). A similar differential distribution of EB1 and EB3 was seen on microtubules extending into the peripheral domain of neuronal growth cones (Figure 4c, d). Fluorescence intensity line plots showed that the average length of EB1 and EB3 distribution along the microtubule lattice was $0.54 \pm 0.01 \mu m$ (mean $\pm$ SEM, $n = 226$) and $0.62 \pm 0.02 \mu m$ (mean $\pm$ SEM, $n = 226$) respectively (Figure 4b).

To determine the difference between the location of EB1 and EB3 at microtubule plus-ends, we measured the distance between the...
peaks of the fluorescence intensity line plots of EB1 and EB3. This showed that EB1 is 152 ± 12 nm (mean ± SEM) ahead of EB3 (n = 72; combined from three independent culture preparations). Since drebrin binds to EB3 but not EB1 (Geraldo et al., 2008), we looked for a spatial association between drebrin distribution and EB3 on microtubule plus-ends in stage I neurons by immunofluorescence. As predicted, this showed that drebrin co-aligns with EB3, but not EB1, at the plus-ends of dynamic microtubules in nascent growth cones (Figure 4e). We confirmed the spatial co-alignment between drebrin and EB3 with fluorescence intensity plots along microtubule plus-ends (Figure 4f).

3.5 | Loss of function of EB3, but not EB1, inhibits neuritogenesis in embryonic rat cortical neurons

