1 **Efa6 protects axons and regulates their growth and branching by inhibiting**
2 **microtubule polymerisation at the cortex**

3

4 Yue Qu*,[1], Ines Hahn*,#,[1], Meredith Lees[1], Jill Parkin[1], André Voelzmann[1], Karel Dorey[1], Alex
5 Rathbone[2], Claire Friel[2], Viki Allan[1], Pilar Okenve-Ramos[3], Natalia Sanchez-Soriano[3],
6 Andreas Prokop[1]

7

8 1) The University of Manchester, Manchester Academic Health Science Centre, Faculty of
9   Biology, Medicine and Health, School of Biology, Manchester, UK
10 2) The University of Nottingham, School of Life Sciences, Faculty of Medicine & Health
11   Sciences, Nottingham, UK
12 3) Department of Cellular and Molecular Physiology, Institute of Translational Medicine,
13   University of Liverpool, Liverpool, United Kingdom

14

15 Running title**:** The role of Efa6 in axon maintenance

16 Key words: *Drosophila*, neurodegeneration, axons, actin, cytoskeleton, microtubules

17

18 *   authors contributed equally
19 #   authors for correspondence:
20   The University of Manchester
21   Faculty of Life Sciences
22   Oxford Road
23   Manchester M13 9PT
24   Tel: +44-(0)161-27-51556
25   Fax: +44-(0)161-27-51505
26   Ines.Hahn@manchester.ac.uk

27

28 **Summary statement (30 words max):**

29 The cortical collapse factor Efa6 inhibits microtubule polymerising outside axonal bundles.
30 Thereby it limits axon growth and branching, but preserves microtubule bundle organisation
31 crucial for axon maintenance.

32

33 **Abstract**
34 Cortical collapse factors affect microtubule (MT) dynamics at the plasma membrane. They
35 play important roles in neurons, as suggested by inhibition of axon growth and regeneration
36 through the Arf activator Efa6 in *C. elegans*, and by neurodevelopmental disorders linked to
37 the mammalian kinesin Kif21A. How cortical collapse factors influence axon growth is little
38 understood. Here we studied them, focussing on the function of *Drosophila* Efa6 in
39 experimentally and genetically amenable fly neurons. First, we show that *Drosophila* Efa6 can
40 inhibit MTs directly without interacting molecules via an N-terminal 18 amino acid motif (MT
41 elimination domain/MTED) that binds tubulin and inhibits microtubule growth *in vitro* and cells.
42 If N-terminal MTED-containing fragments are in the cytoplasm they abolish entire microtubule
43 networks of mouse fibroblasts and whole axons of fly neurons. Full-length Efa6 is membrane-

44 attached, hence primarily blocks MTs in the periphery of fibroblasts, and explorative MTs that
45 have left axonal bundles in neurons. Accordingly, loss of Efa6 causes an increase of
46 explorative MTs: in growth cones, they enhance axon growth, in axon shafts, explorative MTs
47 cause excessive branching, as well as atrophy through perturbations of MT bundles. Efa6
48 over-expression causes the opposite phenotypes. Taken together, our work conceptually links
49 molecular and sub-cellular functions of cortical collapse factors to axon growth regulation and
50 reveals new roles in axon branching and in the prevention of axonal atrophy. Furthermore, the
51 MTED delivers a promising tool that can be used to inhibit MTs in a compartmentalised
52 fashion when fusing it to specifically localising protein domains.

53

Introduction

55 Axons are the cable-like neuronal extensions that wire the nervous system. They are only 0.1-
56 15μm in diameter (Hoffman, 1995), but can be up to a meter long in humans (Debanne et al.,
57 2011; Prokop, 2013a). It is a fascinating challenge to understand how axons can extend over
58 these enormous distances and branch in orderly manners, but also how these delicate
59 structures can be maintained for a lifetime, i.e. many decades in humans. It is not surprising
60 that we gradually lose about half of our axons towards old age (Calkins, 2013; Marner et al.,
61 2003), and that axon decay is a prominent neurodegenerative phenomenon (Adalbert and
62 Coleman, 2012; Fang and Bonini, 2012; Medana and Esiri, 2003; Wang et al., 2012).

63   Essential for axon biology are the parallel bundles of microtubules (MTs) running all
64 along the axon shaft; these bundles provide (1) structural support, (2) highways for life-
65 sustaining cargo transport, and (3) a source of MTs that can leave these bundles to drive
66 morphogenetic changes. Through being organised in this way, MTs essentially drive
67 processes of axon growth, branching and maintenance (Conde and Caceres, 2009; Dent et al.,
68 2011; Hahn et al., 2019; Prokop, 2013a; Voelzmann et al., 2016a). The dynamics of MTs are
69 orchestrated through MT-binding and -regulating proteins, for most of which we know the
70 molecular mechanisms of function. However, such knowledge alone is usually not sufficient to
71 explain their cellular roles.

72   For example, cortical collapse factors are cell surface-associated proteins specifically
73 inhibit MTs that approach the cell periphery. Previous reports suggested important roles for
74 cortical collapse factors in regulating axon growth: the ARF activator Efa6 in *C. elegans*
75 negatively impacts on developmental and regenerative axon growth (Chen et al., 2015; Chen
76 et al., 2011; O'Rourke et al., 2010); the mammalian type 4 kinesin KIF21A also affects axon
77 growth and links to the neurodevelopmental eye movement disorder "congenital fibrosis of
78 extraocular muscles" (OMIM reference #135700; Heidary et al., 2008; Tiab et al., 2004; van
79 der Vaart et al., 2013). However, we can currently only hypothesise how the molecular
80 function of these two collapse factors links to axon growth, most likely by acting in growth
81 cones (GCs).

82   GCs are the amoeboid tip structures where axons extend to wire the nervous system
83 during development or regeneration. The axonal MT bundles terminate in the centre of GCs;
84 from here, single MTs splay into the actin-rich periphery of GCs. These explorative MTs can
85 trigger extension of the entire MT bundle into their direction, thus elongating the axon (Dent et
86 al., 2011; Lowery and van Vactor, 2009; Prokop et al., 2013); their (partial) inhibition through
87 cortical collapse factors could provide a potential mechanism through which cortical collapse
88 factors negatively impact on axon growth.

89   In line with this argumentation, and depending on where cortical collapse factors are
90   present and functionally active, further functional predictions could be made: for example,
91   collateral branching of axons along their shafts has been described to depend on explorative
92   MTs that leave the parallel axonal bundles and polymerise towards the periphery (Kalil and
93   Dent, 2014; Lewis et al., 2013; Tymanskyj et al., 2017; Yu et al., 2008). Cortical collapse
94   factors might therefore be negative regulators of axon branching.

95   Other roles might concern axon maintenance: the model of 'local axon homeostasis'
96   states that the force-enriched environment in axons biases MTs to buckle or project out of the
97   bundle to seed pathological areas of MT disorganisation (Hahn et al., 2019; Prokop, 2016;
98   Voelzmann et al., 2016a). By inhibiting off-track MTs in the axon shaft, cortical collapse
99   factors might prevent such processes, acting in parallel to other bundle-maintaining factors.
100   For example, spectraplakins serve as spacers that keep polymerising MTs away from the
101   cortex by linking the tips of extending MTs to the axonal surface and guiding them into parallel
102   bundles (Alves-Silva et al., 2012). Their deficiency in any organism causes severe MT
103   disorganisation, potentially explaining human dystonin-linked HSAN6 ('type 6 hereditary
104   sensory and autonomic neuropathy''; #614653; Voelzmann et al., 2017). If our hypothesis is
105   correct, loss of cortical collapse factors in axon shafts would also cause MT disorganisation,
106   but through a very different mechanistic route.

107   Here we make use of *Drosophila* neurons as a well-established, powerful model for
108   studying roles of MT regulators (Hahn et al., 2019; Prokop et al., 2013; Sánchez-Soriano et
109   al., 2007). Using *in vitro* and cellular assays, we show that *Drosophila* Efa6 is a cortical
110   collapse factor acting through its N-terminal MT-eliminating domain (MTED). We find that the
111   MTED binds tubulin and blocks MT polymerisation *in vitro* which shows that the effect of the
112   peptide is due to a direct interaction between the peptide and tubulin and does not require any
113   other molecules. By localising to neuronal membranes, it only abolishes explorative MTs. This
114   subcellular role translates into negative regulation of axon growth and branching and the
115   prevention of pathological MT disorganisation, both in cultured neurons and *in vivo*. We
116   propose Efa6 to function as a quality control or axonal maintenance factor that keeps
117   explorative MTs in check, thus playing a complementary role to spectraplakins that prevent
118   MTs from leaving axonal bundles.

119

120   **Methods**

121

122   <u>Fly stocks</u>

123   Loss-of-function mutant stocks used in this study were the two deficiencies uncovering the
124   *Efa6* locus *Df(3R)Exel6273* (94B2-94B11 or 3R:22,530,780..22,530,780) and *Df(3R)ED6091i*
125   (94B5-94C4 or 3R:22,587,681..22,587,681), *shot³* (the strongest available allele of *short stop*)
126   (Kolodziej et al., 1995; Sánchez-Soriano et al., 2009), and the null mutant alleles *Efa6^{KO#1}*,
127   *Efa6^{GX6[w-]}*, *Efa6^{GX6[w+]}* and *Arf51F^{GX16[w-]}* (all genomically engineered precise deletions) (Huang
128   et al., 2009). Gal4 driver lines used were the pan-neuronal lines *sca-Gal4* (strongest in
129   embryos) (Sánchez-Soriano et al., 2010) and *elav-Gal4* (1st and 3rd chromosomal, both
130   expressing at all stages) (Luo et al., 1994), as well as the *ato-Gal4* line expressing in a subset
131   of neurons in the adult brain (Hassan et al., 2000; Voelzmann et al., 2016b). Lines for targeted
132   gene expression were *UAS-Efa6^{RNAi}* (VDRC #42321), *UAS-Gal80^{ts}* (Zeidler et al., 2004), *UAS-*
133   *Eb1-GFP* (Alves-Silva et al., 2012), *UAS-α-tubulin84B* (Grieder et al., 2000) and *UAS-*
134   *tdTomato* (Zschätzsch et al., 2014). Efa6 expression was detected via the genomically

135　engineered *Efa6-GFP* allele, where a GFP was inserted after the last amino acid in exon 14
136　(Huang et al., 2009).

137　*Drosophila* primary cell culture

138　*Drosophila* primary neuron cultures were performed as published previously (Prokop et al.,
139　2012; Qu et al., 2017). In brief, stage 11 embryos were treated for 1 min with bleach to
140　remove the chorion, sterilized for ~30 s in 70% ethanol, washed in sterile Schneider's/FCS,
141　and eventually homogenized with micro-pestles in 1.5 centrifuge tubes containing 21 embryos
142　per 100 µl dispersion medium (Prokop et al., 2012) and left to incubated for 5 min at 37°C.
143　Cells are washed with Schneider's medium (Gibco), spun down for 4 mins at 650 g,
144　supernatant was removed and cells re-suspended in 90 µl of Schneider's medium containing
145　20% fetal calf serum (Gibco). 30 µl drops were placed on cover slips. Cells were allowed to
146　adhere for 90-120 min either directly on glass or on cover slips coated with a 5 µg/ml solution
147　of concanavalin A, and then grown as a hanging drop culture for hours or days at 26°C as
148　indicated.

149　To abolish maternal rescue of mutants, i.e. masking of the mutant phenotype caused by
150　deposition of normal gene product from the healthy gene copy of the heterozygous mothers in
151　the oocyte (Prokop, 2013b), we used a pre-culture strategy (Prokop et al., 2012; Sánchez-
152　Soriano et al., 2010) where cells were kept for 5 days in a tube before they were plated on a
153　coverslip.

154　For the transfection of *Drosophila* primary neurons, a quantity of 70-75 embryos per 100 µl
155　dispersion medium was used. After the washing step and centrifugation, cells were re-
156　suspended in 100 µl transfection medium [final media containing 0.1-0.5 µg DNA and 2 µl
157　Lipofecatmine 2000 (L2000)]. To generate this media, dilutions of 0.1-0.5 µg DNA in 50 µl
158　Schneider's medium and 2 µl L2000 in 50 µl Schneider's medium were prepared, then mixed
159　together and incubated at room temperature for 5-30 mins, before being added to the cells in
160　centrifuge tubes where they were kept for 24 hrs at 26°C. Cells were then treated again with
161　dispersion medium, re-suspended in culture medium and plated out as described above.

162　For temporally controlled knock-down experiments we used flies carrying the driver construct
163　*elav-Gal4*, the knock-down construct *UAS-Efa6-RNAi*, and the temperature-sensitive Gal4
164　inhibitor *UAS-Gal80^{ts}*, all in parallel. At the restrictive temperature of 19°C, Gal80^{ts} blocks
165　Gal4-induced expression of *Efa6-RNAi*, and this repressive action is removed at the
166　permissive temperature of 27°C where Gal80^{ts} is non-functional. Control neurons were from
167　flies carrying only the *Gal4/Gal80* (control 1 in Fig. 8K) or only the *Efa6-RNAi* transgene
168　(control 2).

169

170　Fibroblast cell culture

171　NIH/3T3 fibroblasts were grown in DMEM supplemented with 1% glutamine
172　(Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 10% FCS in culture dishes (100 mm
173　with vents; Fisher Scientific UK Ltd) at 37°C in a humidified incubator at 5% CO_2. Cells were
174　split every 2-3 d, washed with pre-warmed PBS, incubated with 4 ml of Trypsin-EDTA (T-E) at
175　37°C for 5 min, then suspended in 7 ml of fresh culture medium and eventually diluted (1/3-
176　1/20 dilution) in a culture dish containing 10 ml culture media.

177　For transfection of NIH/3T3 cells, 2 ml cell solution (~10^5 cells per ml) were first
178　transferred to 6-well plates, and grown overnight to double cell density. 2 µg of DNA and 2 µl
179　Plus reagent (Invitrogen) were added to 1 ml serum-free media in a centrifuge tube, incubated

180　for 5 mins at RT, then 6 μl Lipofectamine (Invitrogen) were added, and incubated at RT for 25
181　mins. Cells in the 6-well plate were washed with serum-free medium and 25 mins later
182　DNA/Lipofectamine was mixed into the medium (1/1 dilution). Plates were incubated for 3 hrs
183　at 37°C, washed with 2 ml PBS, 400 μl trypsin were added for 5 mins (37°C), then 3 ml
184　complete medium; cells were suspended and added in 1 ml aliquots to 35 mm glass-bottom
185　dishes (MatTek) coated with fibronectin [300 μl of 5 μg/ml fibronectin (Sigma-Aldrich) placed
186　in the center of a MatTek dish for 1 hr at 37°C, then washed with PBS]; 1 ml of medium was
187　added and cells grown for 6 hrs or 24 hrs at 37°C in a CO_2 incubator. For live imaging, the
188　medium was replaced with 2 ml Ham's F-12 medium + 4% FCS.

189

190　Dissection of adult brains

191　To analyse the function of Efa6 in MT bundle integrity in medulla axons *in vivo*, flies were
192　aged at 29°C. Flies were maintain in groups of up to 20 flies of the same gender (Stefana et
193　al., 2017) and changed into new tubes every 3-4 days. Brain dissections were performed in
194　Dulbecco's PBS (Sigma, RNBF2227) after briefly sedating them on ice. Dissected brains with
195　their laminas and eyes attached were placed into a drop of Dulbecco's PBS on MatTek glass
196　bottom dishes (P35G1.5-14C), covered by coverslips and immediately imaged with a 3i
197　Marianas Spinning Disk Confocal Microscope.

198　To measure branching in *ato-Gal4 Drosophila* neurons, adult brains were dissected in
199　Dulbecco's PBS and fixed with 4% PFA for 15 min. Antibody staining and washes were
200　performed with PBS supplemented with 0.3% Triton X-100. Specimens were embedded in
201　Vectashield (VectorLabs).

202

203　Immunohistochemistry

204　Primary fly neurons and fibroblasts were fixed in 4% paraformaldehyde (PFA) in 0.05 M
205　phosphate buffer (PB; pH 7–7.2) for 30 min at room temperature (RT); for anti-Eb1 staining,
206　ice-cold +TIP fix (90% methanol, 3% formaldehyde, 5 mM sodium carbonate, pH 9; stored at -
207　80°C and added to the cells) (Rogers et al., 2002) was added for 10 mins. Adult brains were
208　dissected out of their head case in PBS and fixed with 4% PFA in PBS for 1 hr, followed by a
209　1hr wash in PBT.

210　　　　Antibody staining and washes were performed with PBT. Staining reagents: anti-
211　tubulin (clone DM1A, mouse, 1:1000, Sigma; alternatively, clone YL1/2, rat, 1:500, Millipore
212　Bioscience Research Reagents); anti-DmEb1 (gift from H. Ohkura; rabbit, 1:2000) (Elliott et
213　al., 2005); anti-Elav (mouse, 1:1000, DHB); anti-GFP (goat, 1:500, Abcam); Cy3-conjugated
214　anti-HRP (goat, 1:100, Jackson ImmunoResearch); F-actin was stained with Phalloidin
215　conjugated with TRITC/Alexa647, FITC or Atto647N (1:100 or 1:500; Invitrogen and Sigma).
216　Specimens were embedded in ProLong Gold Antifade Mountant.

217

218　Microscopy and data analysis

219　Standard documentation was performed with AxioCam monochrome digital cameras (Carl
220　Zeiss Ltd.) mounted on BX50WI or BX51 Olympus compound fluorescent microscopes. For
221　the analysis of *Drosophila* primary neurons, we used two well established parameters (Alves-
222　Silva et al., 2012; Sánchez-Soriano et al., 2010): axon length (from cell body to growth cone
223　tip; measured using the segmented line tool of ImageJ) and the degree of MT disorganisation

224   in axons which was either measured as binary score or ratio (percentage of neurons showing
225   obvious MT disorganisation in their axons) or as "MT disorganisation index" (MDI) (Qu et al.,
226   2017): the area of disorganisation was measured using the freehand selection in ImageJ; this
227   value was then divided by axon length (see above) multiplied by 0.5 µm (typical axon diameter,
228   thus approximating the expected area of the axon if it were not disorganised). For Eb1::GFP
229   comet counts, neurons were subdivided into axon shaft and growth cones (GC): the proximal
230   GC border was set where the axon widens up (broader GCs) or where filopodia density
231   increases significantly (narrow GCs). MT loss in fibroblasts was assessed on randomly
232   chosen images of successfully transfected, GFP-expressing fibroblasts, stained for tubulin
233   and actin. Images were derived from at least 2 independent experimental repeats performed
234   on different days, for each of which at least 3 independent culture wells were analysed by
235   taking a minimum of 20 images per well. Due to major differences in plasma membrane
236   versus cytoplasmic localisation of constructs, their expression strengths could not be
237   standardised. Assuming a comparable expression strength distribution, we therefore analyse
238   all transfected cells in the images and assigned them to three categories: MTs intact,
239   damaged or gone (Fig. 3G-G''). To avoid bias, image analyses were performed blindly, i.e. the
240   genotype or treatment of specimens was masked. To analyse ruffle formation in fibroblasts,
241   cells were stained with actin and classified (with or without ruffles).

242   To assess the degree of branching, we measured axonal projections of dorsal cluster
243   neurons in the medulla, which is part of the optic lobe in the adult brain (Hassan et al., 2000;
244   Voelzmann et al., 2016b). These neurons were labelled by expressing *UAS-myr-tdTomato* via
245   the *ato-Gal4* driver either alone (control), together with *UAS-Efa6^RNAi* or together with *UAS-*
246   *Efa6-FL-GFP*. We analysed them in young brains (2-5 d after eclosure of flies from their pupal
247   case) or old brains (15-18 d). Z-stacks of adult fly brains (optic lobe area) were taken with a
248   Leica DM6000 B microscope and extracted with Leica MM AF Premier software. They were
249   imaged from anterior and the number of branches was quantified manually. Branches were
250   defined as the protrusions from the DC neuron axons in the medulla. Branches in fly primary
251   neurons at 5DIV were also counted manually and defined as MT protrusions from main axon.

252   To measure MT disorganisation in the optic lobe of adult flies, *GMR31F10-Gal4*
253   (Bloomington #49685) was used to express *UAS-α-tubulin84B-GFP* (Grieder et al., 2000) in a
254   subset of lamina axons which projects within well-ordered medulla columns (Prokop and
255   Meinertzhagen, 2006). Flies were left to age for 26-27 days (about half their life expectancy)
256   and then their brains were dissected out, mounted in Mattek dishes and imaged using a 3i
257   spinning disk confocal system at the ITM Biomedecial imaging facility at the University of
258   Liverpool. A section of the medulla columns comprising the 4 most proximal axonal terminals
259   was used to quantify the number of swellings and regions with disorganised MTs.

260   Time lapse imaging of cultured primary neurons (in Schneider's/FCS) and fibroblasts
261   (in Ham's F-12/FCS) was performed on a Delta Vision Core (Applied Precision) restoration
262   microscope using a [*100x/1.40 UPlan SAPO (Oil)*] objective and the Sedat Quad filter set
263   (*Chroma #89000*). Images were collected using a Coolsnap HQ2 (Photometrics) camera. The
264   temperature was set to 26°C for fly neurons and 37°C for fibroblasts. Time lapse movies were
265   constructed from images taken every 2 s for 2 mins. To analyse MT dynamics, Eb1::GFP
266   comets were tracked manually using the "manual tracking" plug-in of ImageJ.

267   For statistical analyses, Kruskal–Wallis one-way ANOVA with *post hoc* Dunn's test or
268   Mann–Whitney Rank Sum Tests (indicated as P_{MW}) were used to compare groups, and χ2
269   tests (indicated as P_{X2}) were used to compare percentages. All raw data of our analyses are
270   provided as supplementary Excel/Prism files.

271

Molecular biology

EGFP tags are based on pcDNA3-EGFP or pUAST-EGFP. All *Drosophila melanogaster* efa6 constructs are based on cDNA cloneIP15395 (Uniprot isoform C, intron removed). *Caenorhabditis elegans* efa-6 (Y55D9A.1a) constructs are derived from pCZGY1125-efa-6-pcr8 (kindly provided by Andrew Chisholm). *Homo sapiens* PSD1 (ENST00000406432.5, isoform 202) constructs were PCR-amplified from pLC32-hu-psd1-pcr8 vector (kindly provided by Andrew Chisholm). *Homo sapiens* PSD2 (ENST00000274710.3, isoform 201, 771aa) constructs were PCR-amplified from pLC33-hu-psd2-pcr8 vector (kindly provided by Andrew Chisholm). *Homo sapiens* PSD3 was PCR-amplified from pLC34 hu-psd3-pcr8 vector (kindly provided Andrew Chisholm). Note that the PSD3 cDNA clone is most closely related to isoform 201 (ENST00000286485.12: 513aa) and therefore lacks the putative N-terminus found in isoform 202 (ENST00000327040.12). However, the putative MTED core sequence is encoded in the C-terminal PH domain (Fig.2C), not the potential N-terminus. *Homo sapiens* PSD4 (ENST00000441564.7, isoform 205) was PCR-amplified from pLC35-hu-psd4-pcr8 vector (kindly provided by Andrew Chisholm).The CAAX motif is derived from human KRAS. The *Dm*Efa6-NtermΔSxiP::EGFP (aa1-410) insert was synthesised by GeneArt Express (ThermoFisher). All construct were cloned using standard (SOE) PCR/ligation based methods, and constructs and inserts are detailed in Table T1. To generate transgenic fly lines, *P[acman]M-6-attB-UAS-1-3-4* constructs were integrated into *PBac{yellow[+]-attP-3B}VK00031* (Bloomington line #9748) via PhiC31 mediated recombination (outsourced to Bestgene Inc.).

293

final vector	Source	insert
pcDNA3-EGFP	Addgene	*XhoI*-EGFP-*XbaI*
pUAST-AscI-PacI-EGFP	this study	*KpnI, AscI, PacI*-EGFP-*XbaI*
pUAST-DmEfa6FL-EGFP (aa1-1387)	this study	*KpnI, AscI*-**kozak**-Efa6 (aa1-1387)-**GSGSGS-EGFP**-*PacI, XbaI*
P[acman]M-6-attB-UAS-1-3-4-DmEfa6FL-EGFP (aa1-1387)	this study	*AscI*-**kozak**-*Dm*Efa6 (aa1-1387)-**GSGSGS-EGFP**-*PacI*
pcDNA3.1-DmEfa6FL-EGFP (aa1-1387)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6 (aa1-1387)-**GSGSGS-EGFP**-*PacI, XbaI*
pUAST-DmEfa6ΔCterm-EGFP (aa1-894)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6Δ**Cterm** (aa1-894)-**GSGSGS-EGFP**-*PacI, XbaI*
P[acman]M-6-attB-UAS-1-3-4-DmEfa6ΔCterm-EGFP (aa1-894)	this study	*AscI*-**kozak**-*Dm*Efa6Δ**Cterm** (aa1-894)-**GSGSGS-EGFP**-*PacI*
pcDNA3.1-DmEfa6ΔCterm-EGFP (aa1-894)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6Δ**Cterm** (aa1-894)-**GSGSGS-EGFP**-*PacI, XbaI*
pUAST-DmEfa6-Nterm-EGFP (aa1-410)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-Nterm (aa1-410)-**GSGSGS-EGFP**-*PacI, XbaI*
P[acman]M-6-attB-UAS-1-3-4-DmEfa6-Nterm-EGFP (aa1-410)	this study	*AscI*-**kozak**-*Dm*Efa6-Nterm (aa1-410)-**GSGSGS-EGFP**-*PacI*
pcDNA3.1-DmEfa6-Nterm-EGFP (aa1-410)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-Nterm (aa1-410)-**GSGSGS-EGFP**-*PacI, XbaI*
pUAST-DmEfa6-Nterm-CAAX-EGFP (aa1-410)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-Nterm (aa1-410)-**GSGSGS-EGFP-CAAX[KRAS]**-*PacI, XbaI*
P[acman]M-6-attB-UAS-1-3-4-DmEfa6-Nterm-CAAX-EGFP (aa1-410)	this study	*AscI*-**kozak**-*Dm*Efa6-Nterm (aa1-410)-**GSGSGS-EGFP-CAAX[KRAS]**-*PacI*
pcDNA3.1-DmEfa6-Nterm-CAAX-EGFP (aa1-410)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-Nterm (aa1-410)-**GSGSGS-EGFP-CAAX[KRAS]**-*PacI, XbaI*

pUAST-DmEfa6-NtermΔSxiP-EGFP (aa1-410)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-NtermΔ**SxiP** (aa1-410; **SQIP>AAAA; SRIP>AAAA**)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-DmEfa6-NtermΔSxiP-EGFP (aa1-410)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-NtermΔ**SxiP** (aa1-410; **SQIP>AAAA; SRIP>AAAA**)-**GSGSGS-EGFP**-*PacI, XbaI*
pUAST-DmEfa6-NtermΔMTED-EGFP (aa1-300)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-NtermΔ**MTED** (aa1-300)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-DmEfa6-NtermΔMTED-EGFP (aa1-300)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-NtermΔ**MTED** (aa1-300)-**GSGSGS-EGFP**-*PacI, XbaI*
pUAST-DmEfa6ΔNerm-EGFP (aa851-1387)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6Δ**Nerm** (aa851-1387)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-DmEfa6ΔNerm-EGFP (aa851-1387)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6Δ**Nerm** (aa851-1387)-**GSGSGS-EGFP**-*PacI, XbaI*
pUAST-DmEfa6-MTED-EGFP (aa322-341)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-MTED (aa322-341)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-DmEfa6-MTED-EGFP (aa322-341)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-MTED (aa322-341)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-CeEfa6-FL-EGFP (aa1-816)	this study	*KpnI, AscI*-**kozak**-*Ce*Efa6 (aa1-816)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-CeEfa6-Nterm-EGFP (aa1-152)	this study	*KpnI, AscI*-**kozak**-*Ce*Efa6-Nterm (aa1-152)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-CeEfa6-MTED-EGFP (aa24-42)	this study	*KpnI, AscI*-**kozak**-*Ce*Efa6-MTED (aa24-42)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-HsPSD1-FL-EGFP (aa1-1024)	this study	*KpnI, AscI*-**kozak**-*Hs*PSD1 (aa1-1024)-**GSGSGS**-*NotI*-**EGFP**-*PacI, XbaI*
pcDNA3.1-HsPSD1-Nterm-EGFP (aa1-280)	this study	*KpnI, AscI*-**kozak**-*Hs*PSD1-Nterm (aa1-280)-**GSGSGS**-*NotI*-**EGFP**-*PacI, XbaI*
pcDNA3.1-HsPSD1-MTED-EGFP (aa31-49)	this study	*KpnI, AscI*-**kozak**-*Hs*PSD1-MTED (aa31-49)-**GSGSGS**-*NotI*-**EGFP**-*PacI, XbaI*
pcDNA3.1-HsPSD2-FL-EGFP (aa1-771)	this study	*KpnI, AscI*-**kozak**-*Hs*PSD2 (aa1-771)-**GSGSGS**-*NotI*-**EGFP**-*PacI, XbaI*
pcDNA3.1-HsPSD3-EGFP (aa515-1047)	this study	*KpnI, AscI*-**kozak**-*Hs*PSD3 (aa515-1047)-**GSGSGS**-*NotI*-**EGFP**-*PacI, XbaI*
pcDNA3.1-HsPSD4-FL-EGFP (aa1-1027)	this study	*KpnI, AscI*-**kozak**-*Hs*PSD4 (aa1-1027)-**GSGSGS**-*NotI*-**EGFP**-*PacI, XbaI*
pcDNA3.1-co-HsPSD1-MTED-EGFP (aa31-49)	this study	*KpnI, AscI*-**kozak**-*Hs*PSD1-MTED (aa31-49)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-co-CeEfa6-MTED-EGFP (aa24-42)	this study	*KpnI, AscI*-**kozak**-*Ce*Efa6-MTED (aa24-42)-**GSGSGS-EGFP**-*PacI, XbaI*
pcDNA3.1-co-DmEfa6-MTED-EGFP (aa322-341)	this study	*KpnI, AscI*-**kozak**-*Dm*Efa6-MTED (aa322-341)-**GSGSGS-EGFP**-*PacI, XbaI*
pCS107-DmEfa6-Nterm-EGFP	this study	*NotI*-**kozak**-*Dm*Efa6-Nterm (aa1-410)-**GSGSGS-EGFP**-*StuI*
RFP (Xenopus injection)	tba	tba
pFastBac-His6-MCAK-EGFP-StrepII	tba	His6-MCAK::EGFP-StrepII
pFastBac-His6-DmEfa6ΔCterm-EGFP-StrepII (aa1-894)	this study	His6-*Dm*Efa6Δ**Cterm**::EGFP-StrepII (aa1-894)

294   **Tab. T1**. co=codon optimised; *Dm=Drosphila melanogaster*; *Ce=Caenorhabditis elegans*;
295   *Hs=Homo sapiens*

296

297   *In silico* analyses

298   To generate the **phylogenetic tree** of Efa6/PSD full length isoforms and N-terms of different
299   species (see Fig. S1), their amino acid sequences were aligned using Muscle or ClustalO
300   (Goujon et al., 2010; McWilliam et al., 2013; Sievers et al., 2011). ProtTest (Abascal et al.,

2005; Darriba et al., 2011) was used to determine amino acid frequencies in the protein datasets and to identify the optimal amino acid substitution model to be used for the Bayesian inference (VT+I+G+F). CUDA-Beagle-optimised MrBayes (Ronquist et al., 2012) was run using the VT+I+G+F model [prset statefreqpr=fixed(empirical); lset rates=invgamma] using 5 chains (1 heated) and 9 parallel runs until the runs converged and standard deviation of split frequencies were below 0.015 (0.06 for N-terms); PSRF+ was 1.000 and min ESS was >1300 for the TL, alpha and pinvar parameters. The *Drosophila melanogaster* Sec7-PH domain-containing protein Steppke was used as outgroup in the full length tree. Archaeopteryx (Han and Zmasek, 2009) was used to depict the MrBayes consensus tree showing branch lengths (amino acid substitutions per site) and Bayesian posterior probabilities.