To test whether the EB proteins have a role in neuritogenesis, we used the CRISPR-Cas9 gene editing system to remove either EB1 or EB3 from embryonic cortical neurons. We screened four individual gRNA sequences for each EB protein with the aim to introduce indel mutations. Following transfection with CRISPR plasmids, neurons were cultured for 5 days before re-plating to allow time for loss of EB protein and at the same time follow neuritogenesis. We used quantitative immunoblotting to assess the effects of each plasmid on EB protein levels. This showed that the EB1 gRNA sequences targeting Ex2 and Ex 4.2 did not decrease EB1 in embryonic cortical neurons (not shown), whereas the gRNA sequences targeting Ex3 and Ex4.1 did (Figure 5a, b). Combining gRNA sequences targeting Ex3 and Ex4.1 reduced EB1 levels further (Figure 5a, b). The EB3 gRNA sequences targeting Ex3.1 and Ex3.2 decreased EB3 levels in embryonic cortical neurons (Figure 5c, d), whereas Ex4.1 and Ex4.2 produced no change in EB3 levels (not shown). Combining the EB3 gRNA sequences targeting Ex3.1 and Ex3.2, we found that EB3 was reduced in EB3-transfected neurons compared to vehicle (Con), whereas EB1 levels were not changed. Means from n = 10, neurons in each of n = 3, independent culture preparations are shown. Significant difference (Student’s t test): **, p < 0.01, Con versus BTP2 t = 6.28.
Figure 3  Drebrin over-expression induces supernumerary neurites in embryonic cortical neurons. (a) YFP, wild-type drebrin-YFP (WT), S142A drebrin-YFP (S142A) or S142D drebrin-YFP (S142D) were expressed in embryonic cortical neurons and cultures labelled with antibodies to GFP (which cross-reacts with YFP, green) and drebrin (red). Scale bars, 5 \( \mu \)m. (b) Quantification of the number of neurites in transfected cells. Neurons were sorted into groups having 1–5 (green) or >5 (blue) neurites. Means are from 100 or more neurons in each of \( n = 4 \), independent culture preparations. Significant differences (Two-way Anova with Tukey’s multiple comparison): *** \( p < 0.001 \), \( F = 170.9 \). (c) Drebrin over-expression increases F-actin and pS142 drebrin in neurites. YFP, drebrin-YFP (WT), S142A drebrin-YFP (S142A) or S142D drebrin-YFP (S142D) were expressed in embryonic cortical neurons and cultures labelled with antibodies to pS142 drebrin (red) and with phalloidin (green), to label F-actin. Scale bars, 5 \( \mu \)m. (d) Neurites in drebrin over-expressing embryonic cortical neurons contain abundant dynamic microtubules. Wild-type drebrin-YFP was expressed in embryonic cortical neurons and cultures labelled with antibodies to GFP (which cross-reacts with YFP, green) and tyrosinated \( \alpha \)-tubulin, to label dynamic microtubules (tyr-tub, blue) and with phalloidin (red). Scale bar, 5 \( \mu \)m. (e) The increase in F-actin levels produced by drebrin over-expression depends on S142 phosphorylation. Embryonic cortical cultures were transfected with YFP, wild-type drebrin-YFP (WT), S142A drebrin-YFP (S142A) or S142D drebrin-YFP (S142D) and cultures labelled with antibodies to GFP (which cross-reacts with YFP), drebrin and phalloidin to label F-actin. Means are from 10 or more neurons in each of \( n = 3 \), independent culture preparations. Significant difference (One-way Anova with Dunnett’s multiple comparison): * \( p < 0.05 \), \( F = 4.620 \).
FIGURE 4  EB1 and EB3 are differentially distributed at dynamic microtubule plus-ends in embryonic cortical neurons. (a) Confocal images of a Stage I embryonic cortical neuron immunolabelled with antibodies to EB1 (green, arrows), EB3 (blue, arrowheads) and tyrosinated α-tubulin (tyr-tub, red), to label dynamic microtubules and βIII tubulin (violet) to identify neurons. On single microtubules extending into the lamellipodium, EB1 (arrows) can be seen to occupy a slightly shorter and more distal position at the microtubule plus-end than EB3 (arrowheads). Inset shows enlarged view of EB1 (green) and EB3 (blue) in a lamellipodium. Scale bar, 2 µm and inset 1 µm. (b) Fluorescence intensity line plots showing the length distributions of EB1 (green) and EB3 (blue) at a dynamic microtubule plus-end (tyr-tub, red) in Stage I lamellipodia. (c) Confocal images of growth cones from a Stage I embryonic cortical neuron undergoing neuritogenesis immunolabelled with antibodies to EB1 (arrows, green), EB3 (arrowheads, blue), tyrosinated α-tubulin, to label dynamic microtubules (tyr-tub, red) and βIII tubulin (violet) to identify neurons. Inset shows enlarged view of EB1 (green) and EB3 (blue) in a growth cone. Scale bar, 2 µm and inset 1 µm. (d) Fluorescence intensity line plots showing the length distributions of EB1 (green) and EB3 (blue) at a dynamic microtubule plus-end (tyr-tub, red) in a growth cone of a Stage II neuron. (e) Confocal images of a nascent growth cone from a Stage I embryonic cortical neuron immunolabelled with antibodies to EB1 (green), drebrin (red), EB3 (blue) and tyrosinated α-tubulin (violet), to label dynamic microtubules. Scale bar, 1 µm. (f) Fluorescence intensity line plots showing the length distributions of EB1 (green), drebrin (red) and EB3 (blue) at a dynamic microtubule plus-end (tyr-tub, black) in nascent growth cones from Stage I embryonic cortical neurons.
remain unchanged by EB3 knockdown, as judged by quantitative immunofluorescence and immunoblotting (Figure S2), we conclude that EB3, but not EB1, is involved in neuritogenesis. These results are consistent with our previous findings showing that a dominant negative form of EB3 (EB3 M), but not EB1 M, inhibits neuritogenesis in embryonic cortical neurons (Geraldo et al., 2008).
3.6 | Gain of function of EB3, but not EB1, promotes neuritogenesis and induces supernumerary neurites in embryonic rat cortical neurons

To test whether over-expression of EB proteins affects neuritogenesis, we transfected embryonic cortical neurons with plasmids encoding GFP, EB1-GFP or EB3-mCherry and cultured them for 3 days. Cultures were fixed 3 h after re-plating and immunolabelled with antibodies to GFP or red fluorescent protein (RFP) and βIII-tubulin, to identify neurons, and with phalloidin to label F-actin (Figure 5i). We were careful to include in our analysis only those neurons in which the microtubule organization was normal and EB1-GFP or EB3-GFP were exclusively present at the tips of microtubules, rather than being abnormally distributed along the microtubule lattice as can happen in over-expression of these proteins (Bu & Su, 2003; Ligon et al., 2003; Mimori-Kiyosue et al., 2000). EB3-mCherry but not EB1-GFP promoted neuritogenesis, inducing supernumerary neurites in neurons (Figure 5i, j).