To **identify a potential MTED in PSD1**, previously identified Efa6 MTED motifs (O'Rourke et al., 2010) of 18 orthologues were aligned to derive an amino acid logo. Further orthologues were identified and used to refine the logo. Invariant sites and sites with restricted amino acid substitutions were determined (most prominently MxG-stretch). Stretches containing the invariant MxG stretch were aligned among vertebrate species to identify potential candidates. Berkley's Weblogo server (Crooks et al., 2004) was used to generate amino acid sequence logos for each phylum using MTED (ExxxMxGE/D) and MTED-like (MxGE/D) amino acid stretches.

In vitro analyses

Protein Expression and Purification: *Drosophila* Efa6-ΔCterm was cloned into a modified pFastBac vector containing an N-terminal *His6* tag and C-terminal *eGFP* and *StrepII* tags. Recombinant protein was expressed in Sf9 insect cells for 72 hours using a *Baculovirus* system. The protein was purified via a two-step protocol of Ni-affinity using a 1ml His-Trap column (GE Healthcare) in Ni-affinity buffer [50 mM Tris pH 7.5, 300 mM NaCl, 1mM Mg Cl$_2$, 10 % (v/v) glycerol] and elution with 200mM imidazole, followed by Step-tag affinity chromatography using StepTactin resin (GE Healthcare) in BRB20, 75mM KCl. 0.1% Tween 20, 10% (v/v) glycerol and elution with 5mM desthiobiotin. MTED peptide (Genscript) was shipped as lyophilised powder with a purity of 95.2%. Upon arrival peptide was dissolved in ultrapure water and used directly.

MT binding assays: GMPCPP-stabilised, rhodamine-labeled MTs were adhered to the surface of flow chambers (Helenius et al., 2006). 20 nM Efa6-ΔCterm::GFP (in BRB20 pH 6.9, 75mM KCl, 0.05% Tween20, 0.1 mg/ml BSA, 1% 2-mercaptoethanol, 40mM glucose, 40 mg/ml glucose oxidase, 16 mg/ml catalase) or 20 nM MCAK::GFP (in the same buffer plus 1 mM ATP and 1 mM taxol) was introduced to the MT-containing channel. Images were recorded using a Zeiss Observer.Z1 microscope equipped with a Zeiss Laser TIRF 3 module, QuantEM 512SC EMCDD camera (Photometrics) and 100x objective (Zeiss, alphaPlanApo/1.46NA oil). Images of rhodamine-labeled MTs using a lamp as the excitation source and GFP fluorescence using TIRF illumination via a 488 nm laser were collected as described (Patel et al., 2016). For both rhodamine and GFP imaging an exposure time of 100 ms was used. The mean GFP intensity on individual MTs was determined from the mean pixel intensity of lines drawn along the long-axis of individual microtubules in Fiji (Schindelin et al., 2012). The rhodamine signal was used to locate the position of MTs in the GFP images. Intensity from a region of background was subtracted.

MT depolymerisation assays: GMPCPP-stabilised, rhodamine-labelled MTs were adhered to the surface of flow chambers (Helenius et al., 2006). Images of a field of fluorescent

microtubules were recorded using a Zeiss Observer.Z1 microscope, collecting 1 image every 5 s with an exposure time of 100 ms. Efa6-ΔCterm::GFP (14 nM), MCAK (40 nM) in solution (BRB20 pH 6.9, 75mM KCl, 1mM ATP, 0.05% Tween 20, 0.1 mg/ml BSA, 1% 2-mercaptoethanol, 40mM glucose, 40 mg/ml glucose oxidase, 16 mg/ml catalase) were added to the channel 1 min after acquisition had commenced. Depolymerisation rates were determined from plots of the length of individual microtubules versus time, obtained by thresholding and particle analysis of images using Fiji (Schindelin et al., 2012).

***Xenopus* oocyte assays**: cytosol extracts from *Xenopus* oocytes were obtained as described in (Allan and Vale, 1991). **MT depolymerisation** was assessed in a microscopic flow chamber (Vale and Toyoshima, 1988) where *Xenopus* cytosol (1 µl cytosol diluted with 20 µl acetate buffer) was incubated for 20 min to allow MTs to polymerise. Then cytosol was exchanged by flow through with Efa6-ΔCterm::GFP, MCAK or synthetic MTED peptide (all 20 nM in acetate buffer pH 7.4: 100 mM K-Acetate, 3 mM Mg-Acetate, 5 mM EGTA, 10 mM HEPES), and MT length changes observed by recording 10 random fields via VE-DIC microscopy (Allan, 1993; Allan and Vale, 1991). **MT polymerisation** was analysed in a microscope flow cell containing 9 µl diluted *Xenopus* cytosol (see above) to which 1 µl acetate buffer was added, either alone or containing 20nM MTED. After 10 min, 20 random fields were recorded via VE-DIC microscopy for each condition and the numbers of MTs per field counted.

For the *in vivo* assay, *Xenopus* embryos were injected in one blastomere at the 4-cell stage with 200 ng of mRNA encoding Efa6-Nterm::GFP or mCherry alone. The embryos were imaged at stage 10.25 (Heasman, 2006) with a Leica fluorescent stereoscope.

Microtubule growth assays

30μM porcine brain tubulin (25% rhodamine-labelled) was incubated in 80mM PIPES pH6.9, 5mM $MgCl_2$, 1mM EGTA, 5% DMSO and 1mM GTP at 37°C for 30min in the presence of either no peptide, 30μM MTED peptide or 300μM MTED peptide. The reactions were then diluted 60-fold into BRB80 buffer (80mM PIPES pH6.9, 1mM $MgCl_2$, 1mM EGTA) containing 1mM taxol. Samples were added to channels constructed from poly-lysine coated cover glasses, washed with BRB80, 1mM taxol and imaged by fluorescence microscopy.

Tubulin pull-down assays

MTED peptide was coupled to cyanogen bromide-activated Sepharose beads (GE Healthcare). 30μM porcine brain tubulin was incubated with either peptide-coated or uncoated Sepharose beads in BRB80, 0.2% Tween 20 for 30mins at 20°C. The beads were washed three times with a 2:1 *v/v* ratio of BRB80, 0.2% Tween 20 to beads. An equal volume of 2x Laemmli buffer was added to the washed beads, incubated at 90°C for 5min, spun down and supernatant run on a 12% SDS-PAGE gel.

Results

Efa6 is widely expressed in *Drosophila* neurons and restricts axonal growth

To evaluate the function of Efa6 in neurons, we first determined its expression in the nervous system. We used a genomically engineered fly line in which the endogenous *Efa6* gene was GFP-tagged (*Efa6-GFP*; Huang et al., 2009). These animals widely express Efa6::GFP throughout the CNS at larval and adult stages (Fig. 1F-I). We cultured primary neurons from

391 this fly line to analyse the subcellular distribution of Efa6. In young neurons at 6 hrs *in vitro*
392 (6HIV) and in mature neurons at 5 days *in vitro* (5DIV), Efa6 was localised throughout cell
393 bodies and axons (Fig. 1B, E).

394 We next determined whether *Drosophila* Efa6 has an impact on axon growth, using fly lines
395 with decreased or abolished Efa6 expression: Efa6 knock-down (*Efa6-RNAi*), overlapping
396 deficiencies uncovering the entire *Efa6* gene locus (*Efa6^{Def}*), or different loss-of-function
397 mutant alleles generated by genomic engineering (*Efa6^{KO#1}*, *Efa6^{GX6[w-]}*, *Efa6^{GX6[w+]}*). In all
398 these conditions, axon length at 6 HIV was increased compared to wild-type by at least 20%
399 (Fig. 2D).

400 We then tested whether over-expression of Efa6 would cause the opposite effect, i.e. axon
401 shortening or even loss. For this, we generated a transgenic *UAS-Efa6-FL-GFP* line and, in
402 addition, developed methods to transfect *UAS*-constructs into *Drosophila* primary neurons
403 (see Methods). When expressed pan-neuronally transgenic or transfected full-length *Efa6-*
404 *FL::GFP* localised to cell bodies, axons and growth cones of primary neurons, as similarly
405 observed with the endogenous protein (Figs.1B,C, S3B). The transgenic expression caused a
406 ~20% reduction in axon length, which was increased to ~50% upon transfection (likely due to
407 higher copy numbers of the expression construct ;Fig. 2C, D). Furthermore, we observed an
408 increase in the number of neurons without axons from ~26% in *UAS-GFP*-transfected controls
409 to ~43% in Efa6-FL::GFP-positive neurons (Fig.3B).

410 Together, these results suggest that Efa6 restricts axonal growth, comparable to reports for *C.*
411 *elegans* Efa6 (*Ce*Efa6; Chen et al., 2015; Chen et al., 2011). The loss of whole axons upon
412 Efa6-FL::GFP over-expression might suggest that Efa6 performs its morphogenetic roles by
413 inhibiting MTs.

414

415 <u>Efa6 eliminates peripheral or even entire MT networks in mouse fibroblasts</u>

416 To assess whether the negative impact of Efa6 on axon outgrowth might be through inhibiting
417 MTs, we used NIH3T3 mouse fibroblasts as a heterologous cell system known to provide
418 meaningful readouts for functional studies of *Drosophila* MT regulators (Alves-Silva et al.,
419 2012; Beaven et al., 2015). When fibroblasts were analysed 24 hrs after transfection with
420 *Efa6-FL-GFP*, we found a graded depletion of MT networks depending on Efa6-FL::GFP
421 protein levels (shown and quantified in Fig.S5). At moderate expression levels, Efa6-FL::GFP
422 localised along the circumference and in areas of membrane folds (open arrow heads in
423 Figs.S5B), and MTs tended to be lost predominantly from the cell fringes (curved arrows in
424 Figs.S5B and S7B). At high expression levels, Efa6-FL::GFP became detectable in the
425 cytoplasm and even nucleus (double-chevrons in Fig.S5C), suggesting that membrane-
426 association might become saturated. In these cases, MTs were completely absent (Fig.S5C).
427 When quantifying these MT phenotypes across all transfected fibroblasts, there was a strong
428 increase in MT network defects and depletion upon Efa6-FL::GFP expression as compared to
429 GFP controls (Fig. 3B).

430 When performing live analyses, we consistently observed that growing MTs labelled with
431 EB3::mCherry extended to the very cell fringes of control fibroblasts (Suppl. Movie M1),
432 whereas MTs in fibroblasts transfected with Efa6-FL::GFP showed a very different behaviour:
433 hardly any MTs polymerised into areas along the rim where Efa6 was enriched but stopped at
434 the border, often accompanied by Efa6-FL::GFP accumulation at MT plus ends at the
435 invasion site (Suppl. Movie M2).

436   Taken together, also these data suggest that Efa6 inhibits MTs. The fibroblast experiments
437   suggest that Efa6 is membrane-associated and excludes MTs from this position, and the
438   studies in fly neurons indicate the relevance of such functions for neuronal morphogenesis.
439   The combined use of mouse fibroblasts and *Drosophila* primary neurons provides therefore a
440   robust system with informative readouts for MT loss - ideal to carry out a systematic structure-
441   function analysis of Efa6.

442

443   <u>The N-terminal 18aa motif of Efa6 is essential for microtubule-inhibiting activity of Efa6</u>

444   A detailed analysis of the domain structures of Efa6 proteins from 30 species revealed that C-
445   termini of almost all species contain a putative pleckstrin homology domain (PH; potentially
446   membrane-associating; Macia et al., 2008), a Sec7 domain (potentially activating Arf
447   GTPases; D'Souza-Schorey and Chavrier, 2006; Huang et al., 2009) and a coiled-coil (CC)
448   domain (Franco et al., 1999; Figs.3A, S2). In contrast, the N-termini are mainly unstructured
449   and reveal enormous length differences among species. Accordingly, phylogenetic
450   relationship analyses comparing either full-length or N-terminal Efa6, show that chordate
451   proteins are rather distant from invertebrates, and that arthropods form a clear subgroup
452   within the invertebrates (Fig. S1A,B). None of the identifiable N-terminal domains/motifs is
453   particularly well conserved (details in Fig.S2). For example, the *Drosophila* N-terminus
454   contains (1) a putative PDZ domain (aa16-88; mainly found in insect versions of Efa6), (2) two
455   SxIP motifs (aa 233-6 and 262-5; found primarily in Efa6 of flies, some other insects and
456   molluscs; some vertebrate/mammalian species display derived SxLP motifs), and (3) a motif
457   of 18aa displaying 89% similarity with a motif in the N-terminus of *Ce*Efa6 suggested to be
458   involved in MT inhibition (O'Rourke et al., 2010; conserved in nematodes, arthropods and
459   molluscs).

460   To assess potential roles of the *Drosophila* 18aa motif (from now on referred to as MT
461   elimination domain, MTED), we generated a series of GFP-tagged N-terminal constructs
462   (Fig.3B): *Efa6-ΔCterm-GFP* (encoding the entire N-terminal half upstream of the Sec7
463   domain), *Efa6-Nterm-GFP* (restricting to the N-terminal part containing all the identified
464   functional domains), *Efa6-Nterm^{ΔMTED}-GFP* (lacking the MTED) and *Efa6-MTED-GFP*
465   (encoding only the MTED). All these N-terminal Efa6 variants showed the same localisation
466   pattern throughout neurons (Fig.S3C,D,F,G), and in the cytoplasm and nucleus of fibroblasts
467   (Fig.S4C,D,F,G). Cytoplasmic and nuclear localisations occurred even at low expression
468   levels, indicating that the absent C-terminus (and likely PH domain within) usually mediates
469   membrane association. This nuclear localisation occurs in the absence of any predicted N-
470   terminal nuclear localisation sequences (Figs.3A, S2A), likely reflecting a known artefact of
471   GFP-tagged proteins (Alves-Silva et al., 2012; Seibel et al., 2007).

472   In spite of their very similar localisation patterns, the functional impact of these constructs was
473   clearly MTED-dependent: only constructs containing the MTED (Efa6-ΔCterm::GFP, Efa6-
474   Nterm::GFP and Efa6-MTED::GFP) caused strong axon loss in neurons and MT network
475   depletion in fibroblasts, whereas Efa6-Nterm^{ΔMTED}::GFP behaved like GFP controls (Figs.3B;
476   S3C,D,F,G; S7C,D,G,H).

477   In addition, we assessed potential roles of the two SxIP sites predicted to bind EB proteins
478   (Honnappa et al., 2009; Fig.3A). Accordingly, we found that Efa6-Nterm::GFP tip-tracks and
479   that Efa6-FL::GFP accumulates at sites where EB3-enriched MTs get in contact (Suppl. Mov.
480   M2 and 3). Such binding to EBs at MT plus ends, might enhance Efa6's ability to capture MTs
481   for inhibition. However, when replacing each of the two SxIP motifs by four alanines (Efa6-

482 Nterm$^{\Delta SxIP}$::GFP), the construct still induced a strong axon loss and MT network depletion in
483 fibroblasts (Figs.3B, S3E, S4E, S7F). Similar observations were reported for Kif2C, which
484 clearly tip-tracks MTs through binding EB1, but does not require this property for its MT
485 depolymerising activity (Moore et al., 2005).

486 Taken together, our results pinpoint the MTED as the key mediator of MT-depleting functions
487 of *Drosophila* Efa6, suggesting this function to be conserved between flies and *C. elegans*.

488

489 <u>The MTED is a good predictor of MT-inhibiting function directly affecting MT polymerisation</u>

490 To assess whether the MTED motif is a good predictor for MT-inhibiting capabilities of Efa6
491 family members, we used 12 different constructs comprising: full length versions of (1)
492 *Ce*Efa6, (2) *Drosophila* Efa6 and (3-6) all four human PSDs (Fig. 3C), as well as N-terminal
493 versions of (7) *Ce*Efa6, (8) fly Efa6 and (9) human PSD1 (Fig. 3D). Furthermore, we deduced
494 a MTED consensus sequence from 39 *Efa6* genes (details in Fig. S2C), identified the most
495 likely human MTED-like sequence (position 31-49aa of PSD1; MTED-core in Fig. 3A) and
496 synthesised codon-optimised versions of (10) this human as well as the (11) fly and (12) worm
497 MTEDs (Figs. S2B,C). When transfected into fibroblasts, we found that all 6 fly/worm
498 constructs had strong MT-inhibiting properties, whereas none of the 6 human constructs
499 (PSD1-4 full length, PSD1-Nterm, PSD1-MTED-like) showed MT collapse (Fig.3C-E).
500 Therefore, the presence of a well conserved canonical MTED seems to be a good predictor
501 for MT-inhibiting capabilities of Efa6 proteins.

502 To gain insights into the mechanisms through which MTEDs might act, we carried out a series
503 of *in vitro* experiments. Purified Efa6-Nterm::GFP clearly associated with MTs *in vitro* (Fig.
504 S8A), but failed to reconstitute any MT-inhibiting activity (Fig. S8B). We therefore tested the
505 same protein in *Xenopus* oocyte extract to assess potential co-factor requirements, but saw
506 again no activity (Fig. S8C) - in spite of the fact that injection of a corresponding mRNA into
507 *Xenopus* oocytes caused strong cell division phenotypes (Fig. S8D,E). We suspected
508 problems with recombinant expression of the protein, which is predicted to have large
509 disordered regions, and instead used synthetic MTED peptide. We found that MTED-coated
510 sepharose beads pulled down unpolymerised tubulin (Fig.4B), indicating a direct interaction of
511 the peptide with tubulin. Furthermore, addition of synthetic MTED peptide to a MT growth
512 assay, resulted in strong suppression of MT polymerisation in a dose-dependent manner
513 (Fig.4A).

514 Taken together, the MTED exists primarily in Efa6 homologues of invertebrate species and its
515 presence correlates with MT-inhibiting properties of these proteins. This conclusion is strongly
516 supported by our finding that *Drosophila* MTED directly interferes with MT polymerisation,
517 which can explain why MTs fail to enter Efa6-enriched areas in fibroblasts (Suppl. Mov. M2).

518

519 <u>The C-terminal domain restricts the microtubule-inhibiting activity of Efa6 to the cortex</u>

520 Our structure-function analyses strongly suggested that Efa6 is membrane-associated. This is
521 further supported by a membrane ruffle phenotype we observed in fibroblasts when
522 expressing Efa6-FL::GFP or the C-terminal derivative Efa6-ΔNterm::GFP (Figs.3B; curved
523 open arrows in Figs.S4B,H and S6B,D). Efa6-ΔNterm::GFP had no obvious effects on MT
524 networks (Fig. S7I), and its membrane ruffling phenotype likely reflects an evolutionarily
525 conserved function of the Efa6 C-terminus through its Sec7, PH and/or CC domains (Derrien
526 et al., 2002; Franco et al., 1999; Macia et al., 2008). Accordingly, we find the same membrane

527 ruffling when expressing PSD1-FL::GFP (curved open arrows in Fig. S6E).

528 However, even if the C-terminus plays no active role in the MT inhibition process, it still
529 regulates this function. This is suggested by the Efa6-ΔCterm::GFP and Efa6-Nterm::GFP
530 variants which lack the C-terminus (Fig.3B), fail to associate with the cortex (Fig.S4C,D,F,G),
531 do not cause ruffling (Fig.S6C), but induce MT phenotypes far stronger than Efa6-FL::GFP
532 does (Figs.3B; S7C,D,H *vs* B). To assess whether lack of membrane tethering could explain
533 this phenotypic difference, we generated the Efa6-Nterm::GFP::CAAX variant (Fig.3B) where
534 Efa6-Nterm::GFP is fused to the membrane-associating CAAX domain (Hancock et al., 1991);
535 this addition of CAAX changed the properties of Efa6-Nterm::GFP back to Efa6-FL::GFP-like
536 behaviours (Figs.3B; S4B,I; S7B,E): the hybrid protein localised to the cortex in fibroblasts
537 and had only a moderate MT phenotype, and also the axon loss phenotype was mild. Also in
538 live analyses, the CAAX construct reproduced the effect of excluding MTs from Efa6-N-
539 term::GFP::CAAX-enriched areas (Suppl. Mov. M4). These findings confirm membrane
540 tethering as an important regulatory feature restricting Efa6 function.

541 Taken together, our structure-function data clearly establish *Drosophila* Efa6 as a cortical
542 collapse factor: its N-terminal MTED blocks polymerisation which of MTs at the cortex via the
543 Efa6 C-terminus.

544

545 <u>Efa6 negatively regulates MT polymerisation at the growth cone membrane and in filopodia</u>

546 We next asked how Efa6's cortical collapse function relates to the observed axon growth
547 phenotypes. For this, we focussed on growth cones (GCs) as the sites where axons extend;
548 this extension requires the splaying of MTs from the axonal bundle tip at the base of GCs to
549 explore the actin-rich periphery (Dent et al., 2011; Lowery and van Vactor, 2009; Prokop et al.,
550 2013).

551 In GCs of primary neurons at 6 HIV, loss of Efa6 caused an increase in MT polymerisation
552 events: the total number of Eb1 comets was increased as compared to wild-type controls (Fig.
553 5I). Eb1::GFP comets in *Efa6* mutant neurons frequently persisted when reaching the GC
554 periphery, where they could occasionally be observed to undergo curved extensions along the
555 periphery (Suppl. Mov. M7and SM8). Comet velocity was unaffected (~0.3 μm/s), but the
556 lifetime of Eb1::GFP comets was ~1.4 times longer in mutant GCs, with the dwell time of Eb1
557 comets at the tip of filopodia being increased from 2.10s +/- 0.24 s in wild-type to 6.26s +/-
558 0.40 s in *Efa6* mutant neurons (Fig. 5M); we even observed cases where comets at the tips of
559 filopodia were moving backwards, seemingly pushed back by the retracting filopodial tip
560 (Suppl. Mov. M7 and M8). In agreement with the increased lifetime, more microtubules
561 invaded growth cone filopodia in *Efa6* mutant neurons, as quantified by counting filopodia that
562 contained EB1comets or MTs (Fig.5D,E,J,K; note that the total number of filopodia per GC
563 was in the range of 10-11 for both wild-type and *Efa6*; not shown). Transgenically expressed
564 Efa6-FL::GFP caused the opposite effect, i.e. a reduction in the number of GC filopodia
565 containing Eb1 comets or MTs (Fig. 5G-L; green columns).

566 Next, we investigated MT dynamics in axon shafts. In contrast to GCs, MTs in the axon shaft
567 are organised into bundles, hence kept away from the membrane. Accordingly, neither loss-
568 nor gain-of-function had an obvious effect on Eb1 comet numbers, lifetimes, velocities and
569 directionalities (Fig. 6A-D). However, like in GCs, there was a strong increase in filopodia
570 along the shaft that contained MTs when Efa6 was absent, and a strong decrease when over-
571 expressing Efa6-FL::GFP (Fig. 6E-G).

572 Taken together, our data are consistent with a model in which Efa6 primarily inhibits
573 explorative MTs that leave the axon bundle in either GCs or axon shafts and polymerise
574 towards the cell membrane or into filopodia. Surplus MTs in the periphery of GCs can explain
575 the extra axonal growth we observed (Fig.2).

576

577 <u>Efa6 negatively influences axon branching</u>

578 We hypothesised that an increase in explorative MTs could also cause a rise in axon
579 branching (see Introduction), either by inducing GC splitting through parallel growth events in
580 the same GC (Acebes and Ferrus, 2000), or by seeding new collateral branches along the
581 axon shaft (Kalil and Dent, 2014; Lewis et al., 2013). To test this possibility, we studied mature
582 primary neurons at 5 days *in vitro* (DIV). We found that *Efa6^KO#1* homozygous mutant neurons
583 showed almost double the number of collateral branches as observed in wild-type neurons,
584 whereas expression of Efa6-FL::GFP reduced branching by 21% (Fig.7A-C,E). This reduction
585 is mediated by the Efa6 N-terminus, since expression of Efa6-Nterm::GFP::CAAX caused a
586 similar degree in branch reduction (Fig.7D,E).

587 To extend these studies to neurons *in vivo*, we studied dorsal cluster neurons, a subset of
588 neurons with stereotypic axonal projections in the optic lobe of adult brains (Fig.7F-K, see
589 Methods; Hassan et al., 2000; Voelzmann et al., 2016b). To manipulate Efa6 levels in these
590 neurons, either *Efa6-RNAi* or *Efa6-FL-GFP* was co-expressed with the membrane marker
591 *myr-tdTomato*. Their axon branches were assessed in brains of young and old flies (2-5 d and
592 15-18 d after eclosure from the pupal case, respectively; Figs.7F-K). We found that *Efa6*
593 knock-down in dorsal cluster neurons caused a significant increase in branch numbers by 29%
594 in young and by 38% in old brains, whereas over-expression of Efa6::GFP strongly decreased
595 branch numbers by 33% in young and 28% in old brains, respectively (Fig.7L).

596 In these experiments, Efa6-FL::GFP expression had an intriguing further effect: Only 57% of
597 young brains had any axons in the medulla region, compared to 88% in controls (Figs.7H,M,
598 S9B). However, in the older Efa6-FL::GFP expressing fly brains, the axons were eventually
599 present (Fig.7K,M, S9D). We concluded that this phenotype reflected delayed outgrowth,
600 which is also consistent with the decrease in axon growth observed upon Efa6 over-
601 expression in primary neurons (green bars in Fig.2D).

602 Taken together, our data indicate a physiologically relevant role of Efa6 as negative regulator
603 of axonal branching, mediated through its N-terminus, most likely via its function as cortical
604 collapse factor.

605

606 *Efa6* maintains axonal MT bundle integrity in cultured neurons

607 Apart from changes in growth and branching, we noticed that a significant amount of Efa6-
608 depleted neurons displayed axons with swellings where MTs lost their bundled conformation
609 and were arranged into intertwined, criss-crossing curls instead (Fig.S10; arrowheads in Fig.
610 8D-F). To quantify the strength of this phenotype, we measured the area of MT
611 disorganisation relative to axon length (referred to as 'MT disorganisation index', MDI; Qu et
612 al., 2017). MDI measurements in *Efa6* mutant neurons revealed a mild 1.3 fold increase in MT
613 disorganisation in young neurons which gradually worsened to 2.3 fold at 5 DIV and ~4 fold at
614 10 DIV (Fig.8A-F,I; all normalised to controls).

615 The observed gradual increase in phenotype could be the result of a genuine function of *Efa6*
616 not only during axon growth but also their subsequent maintenance. Alternatively, it could be

617 caused by maternal gene product deposited in the mutant embryos by their heterozygous
618 mothers (Prokop, 2013b); such maternal *Efa6* could mask mutant phenotypes at early stages
619 so that they become apparent only after most Efa6 has degraded. To assess the latter
620 possibility, we used a pre-culture strategy to remove potential maternal Efa6 (see Methods;
621 Prokop et al., 2012; Sánchez-Soriano et al., 2010). When plating neurons after 5 days of pre-
622 culture, we still found a low amount of MT disorganisation in young neurons and a subsequent
623 gradual increase to severe phenotypes over the following days (Fig.8J).

624 This finding argues for a continued role of Efa6 in preventing MT disorganisation during
625 development as well as in mature neurons. To further test this possibility, we used a
626 temperature-based conditional knock-down technique (*elav-GAL4 UAS-Efa6-RNAi UAS-*
627 *Gal80^{ts}* abbreviated to *elav/Efa6^{IR}/Gal80^{ts}*; see Methods): the *elav/Efa6^{IR}/Gal80^{ts}* neurons
628 were grown without knock-down (19°C) for 3 days, a stage at which they have long undergone
629 synaptic differentiation (Küppers-Munther et al., 2004; Prokop et al., 2012); at that point, we
630 found no difference in MT disorganisation between non-induced construct-bearing cells and
631 control neurons (Fig. 8K). After this period, cells were grown for another four days under
632 knock-down conditions (27°C), and then fixed on day seven. At this point, MT disorganisation
633 in the *elav/Efa6^{IR}/Gal80^{ts}* neurons was significantly increased over control neurons (Fig. 8K),
634 indicating that Efa6 is not only required during development but also during later maintenance
635 to prevent MT disorganisation.

636 In contrast to increased MT disorganisation upon functional loss of Efa6, expression of Efa6-
637 FL::GFP or Efa6-Nterm::GFP::CAAX showed a tendency to reduce MT disorganisation even
638 below the baseline levels measured in control cells (cultured in parallel without the expression
639 construct; Fig.8I), arguing that also this role of Efa6 is likely due to the cortical collapse
640 function of Efa6 (see Discussion).

641

642 *Efa6* maintains axonal MT bundle integrity *in vivo*

643 We then assessed whether a role of Efa6 in MT bundle maintenance is relevant *in vivo*. For
644 this, we studied a subset of lamina neurons, which project prominent axons in the medulla of
645 the adult optic lobe (Prokop and Meinertzhagen, 2006). We labelled MTs in these axons by
646 expressing α-*tubulin84B-GFP* either alone (*GMR-tub* controls), or together with *Efa6^{RNAi}* to
647 knock down *Efa6* specifically in these neurons (*GMR-tub-Efa6^{IR}*; see Methods for details).

648 When analysing aged flies at 26-27 days, we found that *Efa6* knock-down caused a doubling
649 in the occurrence of axonal swellings with disorganised axonal MTs: the average of total
650 swellings per column section was increased from 0.3 in controls to 0.65 swellings upon Efa6
651 knock-down; about a third of these contained disorganised MTs (*GMR-tub-Efa6^{IR}*: 0.23 per
652 column section; *GMR-tub*: 0.13; Fig.9). These data demonstrated that our findings in cultured
653 neurons are relevant *in vivo*.

654 We propose therefore that Efa6 provides a quality control mechanism that prevents MT
655 disorganisation by inhibiting only MTs that have escaped axonal bundles. This model would
656 also be consistent with the slow onset and gradual increase of MT disorganisation we
657 observed upon Efa6 deficiency (Fig.8I,J).