3.7 | Dampening microtubule dynamics inhibits growth cone formation and neuritogenesis

We tested the role of dynamic microtubules in neuritogenesis by treating embryonic cortical neurons in culture with the microtubule-targeting drug eribulin, which inhibits microtubule dynamics (Aseyev et al., 2016). Dissociated cortical neurons were cultured in the presence of eribulin over a range of concentrations for 3 h and scored for neurite production using confocal microscopy. Eribulin potently inhibited neuritogenesis in a dose-dependent manner (Figure 6a, b). Lamellipodia with radial F-actin-containing filopodia still form in the presence of eribulin but there is a dramatic reduction in the number of dynamic microtubules entering the lamellipodium compared to controls (Figure 6c, d). In control cultures, 6.2 microtubules per unit length of lamellipodium were found on average, whereas after eribulin treatment, 0.36 microtubules per unit length of lamellipodium were found (Figure 6e). The loss of dynamic microtubules from the lamellipodium most likely explains the inhibition of neuritogenesis produced by eribulin and supports the hypothesis that neuritogenesis depends on microtubule-F-actin coupling.

Since eribulin binds to β-tubulin, blocking the hydrolysis of GTP at the exchangeable β-tubulin site (Dabydeen et al., 2006), we wondered how this might affect binding of EB1 or EB3 to microtubule plus-ends. We treated embryonic cortical neurons in culture with eribulin (10 nM) for 20 or 30 min and then immunolabelled the cultures with antibodies to EB1, EB3 and tyrosinated α-tubulin to label dynamic microtubules (Figure 6f, g). After 20 min of eribulin treatment, microtubules had not been completely lost from the lamellipodium and retained EB1 binding at their plus-ends; however, these microtubules had lost EB3 binding (Figure 6f). After 30 min, microtubules were absent from the lamellipodium and restricted to the peri-nuclear region where EB1-labelled microtubule plus-ends were found (Figure 6g).

4 | DISCUSSION

Cellular responses to homing signals and guidance cues require cells to co-ordinate the dynamic behaviour of their F-actin and...
microtubule cytoskeletons (Rodriguez et al., 2003). Several pathways have been proposed to couple, physically and/or functionally, F-actin to microtubules (Coles & Bradke, 2015). Here, we provide evidence that the drebrin/EB3 pathway has an important role in neuritogenesis of embryonic cortical neurons in culture. We propose that this occurs by enabling the physical coupling of dynamic microtubules to F-actin in filopodia. We show here that loss of drebrin from cortical neurons using CRISPR-Cas9 gene editing inhibits neuritogenesis, whereas drebrin over-expression enhances neuritogenesis. The inhibition of neuritogenesis produced by drebrin knockdown was temporary since, 24 h after re-plating, transfected neurons lacking drebrin had undergone neuritogenesis at comparable levels to controls. This is consistent with recent studies showing that there are no gross brain abnormalities in transgenic mice lacking drebrin (Willmes et al.,
F-actin flow, which is known to oppose microtubule advance, rather. This effect is most likely because of an increase in retrograde
re-organization underlying neuritogenesis. EB1 still localizes to the plus-ends of these microtubules (arrows) whereas EB3 is absent. (g) After eribulin (10 nM) treatment for 30 min, microtubules are confined to a perinuclear region and seldom enter lamellipodia and retain EB1 (arrows) but not EB3 at their plus-ends. Scale bars, 2.5 µm

2017). However, loss of drebrin in some neuronal populations delays neuronal migration during development (Shan et al., 2021). The compensatory mechanisms that are presumably activated in these cases have yet to be identified and it might be the case that the effectiveness of compensatory mechanisms is different for neuritogenesis and neuronal migration. Neuritogenesis might be more cell intrinsic than neuronal migration, which depends on extrinsic cell signalling. We independently confirmed a role for drebrin in neuritogenesis by showing that BTP2, a small molecule inhibitor of drebrin binding to F-actin (Dart et al., 2017; Mancini et al., 2011; Mercer et al., 2010), also inhibits neuritogenesis. Drebrin stabilizes F-actin in cell-free systems (Mikati et al., 2013) and over-expression increases the levels of F-actin (Mizui et al., 2009). We found here that drebrin knockdown reduced the number of dynamic microtubules entering the lamellipodium from the circumferential bundle of microtubules in Stage I neurons. This effect is most likely because of an increase in retrograde F-actin flow, which is known to oppose microtubule advance, rather than the loss of drebrin-EB3-driven cross-linking of F-actin to microtubules. Consistent with this view is recent work showing that drebrin can slow F-actin retrograde flow in growth cones (Meka et al., 2019). It appears, therefore, that one of the functions of drebrin is counteracting F-actin retrograde flow, thereby enabling microtubule advance towards the cell periphery, an early step in the cytoskeletal re-organization underlying neuritogenesis.