658

659 Efa6 and Shot promote MT bundles through complementary mechanisms

660 If Efa6 provides a quality control mechanism that "cleans up" explorative MTs, it should act

661 complementary to other factors that "prevent" explorative MTs by actively keeping them in
662 axonal bundles. Very powerful preventive factors in both mammals and fly are the
663 spectraplakins (Bernier and Kothary, 1998; Dalpe et al., 1998; Voelzmann et al., 2017). In
664 *Drosophila*, spectraplakins are represented by the single *short stop* (*shot*) gene; *shot*
665 deficiency causes a severe increase in axonal off-track MTs and MT disorganisation (Alves-
666 Silva et al., 2012; Qu et al., 2017; Sánchez-Soriano et al., 2009).

667 To study potential mutual enhancement of *Efa6* and *shot* mutant phenotypes, we first
668 determined numbers of MTs in axonal shaft filopodia: both single-mutant conditions showed a
669 strong enhancement of filopodial MTs (blue *vs.* orange bars in Fig.6F,G); this phenotype was
670 substantially further increased in *shot³ Efa6^{GX6[w-]}* double-mutant neurons (orange/blue bars in
671 Fig. 6F,G). *Vice versa*, when transfecting *Efa6-FL-GFP* to boost the hypothesised "cleaning-
672 up" function, the *shot³* mutant phenotype was significantly improved (Fig. 6G).

673 We then tested whether this increase in off-track MTs would correlate with more MT
674 disorganisation. At 6 HIV, *shot³* mutant neurons displayed a 2.4-fold, and *Efa6^{GX6[w-]}* mutant
675 neurons a 1.55-fold increase in MDI (normalised to wild-type); this value was dramatically
676 increased to 6.16 fold in *shot³ Efa6^{GX6[w-]}* double mutant neurons (Fig. 8M). This strongly
677 suggests that Efa6 and Shot do not act through the same mechanism, but perform
678 complementary roles in regulating and maintaining axonal MTs and MT bundles. This
679 conclusion was further confirmed by our finding that transfection of *Efa6-FL-GFP* into *shot³*
680 mutant neurons could alleviate the MDI phenotype (Fig. 8N).

681 Finally, we assessed whether these complementary relationships between Shot and Efa6 are
682 relevant *in vivo*. Since complete loss of Shot is an embryonically lethal condition, we first
683 tested this in culture whether the lack of just one copy of *shot* has an enhancing effect on Efa6
684 deficiency. We found that MT disorganisation phenotypes of *Efa6-RNAi* (blue bar in Fig.8L)
685 and of *shot^{3/+}* heterozygous mutant neurons (orange bar) at 6 HIV were clearly enhanced
686 when both genetic manipulations were combined (orange/blue bar). When testing the same
687 genetic constellations in our optic lobe model, we found that the originally observed increase
688 in MT disorganisation caused by cell-autonomous knock-down of *Efa6* (black arrows and blue
689 bar in Fig.9B,E) was also further enhanced when the same experiment was carried out in a
690 *shot^{3/+}* heterozygous mutant background (black arrows and orange/blue bar in Fig.9C,E).

691 These findings support our conclusion that there is a correlation between off-track MTs and
692 MT disorganisation. Furthermore, they are consistent with a scenario where both Shot and
693 Efa6 regulate axonal MTs but through independent and complementary pathways: Efa6
694 inhibits MTs at the cortex (with peripheral MTs persisting for longer if Efa6 is absent), whereas
695 Shot actively maintains MTs in bundles (with more MTs going off-track if Shot is absent) - and
696 both these functions complement each other during MT bundle maintenance (see further
697 details in the Discussion).

698

699 **Discussion**

700 Cortical collapse factors are important microtubule regulators relevant for axon morphology

701 Axons are the structures that wire our brain and body and are therefore fundamental to
702 nervous system function. To understand how axons are formed during development, can be
703 maintained in a plastic state thereafter, and why they deteriorate in pathological conditions, we
704 need to improve our knowledge of axonal cell biology (Hahn et al., 2019). The MT bundles
705 that form the core of axons are an essential aspect of this cell biology, and understanding how

706 these bundles are regulated and contribute to axon morphogenesis will provide essential
707 insights into axon development and maintenance (Voelzmann et al., 2016a). Here we have
708 addressed fundamental contributions made by cortical collapse factors. We started from
709 reports that two such factors from distinct protein families both negatively impact on axon
710 growth in species as diverse as *C. elegans* (*Ce*Efa6; Chen et al., 2015; Chen et al., 2011) and
711 mouse (Kif21A; van der Vaart et al., 2013).

712 We found that *Dm*Efa6 likewise acts as a negative regulator of axon growth. We demonstrate
713 that fly Efa6 is a cortical collapse factor, inhibiting MTs primarily via the 18 aa long MTED.
714 Since the MTED is the only shared motif with *Ce*Efa6 in an otherwise entirely divergent N-
715 terminus (Fig. 3C), this clearly demonstrates that the MTED is functionally conserved between
716 both species (Chen et al., 2015; Chen et al., 2011; O'Rourke et al., 2010).

717 Capitalising on *Drosophila* neurons as a conceptually well-established model (Prokop et al.,
718 2013; Voelzmann et al., 2016a), we went on to demonstrate two novel roles for Efa6: as a
719 negative regulator of axon branching and a quality control factor maintaining MT bundle
720 organisation. To perform these functions, Efa6 does not affect the dynamics of MTs contained
721 within the central axonal bundles, but it inhibits mainly those MTs that leave these bundles
722 (Fig.10A). By inhibiting explorative MTs in GCs, it negatively impacts on a key event
723 underlying axon growth (explained below; yellow arrows in Fig.10C). By inhibiting off-track
724 MTs in the axon shaft, it tones down the machinery that seeds new interstitial branches (red
725 arrow in Fig.10C), but also prevents these MTs from going astray and cause MT
726 disorganisation (curled MTs in Fig.10C).

727 Therefore, our work provides conceptual understanding of cortical collapse factors, which can
728 explain how their molecular functions and subcellular roles in MT regulation link to their
729 reported axonal growth phenotypes during development and regeneration (Chen et al., 2015;
730 Chen et al., 2011; Heidary et al., 2008; van der Vaart et al., 2013), and to their additional
731 functions in axon branching and maintenance reported here. Apart from existing links of
732 cortical collapse factors to neurodevelopmental disorders (Heidary et al., 2008; Tiab et al.,
733 2004; van der Vaart et al., 2013), we would therefore predict future links also to
734 neurodegeneration.

735

736 <u>Roles of Efa6 during axonal growth</u>

737 During axon growth, MTs constantly polymerise towards the periphery of GCs; the advance of
738 many of these MTs is inhibited at the leading edge, and our work shows that cortical collapse
739 factors are key mediators to this end. Only a fraction of MTs enters filopodia, potentially
740 helped by active guidance mechanisms such as MT-actin cross-linkage (e.g. through
741 spectraplakins, tau, drebrin-EB3 ;Alves-Silva et al., 2012; Biswas and Kalil, 2018; Geraldo et
742 al., 2008). The widely accepted protrusion-engorgement-consolidation model of axon growth
743 proposes that stabilised MTs in filopodia can seed axon elongation events (Aletta and Greene,
744 1988; Goldberg and Burmeister, 1986; Prokop et al., 2013). This model is consistent with our
745 findings for Efa6. Thus loss of Efa6 can contribute to enhanced axon growth in two ways:
746 firstly, through allowing more MTs to enter filopodia; secondly, by allowing them to dwell in
747 filopodia for longer, thus enhancing the likelihood of their stabilisation (yellow arrows in
748 Fig.10C). This scenario can explain why loss of *Efa6* in *C. elegans* improves axon re-growth
749 after injury and growth overshoot during development (Chen et al., 2015; Chen et al., 2011),
750 and why the upregulation of Kif21A levels in GCs causes stalled axon growth (van der Vaart
751 et al., 2013).

752

Roles of Efa6 during axonal branching

754 As explained in the introduction, axon branching can occur via GC split, in that diverging MTs
755 get stabilised in parallel in the same GC (Acebes and Ferrus, 2000; yellow arrows in Fig.10C).
756 Alternatively, it can occur through interstitial branching which involves the active generation
757 (e.g. through MT severing) and then stabilisation of off-track MTs (Kalil and Dent, 2014; Lewis
758 et al., 2013; Tymanskyj et al., 2017; Yu et al., 2008). Both models agree with our observations
759 in Efa6-deficient/over-expressing neurons: we find greater/lower numbers of MTs in GC and
760 shaft filopodia at 6 HIV, which then correlate with enhanced/reduced axonal branch numbers
761 in mature neurons (red arrow in Fig.10C).

762 If interstitial branch formation is negatively regulated by *Efa6,* this poses the question as to
763 whether Efa6 has to be actively down-regulated in healthy neurons for branching to occur;
764 Efa6 could either be physically removed from future branch points (Chen et al., 2015) or its
765 MT inhibition function could be switched off. We believe that no such regulation is required
766 because Efa6 seems to be in a well-balanced equilibrium. Enough Efa6 appears to be present
767 to inhibit occasional, likely accidental off-track MTs; this capacity is surpassed when the
768 number of off-track MTs is actively increased, for example through MT severing proteins
769 during axonal branch formation (Yu et al., 2008). Such a saturation model is supported by our
770 experiments with *shot* (Fig.6F,G): filopodial MT numbers are elevated in *shot* mutant neurons,
771 although Efa6 is present and functional (as demonstrated by the further increase in filopodial
772 MT numbers in *shot Efa6* double-mutant neurons; Fig.10D,E). This strongly suggests that
773 Efa6 function occurs at a level that is easily saturated when increasing the number of
774 explorative MTs.

775

Roles of Efa6 during axonal MT bundle maintenance

777 Axonal MT disorganisation in Efa6-deficient neurons occurs gradually and can even be
778 induced by knock-down of Efa6 at mature stages (Fig.8K). Therefore, Efa6 appears to prevent
779 MT disorganisation during axon development and maintenance, as is consistent with its
780 continued expression in the nervous system (Fig.1). Such a continued role makes sense in a
781 scenario where MT bundles remain highly dynamic throughout a neuron's lifetime, constantly
782 undergoing polymerisation to drive renewal processes that prevent senescence (Hahn et al.,
783 2019; Voelzmann et al., 2016a).

784 Based on these findings, we propose Efa6 to act as a quality control or maintenance factor
785 within our model of "local axon homeostasis" (Hahn et al., 2019; Voelzmann et al., 2016a).
786 This model states that MTs in the force-enriched environment of axons have a tendency to go
787 off-track and curl up (Pearce et al., 2018), thus potentially seeding MT disorganisation.
788 Different classes of MT-binding regulators, amongst them spectraplakins, prevent this by
789 actively promoting the bundled conformation (Voelzmann et al., 2017). We propose that
790 cortical collapse factors act complementary in that they do not actively maintain MTs in
791 bundles, but inhibit those MTs that have escaped the bundling mechanisms (Hahn et al.,
792 2019).

793 In this scenario, MTs are protected from cortical collapse as long as they are actively
794 maintained in axonal bundles; this can explain the long known conundrum of how axonal MTs
795 extend hundreds of micrometres in relative proximity to the cell cortex in axons, whereas in
796 non-neuronal cells cortical proximity of MTs tends to trigger either their inhibition or tethered

797   stabilisation (Fukata et al., 2002; Kaverina et al., 1998).

798

Evolutionary and mechanistic considerations of Efa6 function

800   We found that the MTED motif correlates well with MT inhibiting functions of Efa6 family
801   members, whereas the rest of the N-terminus bears no obvious further reminiscence. Our
802   experiments with N-terminal protein and synthetic MTED peptide, both reveal association with
803   MTs/tubulin. The MTED strongly interferes with MT polymerisation. Future co-crystallisation
804   experiments are required to reveal how the MTED works. Given its small size we hypothesise
805   that it simply blocks assembly, rather than acting via more complex mechanisms such as
806   active promotion of depolymerisation (e.g. kinesin-8 and -13, XMap215; Al-Bassam and
807   Chang, 2011; Brouhard and Rice, 2014) or severing (e.g. spastin, katanin, fidgetin; McNally
808   and Roll-Mecak, 2018; Sharp and Ross, 2012).

809   In any case, the small size of MTEDs might come in handy as experimental tools to inhibit
810   MTs, potentially displaying complementary properties to existing genetic tools such as the
811   kinesin-13 Kif2C (Moore et al., 2005; Schimizzi et al., 2010), stathmin (Marklund et al., 1996)
812   or spastin (Eckert et al., 2012). Importantly, the experiments with the CAAX domain have
813   shown that Efa6's MT inhibiting function can be targeted to specific subcellular compartments
814   clearing them of MTs, thus opening up a wide range of future applications.

815   Interestingly, the MT-inhibiting role of Efa6 seems not to be conserved in chordates (Fig.2A).
816   However, roles of cortical collapse factors in neurons seem to have been taken over by other
817   proteins such as the kinesin-4 family member Kif21A. The CFEOM1-linked Kif21A^{R954W}
818   mutation causes the protein to relocate from the axon shaft to the growth cone of cultured
819   hippocampal neurons (van der Vaart et al., 2013). In consequence, increased Kif21A levels in
820   GCs cause reduced axon growth - and we observed the same with Efa6 over-expression
821   (green bars in Fig.2D). The decreased levels of Kif21A in proximal axons correlate with a local
822   increase in side branches - and we observed the same with Efa6 loss of function (blue bars in
823   Fig.7E, L).

824   Finally, we found that the C-terminal domains of Efa6 might display some degree of functional
825   conservation. So far, work on mammalian PSDs has revealed functions for C-terminal
826   domains in regulating ARF6, ARF1 or ARL14 during actin cytoskeletal reorganisation and
827   membrane ruffling, tumour formation and immune regulation (Derrien et al., 2002; Paul et al.,
828   2011; Pils et al., 2005). Our finding that PSD1 and C-terminal Efa6 constructs cause similar
829   membrane ruffling phenotypes in fibroblasts (Figs.S4 and S6), suggests that some conserved
830   functions reside in this region and might further contribute, together with N-terminally
831   mediated MT inhibition, to the neuronal or non-neuronal defects that cause semi-lethality
832   displayed by *Efa6* mutant flies (data not shown).

833

Conclusions and future perspectives

835   We propose that Efa6 acts as a cortical collapse factor which is important for the regulation of
836   axonal MTs and relevant for axon growth, maintenance and branching. Although this function
837   of Efa6 is evolutionarily not widely conserved, our findings provide a helpful paradigm for
838   studies of other classes of cortical collapse factors also in mammalian neurons. Promising
839   research avenues will be to refine our mechanistic understanding of how Efa6 blocks MT
840   polymerisation, not only to better understand how it can be regulated in axons, but also to
841   better exploit MTEDs as molecular tools in cell biological research.

842

Acknowledgements

856

References

858

Abascal, F., Zardoya, R., and Posada, D. (2005). ProtTest: selection of best-fit models of protein evolution. Bioinformatics *21*, 2104-2105.

Acebes, A., and Ferrus, A. (2000). Cellular and molecular features of axon collaterals and dendrites. Trends Neurosci *23*, 557-565.

Adalbert, R., and Coleman, M.P. (2012). Axon pathology in age-related neurodegenerative disorders. Neuropathol Appl Neurobiol *39*, 90–108.

Al-Bassam, J., and Chang, F. (2011). Regulation of microtubule dynamics by TOG-domain proteins XMAP215/Dis1 and CLASP. Trends Cell Biol *21*, 604-614.