Since drebrin binds F-actin and simultaneously directly to the +TIP protein EB3 when it is bound to the plus-end of dynamic microtubules entering filopodia, we tested the role of EB3 in neuritogenesis (Geraldo et al., 2008). Loss of EB3, but not EB1, a related +TIP protein that does not bind to drebrin (Geraldo et al., 2008), inhibited neuritogenesis. Conversely, EB3, but not EB1, over-expression enhances neuritogenesis and leads to supernumerary neurites. Previously, we showed that EB1 and EB3 are differentially distributed at the plus-ends of microtubules in human prostate cancer cell lines (Dart et al., 2017). This finding has been independently confirmed recently for two unrelated mammalian cell lines (Roth et al., 2019). Here, we found that EB1 and EB3 are also differentially distributed at the plus-ends of microtubules extending into lamellipodia in Stage I cultured embryonic cortical neurons and in growth cones. EB1 recognizes GTP/GDP-Pi-bound tubulin in the so-called “GTP-cap” region at the microtubule plus-end (Guesdon et al., 2016). The more proximal microtubule binding of EB3 suggests that EB3 recognizes some feature of the plus-end of microtubules that is distinct from that recognized by EB1. This feature is probably not GDP-bound tubulin since, unlike doublecortin which does recognize GDP-tubulin, EB3 does not distribute along the length of the microtubule lattice (Ettinger et al., 2016; Roth et al., 2019). The structural differences between EB1 and EB3 that underlie their differential binding to microtubules remain unknown. The differential localization of these two +TIP proteins on the same microtubule has implications for the functional consequences of drebrin binding to EB3. One consequence of the more distal location of EB1 on the microtubule lattice is that microtubule growth, sustained by EB1 binding, would probably not be impeded when microtubules are cross-linked through the drebrin/EB3 pathway to actin filaments at the base of a filopodium (Figure 7). As the microtubule extends into the filopodium by plus-end assembly, iterative proximo-distal addition of drebrin/EB3-mediated cross-links would produce a zipperpering effect of the microtubule to F-actin in the filopodium (Figure 7). Consistent with a zipperpering effect between microtubules and F-actin mediated by drebrin/EB3 cross-linking, we have previously observed a dynamic, proximo-distal extension of drebrin into filopodia (Geraldo et al., 2008) and here we found that drebrin co-aligns with EB3, but not EB1, at the plus-ends of dynamic microtubules in nascent growth cones.

Treatment of embryonic cortical neurons with the microtubule-targeting drug eribulin (Aseyev et al., 2016) reduced the number of dynamic microtubules and curtailed their extension into lamellipodia. Reduction of microtubule extension into the lamellipodium was associated with a loss of EB3, but not EB1, from microtubule plus-ends, consistent with EB3 recognizing a distinct conformation of the microtubule lattice from that recognized by EB1 (Dart et al., 2017; Roth et al., 2019). Concomitantly, eribulin produced an inhibition of