Aletta, J.M., and Greene, L.A. (1988). Growth cone configuration and advance: a time-lapse study using video-enhanced differential interference contrast microscopy. J Neurosci *8*, 1425-1435.

Allan, V.J. (1993). Assay of membrane motility in interphase and metaphase Xenopus extracts. Methods Cell Biol *39*, 203-226.

Allan, V.J., and Vale, R.D. (1991). Cell cycle control of microtubule-based membrane transport and tubule formation in vitro. J Cell Biol *113*, 347-359.

Alves-Silva, J., Sánchez-Soriano, N., Beaven, R., Klein, M., Parkin, J., Millard, T., Bellen, H., Venken, K.J.T., Ballestrem, C., Kammerer, R.A.*, et al.* (2012). Spectraplakins promote microtubule-mediated axonal growth by functioning as structural microtubule-associated proteins and EB1-dependent +TIPs (Tip Interacting Proteins). J Neurosci *32*, 9143-9158.

Beaven, R., Dzhindzhev, N.S., Qu, Y., Hahn, I., Dajas-Bailador, F., Ohkura, H., and Prokop, A. (2015). *Drosophila* CLIP-190 and mammalian CLIP-170 display reduced microtubule plus end association in the nervous system. Mol Biol Cell *26*, 1491-1508.

Bernier, G., and Kothary, R. (1998). Prenatal onset of axonopathy in Dystonia musculorum mice. Dev Genet *22*, 160-168.

Bettencourt da Cruz, A., Schwarzel, M., Schulze, S., Niyyati, M., Heisenberg, M., and Kretzschmar, D. (2005). Disruption of the MAP1B-related protein FUTSCH leads to changes in the neuronal cytoskeleton, axonal transport defects, and progressive neurodegeneration in *Drosophila*. Mol Biol Cell *16*, 2433-2442.

Biswas, S., and Kalil, K. (2018). The microtubule-associated protein tau mediates the organization of microtubules and their dynamic exploration of actin-rich lamellipodia and filopodia of cortical growth cones. J Neurosci *38*, 291-307.

Brouhard, G.J., and Rice, L.M. (2014). The contribution of aß-tubulin curvature to microtubule dynamics. J Cell Biol *207*, 323-334.

Calkins, D.J. (2013). Age-Related Changes in the Visual Pathways: Blame It on the AxonAge-Related Changes in the Visual Pathways. Invest Ophthalmol Vis Sci *54*, ORSF 37-41.

Chen, L., Chuang, M., Koorman, T., Boxem, M., Jin, Y., and Chisholm, A.D. (2015). Axon injury triggers EFA-6 mediated destabilization of axonal microtubules via TACC and doublecortin like kinase. eLife *4*, e08695.

Chen, L., Wang, Z., Ghosh-Roy, A., Hubert, T., Yan, D., O'Rourke, S., Bowerman, B., Wu, Z., Jin, Y., and Chisholm, A.D. (2011). Axon regeneration pathways identified by systematic genetic screening in *C. elegans*. Neuron *71*, 1043-1057.

Conde, C., and Caceres, A. (2009). Microtubule assembly, organization and dynamics in axons and dendrites. Nat Rev Neurosci *10*, 319-332.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004). WebLogo: a sequence logo generator. Genome Res *14*, 1188-1190.

D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. Nature Rev Molec Cell Biol *7*, 347.

Dalpe, G., Leclerc, N., Vallee, A., Messer, A., Mathieu, M., De Repentigny, Y., and Kothary, R. (1998). Dystonin is essential for maintaining neuronal cytoskeleton organization. Mol Cell Neurosci *10*, 243-257.

Darriba, D., Taboada, G.L., Doallo, R., and Posada, D. (2011). ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics *27*, 1164-1165.

Debanne, D., Campanac, E., Bialowas, A., Carlier, E., and Alcaraz, G. (2011). Axon physiology. Physiol Rev *91*, 555-602.

Dent, E.W., Gupton, S.L., and Gertler, F.B. (2011). The growth cone cytoskeleton in axon outgrowth and guidance. Cold Spring Harb Perspect Biol *3*, a001800.

Derrien, V., Couillault, C., Franco, M., Martineau, S., Montcourrier, P., Houlgatte, R., and Chavrier, P. (2002). A conserved C-terminal domain of EFA6-family ARF6-guanine nucleotide exchange factors induces lengthening of microvilli-like membrane protrusions. Journal of cell science *115*, 2867-2879.

Eckert, T., Le, D.T.-V., Link, S., Friedmann, L., and Woehlke, G. (2012). Spastin's microtubule-binding properties and comparison to katanin. PLoS One *7*, e50161.

Elliott, S.L., Cullen, C.F., Wrobel, N., Kernan, M.J., and Ohkura, H. (2005). EB1 is essential during *Drosophila* development and plays a crucial role in the integrity of chordotonal mechanosensory organs. Mol Biol Cell *16*, 891-901.

Fang, Y., and Bonini, N.M. (2012). Axon degeneration and regeneration: insights from *Drosophila* models of nerve injury. Annu Rev Cell Dev Biol *28*, 575-597.

Franco, M., Peters, P.J., Boretto, J., van Donselaar, E., Neri, A., D'Souza-Schorey, C., and Chavrier, P. (1999). EFA6, a sec7 domain-containing exchange factor for ARF6, coordinates membrane recycling and actin cytoskeleton organization. EMBO J *18*, 1480-1491.

Fukata, M., Watanabe, T., Noritake, J., Nakagawa, M., Yamaga, M., Kuroda, S., Matsuura, Y., Iwamatsu, A., Perez, F., and Kaibuchi, K. (2002). Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. Cell *109*, 873–885.

Geraldo, S., Khanzada, U.K., Parsons, M., Chilton, J.K., and Gordon-Weeks, P.R. (2008). Targeting of the F-actin-binding protein drebrin by the microtubule plus-tip protein EB3 is required for neuritogenesis. Nat Cell Biol *10*, 1181-1189.

Goldberg, D.J., and Burmeister, D.W. (1986). Stages in axon formation: observations of growth of *Aplysia* axons in culture using video-enhanced contrast-differential interference contrast microscopy. J Cell Biol *103*, 1921-1931.

939   Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., and Lopez, R. (2010).
940   A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res *38*, W695-
941   699.

942   Grieder, N.C., de Cuevas, M., and Spradling, A.C. (2000). The fusome organizes the
943   microtubule network during oocyte differentiation in *Drosophila*. Development *127*, 4253-
944   4264.

945   Hahn, I., Fuss, B., Peters, A., Werner, T., Sieberg, A., Gosejacob, D., and Hoch, M. (2013).
946   The *Drosophila* Arf GEF Steppke controls MAPK activation in EGFR signaling. Journal of
947   cell science *126*, 2470-2479.

948   Hahn, I., Voelzmann, A., Liew, Y.-T., Costa-Gomes, B., and Prokop, A. (2019). The model of
949   local axon homeostasis - explaining the role and regulation of microtubule bundles in axon
950   maintenance and pathology bioRxiv, 10.1101/577320

951   Han, M.V., and Zmasek, C.M. (2009). phyloXML: XML for evolutionary biology and
952   comparative genomics. BMC Bioinformatics *10*, 356.

953   Hancock, J.F., Cadwallader, K., Paterson, H., and Marshall, C.J. (1991). A CAAX or a CAAL
954   motif and a second signal are sufficient for plasma membrane targeting of ras proteins.
955   EMBO J *10*, 4033-4039.

956   Hassan, B.A., Bermingham, N.A., He, Y., Sun, Y., Jan, Y.N., Zoghbi, H.Y., and Bellen, H.J.
957   (2000). *atonal* regulates neurite arborization but does not act as a proneural gene in the
958   *Drosophila* brain. Neuron *25*, 549-561.

959   Heasman, J. (2006). Patterning the early Xenopus embryo. Development *133*, 1205-1217.

960   Heidary, G., Engle, E.C., and Hunter, D.G. (2008). Congenital Fibrosis of the Extraocular
961   Muscles. Seminars in Ophthalmology *23*, 3-8.

962   Helenius, J., Brouhard, G., Kalaidzidis, Y., Diez, S., and Howard, J. (2006). The
963   depolymerizing kinesin MCAK uses lattice diffusion to rapidly target microtubule ends.
964   Nature *441*, 115-119.

965   Hinrichs, M.H., Jalal, A., Brenner, B., Mandelkow, E., Kumar, S., and Scholz, T. (2012). Tau
966   protein diffuses along the microtubule lattice. J Biol Chem *287*, 38559-38568.

967   Hoffman, P.N. (1995). Review : The Synthesis, Axonal Transport, and Phosphorylation of
968   Neurofilaments Determine Axonal Caliber in Myelinated Nerve Fibers. The Neuroscientist
969   *1*, 76-83.

970   Honnappa, S., Gouveia, S.M., Weisbrich, A., Damberger, F.F., Bhavesh, N.S., Jawhari, H.,
971   Grigoriev, I., van Rijssel, F.J., Buey, R.M., Lawera, A.*, et al.* (2009). An EB1-binding motif
972   acts as a microtubule tip localization signal. Cell *138*, 366-376.

973   Huang, J., Zhou, W., Dong, W., Watson, A.M., and Hong, Y. (2009). From the Cover:
974   Directed, efficient, and versatile modifications of the *Drosophila* genome by genomic
975   engineering. Proc Natl Acad Sci U S A *106*, 8284-8289.

976   Kalil, K., and Dent, E.W. (2014). Branch management: mechanisms of axon branching in the
977   developing vertebrate CNS. Nat Rev Neurosci *15*, 7-18.

978   Kaverina, I., Rottner, K., and Small, J.V. (1998). Targeting, capture, and stabilization of
979   microtubules at early focal adhesions. J Cell Biol *142*, 181-190.

980   Kolodziej, P.A., Jan, L.Y., and Jan, Y.N. (1995). Mutations that affect the length, fasciculation,
981   or ventral orientation of specific sensory axons in the *Drosophila* embryo. Neuron *15*, 273-
982   286.

983   Krieg, M., Stühmer, J., Cueva, J.G., Fetter, R., Spilker, K., Cremers, D., Shen, K., Dunn, A.R.,
984   and Goodman, M.B. (2017). Genetic defects in β-spectrin and tau sensitize *C. elegans*
985   axons to movement-induced damage via torque-tension coupling. Elife *6*, e20172.

986   Küppers-Munther, B., Letzkus, J., Lüer, K., Technau, G., Schmidt, H., and Prokop, A. (2004).
987   A new culturing strategy optimises *Drosophila* primary cell cultures for structural and
988   functional analyses. Dev Biol *269*, 459-478.

Lewis, T.L., Jr., Courchet, J., and Polleux, F. (2013). Cell biology in neuroscience: Cellular and molecular mechanisms underlying axon formation, growth, and branching. J Cell Biol *202*, 837-848.

Lowery, L.A., and van Vactor, D. (2009). The trip of the tip: understanding the growth cone machinery. Nat Rev Mol Cell Biol *10*, 332-343.

Luo, L., Liao, Y.J., Jan, L.Y., and Jan, Y.N. (1994). Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev *8*, 1787-1802.

Macia, E., Partisani, M., Favard, C., Mortier, E., Zimmermann, P., Carlier, M.F., Gounon, P., Luton, F., and Franco, M. (2008). The pleckstrin homology domain of the Arf6-specific exchange factor EFA6 localizes to the plasma membrane by interacting with phosphatidylinositol 4,5-bisphosphate and F-actin. J Biol Chem *283*, 19836-19844.

Marklund, U., Larsson, N., Gradin, H.M., Brattsand, G., and Gullberg, M. (1996). Oncoprotein 18 is a phosphorylation-responsive regulator of microtubule dynamics. EMBO J *15*, 5290-5298.

Marner, L., Nyengaard, J.R., Tang, Y., and Pakkenberg, B. (2003). Marked loss of myelinated nerve fibers in the human brain with age. J Comp Neurol *462*, 144-152.

McNally, F.J., and Roll-Mecak, A. (2018). Microtubule-severing enzymes: From cellular functions to molecular mechanism. J Cell Biol *217*, 4057-4069.

McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y.M., Buso, N., Cowley, A.P., and Lopez, R. (2013). Analysis Tool Web Services from the EMBL-EBI. Nucleic Acids Res *41*, W597-600.

Medana, I.M., and Esiri, M.M. (2003). Axonal damage: a key predictor of outcome in human CNS diseases. Brain *126*, 515-530.

Moore, A.T., Rankin, K.E., von Dassow, G., Peris, L., Wagenbach, M., Ovechkina, Y., Andrieux, A., Job, D., and Wordeman, L. (2005). MCAK associates with the tips of polymerizing microtubules. J Cell Biol *169*, 391-397.

O'Rourke, S.M., Christensen, S.N., and Bowerman, B. (2010). *Caenorhabditis elegans* EFA-6 limits microtubule growth at the cell cortex. Nat Cell Biol *12*, 1235-1241.

Patel, J.T., Belsham, H.R., Rathbone, A.J., Wickstead, B., Gell, C., and Friel, C.T. (2016). The family-specific alpha4-helix of the kinesin-13, MCAK, is critical to microtubule end recognition. Open Biol *6*.

Paul, P., van den Hoorn, T., Jongsma, Marlieke L.M., Bakker, Mark J., Hengeveld, R., Janssen, L., Cresswell, P., Egan, David A., van Ham, M., ten Brinke, A*., et al.* (2011). A Genome-wide Multidimensional RNAi Screen Reveals Pathways Controlling MHC Class II Antigen Presentation. Cell *145*, 268-283.

Pearce, S.P., Heil, M., Jensen, O.E., Jones, G.W., and Prokop, A. (2018). Curvature-sensitive kinesin binding can explain microtubule ring formation and reveals chaotic dynamics in a mathematical model. Bull Math Biol *80*, 3002-3022.

Pils, D., Horak, P., Gleiss, A., Sax, C., Fabjani, G., Moebus, V.J., Zielinski, C., Reinthaller, A., Zeillinger, R., and Krainer, M. (2005). Five genes from chromosomal band 8p22 are significantly down-regulated in ovarian carcinoma. Cancer *104*, 2417-2429.

Ponting, C.P., Phillips, C., Davies, K.E., and Blake, D.J. (1997). PDZ domains: targeting signalling molecules to sub-membranous sites. Bioessays *19*, 469-479.

Prokop, A. (2013a). The intricate relationship between microtubules and their associated motor proteins during axon growth and maintenance. Neur Dev *8*, 17.

Prokop, A. (2013b). A rough guide to *Drosophila* mating schemes. figshare, dx.doi.org/10.6084/m6089.figshare.106631.

Prokop, A. (2016). Fruit flies in biological research. Biological Sciences Review *28,* 10-14.

Prokop, A., Beaven, R., Qu, Y., and Sánchez-Soriano, N. (2013). Using fly genetics to dissect the cytoskeletal machinery of neurons during axonal growth and maintenance. J Cell Sci *126*, 2331-2341.

Prokop, A., Küppers-Munther, B., and Sánchez-Soriano, N. (2012). Using primary neuron cultures of *Drosophila* to analyse neuronal circuit formation and function. In The making and un-making of neuronal circuits in *Drosophila*, B.A. Hassan, ed. (New York, Humana Press), pp. 225-247.

Prokop, A., and Meinertzhagen, I.A. (2006). Development and structure of synaptic contacts in *Drosophila*. Semin Cell Dev Biol *17*, 20-30.

Qu, Y., Hahn, I., Webb, S.E.D., Pearce, S.P., and Prokop, A. (2017). Periodic actin structures in neuronal axons are required to maintain microtubules. Mol Biol Cell *28* 296-308.

Rogers, S.L., Rogers, G.C., Sharp, D.J., and Vale, R.D. (2002). *Drosophila* EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. J Cell Biol *158*, 873-884.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., and Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol *61*, 539-542.

Sánchez-Soriano, N., Gonçalves-Pimentel, C., Beaven, R., Haessler, U., Ofner, L., Ballestrem, C., and Prokop, A. (2010). *Drosophila* growth cones: a genetically tractable platform for the analysis of axonal growth dynamics. Dev Neurobiol *70*, 58-71.

Sánchez-Soriano, N., Tear, G., Whitington, P., and Prokop, A. (2007). *Drosophila* as a genetic and cellular model for studies on axonal growth. Neural Develop *2*, 9.

Sánchez-Soriano, N., Travis, M., Dajas-Bailador, F., Goncalves-Pimentel, C., Whitmarsh, A.J., and Prokop, A. (2009). Mouse ACF7 and *Drosophila* Short stop modulate filopodia formation and microtubule organisation during neuronal growth. Journal of cell science *122*, 2534-2542.

Schimizzi, G.V., Currie, J.D., and Rogers, S.L. (2010). Expression levels of a kinesin-13 microtubule depolymerase modulates the effectiveness of anti-microtubule agents. PLoS One *5*, e11381.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.

Seibel, N.M., Eljouni, J., Nalaskowski, M.M., and Hampe, W. (2007). Nuclear localization of enhanced green fluorescent protein homomultimers. Analytical Biochemistry *368*, 95-99.

Sharp, D.J., and Ross, J.L. (2012). Microtubule-severing enzymes at the cutting edge. Journal of cell science *125*, 2561-2569.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J.*, et al.* (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol *7*, 539.

Stefana, M.I., Driscoll, P.C., Obata, F., Pengelly, A.R., Newell, C.L., MacRae, J.I., and Gould, A.P. (2017). Developmental diet regulates *Drosophila* lifespan via lipid autotoxins. Nat Commun *8*, 1384.

Tiab, L., Manzi, V.d.A., Borruat, F.-X., Munier, F., and Schorderet, D. (2004). Mutation analysis of KIF21A in congenital fibrosis of the extraocular muscles (CFEOM) patients. Ophthalmic Genetics *25*, 241-246.

Tymanskyj, S.R., Yang, B., Falnikar, A., Lepore, A.C., and Ma, L. (2017). MAP7 regulates axon collateral branch development in dorsal root ganglion neurons. J Neurosci *37*, 1648-1661.

Vale, R.D., and Toyoshima, Y.Y. (1988). Rotation and translocation of microtubules in vitro induced by dyneins from *Tetrahymena* cilia. Cell *52*, 459-469.

1089 van der Vaart, B., van Riel, Wilhelmina E., Doodhi, H., Kevenaar, Josta T., Katrukha,
1090 Eugene A., Gumy, L., Bouchet, Benjamin P., Grigoriev, I., Spangler, Samantha A., Yu,
1091 Ka L.*, et al.* (2013). CFEOM1-associated kinesin KIF21A is a cortical microtubule growth
1092 inhibitor. Developmental Cell *27*, 145-160.

1093 Voelzmann, A., Hahn, I., Pearce, S., Sánchez-Soriano, N.P., and Prokop, A. (2016a). A
1094 conceptual view at microtubule plus end dynamics in neuronal axons. Brain Res Bulletin
1095 *126*, 226-237.

1096 Voelzmann, A., Liew, Y.-T., Qu, Y., Hahn, I., Melero, C., Sánchez-Soriano, N., and Prokop, A.
1097 (2017). *Drosophila* Short stop as a paradigm for the role and regulation of spectraplakins.
1098 Sem Cell Dev Biol *69*, 40-57.

1099 Voelzmann, A., Okenve-Ramos, P., Qu, Y., Chojnowska-Monga, M., del Caño-Espinel, M.,
1100 Prokop, A., and Sánchez-Soriano, N. (2016b). Tau and spectraplakins promote synapse
1101 formation and maintenance through Jun kinase and neuronal trafficking. eLife *5*, e14694.

1102 Wang, J.T., Medress, Z.A., and Barres, B.A. (2012). Axon degeneration: molecular
1103 mechanisms of a self-destruction pathway. J Cell Biol *196*, 7-18.

1104 Yu, W., Qiang, L., Solowska, J.M., Karabay, A., Korulu, S., and Baas, P.W. (2008). The
1105 microtubule-severing proteins spastin and katanin participate differently in the formation of
1106 axonal branches. Mol Biol Cell *19*, 1485-1498.

1107 Zeidler, M.P., Tan, C., Bellaiche, Y., Cherry, S., Hader, S., Gayko, U., and Perrimon, N.
1108 (2004). Temperature-sensitive control of protein activity by conditionally splicing inteins.
1109 Nat Biotech *22*, 871-876.

1110 Zschätzsch, M., Oliva, C., Langen, M., De Geest, N., Ozel, M.N., Williamson, W.R., Lemon,
1111 W.C., Soldano, A., Munck, S., Hiesinger, P.R.*, et al.* (2014). Regulation of branching
1112 dynamics by axon-intrinsic asymmetries in Tyrosine Kinase Receptor signaling. Elife *3*,
1113 e01699.

1114

1115 **Figures**

1116

1117 **Fig. 1** Efa6 is expressed throughout neurons at all developmental stages

1118 A-E) Images of primary *Drosophila* neurons at 6HIV or 5DIV (at indicated bottom right),
1119 stained for tubulin (magenta) and GFP (green); control neurons are wild-type (wt) or express
1120 *elav-Gal4*-driven nuclear GFP (*elav / nl-GFP*), whereas further neurons are either derived
1121 from the endogenously tagged Efa6::GFP line or express Efa6-FL::GFP under the control of
1122 sca-Gal4 (*sca / Efa6-FL::GFP*); asterisks indicate cell bodies and arrow the axon tips. F-I)
1123 Late larval CNSs at about 4d of development from egg lay (L3; F,G) and adult CNSs from 10d
1124 old flies (H,I) derived from control wild-type animals (wt) or the Efa6::GFP line (*Efa6::GFP*),
1125 stained for GFP and actin (Phalloidin, only larval preparations); OL, optic lobe; Br, central
1126 brain; vNC, ventral nerve cord. Scale bar in A represent 15µm in A-C, 25µm in D and E, 75µm
1127 in F and G, 130µm in H and I.

1128

1129 **Fig. 2**. Efa6 regulates axonal length in primary *Drosophila* neurons. Examples of primary
1130 *Drosophila* neurons at 6HIV (A-C), all stained for actin (magenta) and tubulin (green); neurons
1131 are either wild-type controls (A), Efa6-deficient (B), expressing Efa6-FL::GFP (C); asterisks
1132 indicate cell bodies, arrows point at axon tips; the scale bar in C represents 10µm.
1133 Quantification of axon lengths at 6HIV (D); different genotypes are colour-coded: grey, wild-
1134 type controls; blue, different *Efa6* loss-of-function conditions; green, neurons over-expressing
1135 Efa6 variants; data represent fold-change relative to wild-type controls (indicated as horizontal
1136 dashed "ctrl" line); they are shown as single data points and a bar indicating mean ± SEM

1137   data; P values from Mann-Whitney tests are given above each column, sample numbers at
1138   the bottom of each bar. For raw data see T2, which can be downloaded here:
1139   w.prokop.co.uk/Qu+al/RawData.zip.

1140

1141   **Fig. 3**. Efa6 domain and motif requirements for MT inhibition in neurons and fibroblasts. A)
1142   Schematics of *Drosophila melanogaster (Dm)* Efa6 (isoform C, CG31158), *Caenorhabditis*
1143   *elegans (Ce)* Efa6 (isoform Y55D9A.1a) and *Homo sapiens* (human) PSD1 (isoform 201/202,
1144   NP_002770.3), illustrating the positions (numbers indicate first and last residues) of the
1145   putative PSD95-Dlg1-ZO1 domain [PDZ; expected to anchor to transmembrane proteins
1146   (Ponting et al., 1997), but not mediating obvious membrane association in fibroblasts:
1147   Figs.S4C,D], SxIP/SxLP motifs (SRIP, SQIP, SALP, SSLP), the MT-binding domain (MTED),
1148   SEC7 domain, plekstrin homology (PH) domain and coiled-coil domain (CC). B) Schematics
1149   on the left follow the same colour code and show the *Dm*Efa6 constructs used in this study
1150   (dashed red lines indicate the last/first residue before/behind the truncation). Bar graphs on
1151   the right show the impact that transfection of these constructs had on axon loss in primary
1152   *Drosophila* neurons (dark grey in left graph) and on MT loss in fibroblasts (dark grey or black
1153   as indicated; for respective images see F and G below). Analogous fibroblast experiments as
1154   performed with *Drosophila* constructs were performed with full length constructs of *C. elegans*
1155   Efa6 and human PSDs (C), with N-terminal constructs (D) or synthetic MTEDs (E) of *Dm* and
1156   *Ce*Efa6 and of human PSD1. Throughout this figure, construct names are highlighted in red
1157   for *Drosophila*, light blue for *C. elegans* and yellow for *Homo sapiens*; all graph bars indicate
1158   percentages of neurons with/without axons (light/dark grey) and of fibroblasts with normal,
1159   reduced or absent MTs (light, medium, dark grey, respectively); numbers in the left end of
1160   each bar indicate sample numbers, on the right end the P values from Chi^2 tests relative to
1161   GFP controls; numbers on the right of bars in B compare some constructs to Efa6-FL::GFP,
1162   as indicated by black lines. F-F'') Primary neurons expressing Efa6-FL::GFP transgenically
1163   and stained for tubulin (asterisks, cell bodies; white arrows, axon tips; open arrow, absent
1164   axon). G-G'') Fibroblasts expressing Efa6-FL::GFP and stained for tubulin; curved arrows
1165   indicate areas where MTs are retracted from the cell periphery; grey dots in F-G'' indicate the
1166   phenotypic categories for each neuron and fibroblasts, as used for quantitative analyses in the
1167   graphs above. Scale bar at bottom of F refers to 10μm in F and 25μm in G. For raw data see
1168   T3, which can be downloaded here: w.prokop.co.uk/Qu+al/RawData.zip.

1169

1170   **Fig. 4.** EFA6 peptide interacts directly with α/β-tubulin and inhibits microtubule growth. A) End
1171   point of microtubule growth assay in the presence of no MTED peptide, or a 1:1 and 10:1
1172   molar ratio of MTED peptide to tubulin. Scale bar in A refers to 10 μm. B) Pull-down of tubulin
1173   by sepharose beads coated with MTED peptide via cyanogen bromide coupling and control
1174   beads with no MTED peptide attached.

1175

1176   **Fig. 5**. Efa6 regulates MT behaviours in GCs. A-H') Examples of primary neurons at 6HIV
1177   which are either wild-type controls (top), Efa6-deficient (middle) or expressing Efa6-FL::GFP
1178   (bottom); neurons were either imaged live for Eb1::GFP (green in A,D) or fixed and labelled
1179   for Eb1 and tubulin (B,E,G; as colour-coded) or actin and tubulin (C,F,H; as colour coded;
1180   tubulin shown as single channel image on the right); asterisks indicate cell bodies, white
1181   arrows the tips of GCs, open arrows the tips of MT bundles and arrow heads MTs or Eb1
1182   comets in filopodial processes; the GC in G is outlined with a white dashed line; scale bar in D

represents 5µm in all images. I-M) Quantitative analyses of MT behaviours in GCs of such neurons as indicated above each graph. Different genotypes are colour-coded: grey, wild-type controls; blue, different *Efa6* loss-of-function conditions; green, neurons over-expressing Efa-FL. The graph in L shows percentages of neurons without any MTs in shaft filopodia (dark shade) versus neurons with MTs in at least one filopodium (light shade; P values above bars assessed via Chi^2 tests), whereas all other graphs show single data points and a bar indicating mean ± SEM, all representing fold-increase relative to wild-type controls (indicated as horizontal dashed "ctrl" line; P values above columns from Mann-Whitney tests. The control values in M (dashed line) equate to an Eb1 comet life-time of 2.10s ± 0.24SEM in filpododia and 5.04s ± 0.60SEM in growth cones, and a comet velocity of 0.136 µm/s ± 0.01SEM.Throughout the figure, sample numbers are shown at the bottom of each bar and data obtained from live analyses with Eb1::GFP are framed in red. For raw data see T4, which can be downloaded here: w.prokop.co.uk/Qu+al/RawData.zip.

Fig. 6. Loss of Efa6 promotes MT entry into axon shaft filopodia. Quantitative analyses of MT behaviours in axon shafts, as indicated above each graph; bars are colour-coded: grey, controls; blue, different *Efa6* mutant alleles; green, neurons over-expressing Efa-FL::GFP or Efa6::CAAX::GFP; orange, *shot* mutant allele; red outlines indicate live imaging data, all others were obtained from fixed specimens; numbers at the bottom of bars indicate sample numbers, above bars P values. A-C,G) Fold-changes relative to wild-type controls (indicated as horizontal dashed "ctrl" line) shown as single data points and a bar indicating mean ± SEM; P values were obtained via Mann-Whitney tests; control values (dashed line) in B and C equate to an Eb1 comet lifetime of 7.18 s ± 0.35 SEM and a velocity of 0.169 µm/s ± 0.01SEM. D-F) Binary parameters (light *versus* dark shades as indicated) provided as percentages; P values were obtained via Chi^2 tests. For raw data see T5, which can be downloaded here: w.prokop.co.uk/Qu+al/RawData.zip.

Fig. 7. Efa6 regulates axon branching in in primary *Drosophila* neurons and adult fly brains. Examples of primary *Drosophila* neurons at 5DIV (A-D), all stained for actin (magenta) and tubulin (green); neurons are either wild-type controls (A), Efa6-deficient (B), expressing Efa6-FL::GFP (C), or expressing Efa6-Nterm-CAAX::GFP (D); asterisks indicate cell bodies, arrows point at axon tips, arrow heads at axon branches, the curved arrow at an area of MT disorganisation; the scale bar in C represents 20µm. Quantification of axonal branch numbers (E); different genotypes are colour-coded: grey, wild-type controls; blue, different *Efa6* loss-of-function conditions; green, neurons over-expressing Efa6 variants; data represent fold-change relative to wild-type controls (indicated as horizontal dashed "ctrl" line); they are shown as single data points and a bar indicating mean ± SEM data; P values from Mann-Whitney tests are given above each column, sample numbers at the bottom of each bar. F-K) Brains (medulla region of the optic lobe in oblique view) of young (2-5 d after eclosure; top) and old flies (15-18 d; bottom) driving *UAS-myr-tdTomato* via the *ato-Gal4* driver in dorsal cluster neurons (example neurons are traced in magenta for axons and green for side branches); flies are either wild-type controls (left) or display *ato-Gal4*-driven knock-down of Efa6 (middle) or over-expression of Efa6-FL::GFP (right). L,M) Quantification of data for wild-type (wt; grey), Efa6 knock-down (blue) and Efa6-FL::GFP over-expression (green): L) shows the number of primary branches per axon as fold-change relative to wild-type controls (indicated as horizontal dashed "ctrl" line; data are shown as bars indicating mean ± SEM accompanied by single data points, all normalised to wt); M) displays the number of medullas with axons (light

1230   colours) or without axons (dark colours) shown as a percentages in young and old flies. In all
1231   graphs, sample numbers at the bottom (in L number of medullas before and number of axons
1232   after slash) and P values from Mann-Whitney (G) or Chi^2 tests (H) above each column. Scale
1233   bar in D represents 60µm in all figures. For raw data see T6, which can be downloaded here:
1234   w.prokop.co.uk/Qu+al/RawData.zip.