**FIGURE 6** The microtubule-targeting drug eribulin inhibits neuritogenesis in embryonic cortical neurons. (a) Confocal images of embryonic cortical cultures treated with vehicle (Control) or eribulin (10 nM) for 3 h and labelled with phalloidin (green) and antibodies to drebrin (red) and tyrosinated α-tubulin (blue), to label dynamic microtubules. In control cultures, neurones have undergone neuritogenesis and extended neurites (arrowheads) whereas after eribulin treatment neuritogenesis is inhibited and far fewer neurones have produced neurites (arrowheads). Scale bar, 20 µm. (b) Concentration-dependent inhibition of neuritogenesis by eribulin. Neurones were labelled as in (a) and scored as either Stage I or II. Histogram shows the proportion of neurones with neurites following treatment with eribulin. Means from n = 3–4 independent culture preparations are shown. One-hundred neurones were scored in each culture preparation for each experimental condition. Significant difference (One-way Anova with Dunnett’s multiple comparison): *p < 0.01 **p < 0.001, F = 15.71. (c, d) Confocal images of embryonic cortical cultures treated with vehicle (Control) or eribulin (10 nM) for 30 min and labelled with phalloidin, to label F-actin (green) and antibodies to tyrosinated α-tubulin, to label dynamic microtubules (dynamic Mts, blue). Scale bars, 2 µm. (c) In control cultures, dynamic microtubules extend into the lamellipodium (arrows) and alongside filopodia (arrowhead). (d) After eribulin (10 nM) treatment, dynamic microtubules (arrows) are seldom found in the lamellipodium. (e) Quantification of the number of dynamic microtubules in unit length (10 µm) of lamellipodium in embryonic cortical cultures treated with vehicle (Con) or eribulin (10 nM) for 30 min. Means from n = 3 independent culture preparations are shown. Ten neurones were scored in each culture preparation for each experimental condition. Significant difference (Student’s t test): *p < 0.05, t = 3.24. (f) Confocal images of embryonic cortical cultures treated with eribulin (10 nM) for 20 min and labelled with antibodies to EB1, EB3 and tyrosinated α-tubulin, to label dynamic microtubules. The number of microtubules entering the lamellipodium is reduced and they are shorter than normal. EB1 still localizes to the plus-ends of these microtubules (arrows) whereas EB3 is absent. (g) After eribulin (10 nM) treatment for 30 min, microtubules are confined to a perinuclear region and seldom enter lamellipodia and retain EB1 (arrows) but not EB3 at their plus-ends. Scale bars, 2.5 µm
FIGURE 7 Model illustrating the zipper effect of F-actin/microtubule cross-linking through the drebrin/EB3 pathway. Dynamic microtubules (green bar) entering filopodia have EB1 (yellow) and EB3 (red) bound at their plus-ends (+), EB1 occupying a more distal position on the microtubule than EB3 (this report). Drebrin (turquoise) is bound to the proximal (minus) ends of F-actin in filopodia and cross-links F-actin to microtubules by binding to EB3 on the microtubule plus-end (Geraldo et al., 2008; Worth et al., 2013). As the microtubule extends into the filopodium, driven by plus-end assembly of α/β tubulin dimers, additional EB3 molecules binding to the growing microtubule become available for cross-linking with drebrin and this zips up the microtubule to F-actin in a proximo-distal direction.
neuritogenesis, supporting the previously proposed role of dynamic microtubules (Flynn, 2013). The drebrin/EB3 pathway has been shown to play a role in neuronal migration (Dun et al., 2012; Sonego et al., 2015; Tanabe et al., 2014; Trivedi et al., 2017) and in other cellular contexts where dynamic microtubules are interacting with F-actin (Bazellieres et al., 2012; Kasiulis et al., 2017; Ketschek et al., 2016; Merriam et al., 2013), suggesting that the pathway is canonical. There is evidence that the drebrin/EB3 pathway is regulated by phosphorylation (Gordon-Weeks, 2017). Drebrin exists in a closed conformation, in which one of the two F-actin-binding domains is occluded and an open conformation in which both F-actin-binding domains are available to bind F-actin. Phosphorylation of drebrin by Cdk5 at S142 switches drebrin from the closed to the open conformation and exposes the EB3 binding site (Worth et al., 2013). During neuritogenesis, pS142 drebrin accumulates in filopodia of nascent growth cones (Worth et al., 2013) and here we show that S142 phospho-mimetic mutants of drebrin are more efficient in inducing supernumerary neurites than wild-type drebrin, whereas phospho-dead mutants inhibit neuritogenesis. Interestingly, although all stage I neurons express drebrin, only a sub-population, presumably those in the early stages of neuritogenesis, have pS142 drebrin (Worth et al., 2013). After neuritogenesis, pS142 drebrin remains uniquely localized to all growth cones (Worth et al., 2013). Taken together, these findings suggest that S142 phosphorylation of drebrin regulates the interaction of drebrin with F-actin and EB3, and thereby influences the zippering of F-actin to microtubules in filopodia that underlies neuritogenesis.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

P.G-W. designed the study, obtained the funding, wrote the manuscript and supervised and co-ordinated the project. TP conducted the experiments. TP and FO designed and produced the CRISPR-Cas9 plasmids. FB and P.G-W performed the eribulin experiments.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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