1235

1236   **Fig. 8**. Efa6 helps to maintain axonal MT bundles in *Drosophila* neurons. A-H) Images of
1237   primary neurons at 6HIV (left), 5DIV (middle) and 10DIV (right), stained for tubulin (green) and
1238   actin (magenta), derived from embryos that were either wild-type (wt, A-C), *Efa6* null mutant
1239   (D-F), homozygous for *shot^3* (G) or *shot^3 Efa6$^{GX6[w-]}$* double-mutant (shot Efa6, H); arrows
1240   point at axon tip, arrow heads at areas of MT disorganisation, and asterisks indicate the cell
1241   bodies; the scale bar in A represents 10µm for 6HIV neurons and 25µm for 5DIV and 10DIV
1242   neurons. I-N) Quantitative analyses of MT disorganisation (measured as MT disorganisation
1243   index, MDI) in different experimental contexts (as indicated above graphs); different
1244   genotypes are colour-coded: grey, wild-type controls; blue, *Efa6* loss-of-function; light/dark
1245   orange, *shot^3* in hetero-/homozygosis; green, neurons over-expressing Efa-FL::GFP or Efa6-
1246   NtermCAAX::GFP; data are shown as single data points and a bar indicating mean ± SEM, all
1247   representing fold-change relative to wild-type controls (indicated as horizontal dashed "ctrl"
1248   line); P values from Mann-Whitney tests in I, J) and Kruskal–Wallis one-way ANOVA with *post
1249   hoc* Dunn's test in K-N) are given above each column, sample numbers at the bottom of each
1250   bar. Control 1 in (K) is *tub-Gal80, elav-Gal4* alone and control 2 *UAS-Efa6RNAi*. For raw data
1251   see T7, which can be downloaded here: w.prokop.co.uk/Qu+al/RawData.zip.

1252

1253   **Fig. 9** Efa6 is required for axonal MT bundle maintenance in adult fly brains. A-C) Medulla
1254   region of adult brains at 26-27 days after eclosure, all carrying the *GMR31F10-Gal4* driver and
1255   *UAS-α-tubulin84B-GFP* (*GMR-tub*) which together stain MTs in a subset of lamina neuron
1256   axons that terminate in the medulla. The other specimens co-express *Efa6-RNAi* either alone
1257   (*GMR-tub-Efa6^{IR}* in B) or combined with a *shot$^{3/+}$* heterozygous mutant background (*GMR-tub-
1258   Efa6^{IR} shot$^{3/+}$* in C). White/black arrows indicate axonal swellings without/with MT
1259   disorganisation; rectangles outlined by red dashed lines are shown as 2.5 fold magnified
1260   insets where white arrow heads point at disorganised MTs; the scale bar in A represents
1261   15µm in all images. D, E) Quantitative analyses of all axonal swelling (D) or swellings with MT
1262   disorganisation (E); different genotypes are colour-coded as indicated; bars show mean ±
1263   SEM, all representing fold-change relative to wild-type controls (indicated as horizontal
1264   dashed line); P values from Kruskal–Wallis one-way tests are given above each column,
1265   sample numbers at the bottom of each bar. For raw data see table T8, which can be
1266   downloaded here: w.prokop.co.uk/Qu+al/RawData.zip.

1267

1268   **Fig. 10**. A model of the suggested roles of Efa6. **A**) The model of local axon homeostasis
1269   (Prokop, 2016; Voelzmann et al., 2016a) states that axonal MTs (green bars) are actively kept
1270   in bundles; for example, their continued polymerisation (1) mediated by plus end machinery
1271   (blue circle) is guided by spectraplakins (here Shot) along cortical actin into parallel bundles
1272   (2), or MTs are kept together through cross-linkage (brown "L"; 4) (Bettencourt da Cruz et al.,
1273   2005; Krieg et al., 2017); here we propose that MTs accidentally leaving the bundle become
1274   vulnerable (orange circles) through inhibition (red "T") by cortically anchored Efa6. **B**) In
1275   normal neurons, polymerising MTs in bundles (dark blue circles) are protected by Shot

1276 (marine lines) and MT-MT cross-linkers (brown rectangles), whereas MTs approaches the
1277 membrane (orange arrow heads) either by splaying out in GCs or leaving the bundle in the
1278 shaft (orange arrow heads) become vulnerable (orange circles) to inhibition by Efa6 (light
1279 green/red dashes) both along the cortex and in filopodia **C**) Upon Efa6 deficiency, MTs
1280 leaving bundles or entering GCs are no longer subjected to Efa6-mediated cortical inhibition
1281 (blue arrow heads) and can cause gradual build-up of MT disorganisation; when entering shaft
1282 filopodia they can promote interstitial branch formation (red arrow), when entering GC
1283 filopodia they can promote axon growth or even branching through GC splitting (yellow
1284 arrows). **D**) Far more MTs leave the bundles in *shot* mutant neurons, but a good fraction of
1285 them is inhibited by Efa6 (increased number of orange arrow heads). **E**) In the absence of
1286 both Shot and Efa6, more MTs leave the bundles, but there is no compensating cortical
1287 inhibition (increased number of blue arrow heads), so that the MT disorganisation phenotype
1288 worsens**.**

1289
1290

Supplementary materials

Fig. S1. Phylogentic tree analysis of Efa6. Bayesian phylogenetic analysis of full length (A) or N-termini (B) of Efa6 orthologues. Sequences were aligned using Muscle or ClustalO and posterior probabilities and branch lengths calculated using MrBayes. Branch length scale is indicated; blue numbers show posterior probabilities of each branch split. For the full length tree, *Drosophila steppke* (*step*) was used as outgroup (Hahn et al., 2013). In both full length and N-terminus analyses, chordates (cream colour) split off very early from Efa6 versions of other species, in line with an early speciation event separating both groups before the vertebrate multiplication events took place. Phyla are highlighted in different colours, gene symbols and/or accession numbers are given after the species names.

Fig. S2. Efa6 N-terminal domains vary amongst different phyla. A) Domain annotations in 56 Efa6 orthologues via EMBL SMART and Uniprot. Phyla are colour-coded as in Fig.S1. Note that there is a strong variation of lengths and domain composition in particular of the N-terminus. The putative PDZ domain seems to be a feature mainly of insect versions of Efa6 and is absent from any analysed chordate orthologues; MTED and MTED-like sequences cannot be consistently identified in all Efa6 orthologues and are very divergent in chordate Efa6/PSD versions. SxIP/SxLP sites [flanked by positive charges as would be expected of functional motifs (Honnappa et al., 2009)] are found in the N-terminal half of only a subset of Efa6 versions in nematodes (e.g. *C. elegans*), insects (in particular flies, e.g. *D. melanogaster*) and molluscs, and even fewer in chordates; in mouse, alpaca and cat SxIP/SxLP sites are flanked by negative charges. B) To determine a potential MTED consensus sequence, 37 sequences of molluscs, nematodes, arthropods and putative MTED sequences of mammalian PSD1-4 were grouped according to phylum; consensus sequences were depicted using Berkley's Weblogo online server (default colour scheme). Amino acid positions identical to *D. melanogaster* and *C. elegans* MTED are highlighted (faint yellow).

Fig.S3. Localisation of Efa6 constructs in primary neurons. Images show transfected primary *Drosophila* neurons at 18HIV stained for tubulin (magenta) and GFP (green and in greyscale below the colour image). Cell bodies are indicated by asterisks, axon tips by arrows. The transfected constructs are indicated top right following the nomenclature explained in Fig.3B. Scale bar in A represents 10µm for all figures shown.

Fig.S4. Efa6 constructs localisations in fibroblasts. Images show fibroblasts which are all stained for GFP (green) and either for actin or MTs (magenta); GFP and actin/MTs shown as single channels in greyscale below the colour image, 24hrs after transfection with control (GFP) or Efa6-derived constructs (nomenclature as explained in Fig.3B, but leaving out the "Efa6"-prefix and "::GFP"-postfix, as indicated top right. Double chevrons indicate nuclear localisation, arrow heads membrane localisation apparent at cell edges and curved arrows membrane ruffles. Scale bar in A represents 10µm in all images.

Fig.S5. MT inhibition by Efa6-FL is concentration-dependent in fibroblasts. **A-C)** Representative images of fibroblasts stained for GFP and tubulin (green and magenta, respectively; both shown as single channels in greyscale below the colour image). Images

1336   were taken 24hrs after transfection with *Efa6-FL::GFP*, assessed for GFP intensity (plotted on
1337   the ordinate in **D**; examples for low, moderate and high expression are given in the left, middle
1338   and right columns, respectively) and then grouped with respect to their MT phenotypes into
1339   "MTs intact" (light grey), "mild MT defects" (medium grey) or "MTs gone" (black), as indicated
1340   by greyscale circles in the lowest row of A-C and the abscissa of D. Arrow heads point at GFP
1341   accumulation at membrane edges, white curved arrows indicate areas of the cell from where
1342   MTs have retracted, open arrows point at retraction fibres and the double chevron indicates
1343   the nucleus position and signs of nuclear GFP localisation. Scale bar in A represents 10μm in
1344   all images. For raw data see T9, which can be downloaded here:
1345   w.prokop.co.uk/Qu+al/RawData.zip.

1346

1347   **Fig. S6**. Conserved functions of the Efa6 C-terminus in membrane ruffle formation. **A-E)**
1348   Representative images of fibroblasts stained for GFP and actin (green and magenta,
1349   respectively; both shown as single channels in greyscale below the colour image). Images
1350   were taken 24hrs after transfection with different constructs (indicated top right): control vector
1351   (GFP; A), Efa6-derived constructs (B-D; nomenclature as explained in Fig.3B, but leaving out
1352   the "Efa6"-prefix and "::GFP"-postfix) or PSD1-FL::GFP (E). To the right of each image, a
1353   selected area is displayed with 2.5-fold magnification, showing dotted actin- and GFP-stained
1354   ruffles (curved open arrows) in B, D and E, but not A and C; ruffle formation has been
1355   quantified and is shown as a bar graph in **F**. All graph bars indicate percentages of fibroblasts
1356   with/without membrane ruffles (dark/light grey); P values on top of bars are from Chi^2 tests
1357   relative to GFP controls. As shown, ruffle phenotype were never observed with any N-terminal
1358   Efa6 variants but are reproduced with the Efa6-ΔNterm::GFP variant (comprising the C-
1359   terminal Sec7, PH and CC domains; Fig. 3B). Scale bar in the top image of A represents 10
1360   μM for all fibroblasts shown. For raw data see T10, which can be downloaded here:
1361   w.prokop.co.uk/Qu+al/RawData.zip.

1362

1363   **Fig. S7.** Representative MT phenotypes induced by the different constructs in transfected
1364   fibroblasts. Fibroblasts 24hrs after transfection with different control (GFP) or Efa6-derived
1365   constructs as indicated top right in each image (nomenclature as explained in Fig.3B, but
1366   leaving out the "Efa6"-prefix and "::GFP"-postfix). Cells were stained for tubulin (black; images
1367   shown as inverted greyscale) and classified as "no MT defects" (light grey), "mild MT defects"
1368   (medium grey) or "MTs gone" (black), as indicated by greyscale boxes bottom left of each
1369   image; curved arrows indicate peripheral MT depletion. Each image represents the most
1370   prominent phenotype for each respective construct. Scale bar at the bottom right in H
1371   represents 25μm in all images.

1372

1373   **Fig. S8.** *In vitro* attempts to resolve the MT inhibition mechanism of Efa6. A) To determine
1374   whether Efa6 directly affects MT stability, we recombinantly expressed Efa6-ΔCterm::GFP in
1375   Sf9 cells, purified the protein and observed its interaction with MTs using total internal
1376   reflection fluorescence (TIRF) microscopy in a low ionic strength buffer (BRB20, 75mM KCl);
1377   images on the left show three examples of kymographs of MT lattices decorated with Efa6-
1378   ΔCterm::GFP, which displays a mixture of stationary molecules and diffusive interactions
1379   typical of non-translocating MT-associated proteins (Helenius et al., 2006; Hinrichs et al.,
1380   2012); bar charts (right) show quantification of the amount of interacting protein: background
1381   signal from MTs alone, Efa6-ΔCterm::GFP (20nM) and the non-translocating kinesin

1382   MCAK::GFP (20nM) as positive control; at the same protein concentration, over 2-fold more
1383   molecules of MKAC typically interact with MTs; all graphs in this figure show individual data
1384   points and bars representing mean ± SEM; numbers above bars show P values obtained from
1385   Mann–Whitney Rank Sum statistical analyses, numbers in bars the respective sample
1386   numbers. B) Kymographs (left) show individual fluorescently-labelled GMPCPP-stabilised MTs
1387   *in vitro* (Patel et al., 2016), either alone (MTs only; basal depolymerisation rate, n=7) or in the
1388   presence of 14 nM Efa6-ΔCterm::GFP (n=8) or 40nM MCAK::GFP (n=18); the bar chart (right)
1389   quantifies the induced depolymerisation rates; using two different purifications of Efa6-
1390   ΔCterm::GFP on three separate occasions, we saw no evidence of MT depolymerisation
1391   above the basal level of depolymerisation typically observed in these assays, whereas parallel
1392   control experiments with mitotic centromere-associated kinesin/MCAK showed MT
1393   depolymerisation rates typical of this kinesin. C) To assess whether MT destabilisation might
1394   require additional cytoplasmic factors, we used *Xenopus* oocyte extracts: phase contrast
1395   images show MTs in *Xenopus* oocyte extracts (after they had been allowed to polymerise for
1396   20 min) and then showing stills from before, 10s after and 120s after washing in 20nM
1397   MCAK::GFP (as positive control) or 20nM Efa6-ΔCterm::GFP; squares outlined by dashed
1398   white lines are shown magnified in insets revealing MTs; MTs clearly vanish upon treatment
1399   with MCAK, but counts of MTs did not reveal any obvious effects on MTs with Efa6-
1400   ΔCterm::GFP. D,E) *RFP* controls and *Efa6-Nterm::GFP* expression constructs were injected
1401   into *Xenopus* embryos at the 4-cell stage and analysed 24 hrs later; only the *Efa6* construct
1402   caused a strong suppression of cell division, as indicated by the presence of very large cells
1403   (arrows) and pigmentation defects (curved arrows) at the site of injection, suggesting that
1404   Efa6-Nterm::GFP is functional when expressed in the *Xenopus* context. Scale bar (in top left
1405   image) represents 3 μm in C (2.5 fold magnified insets), and 1400 μm / 350 μm / 140 μm in
1406   left / middle / right images of D and E, respectively. For raw data see T11, which can be
1407   downloaded here: w.prokop.co.uk/Qu+al/RawData.zip.

1408

1409   **Fig. S9**. *ato-Gal4*-driven Efa-FL::GFP expression in adult brains. Brains (region of the optic
1410   lobe in oblique view; white dashed line indicating the lower edge of the medulla) of young (2-5
1411   d after eclosure; top) and old flies (15-18 d; bottom) which are either from wild-type controls or
1412   from flies driving *UAS-myr-tdTomato* and *UAS-Efa6-FL-GFP* via the *ato-Gal4* driver in dorsal
1413   cluster neurons; specimens are stained for GFP and arrows point at stained; for myr-dtTomato
1414   staining of brains from these experiments see Fig.7F-K. Scale bar in D represents 60μm in all
1415   images.

1416   **Fig. S10**. Efa6 mutant primary neurons show MT disorganisation. Quantitative analyses of MT
1417   disorganisation measured as binary score: percentages of neurons with bundled MTs
1418   throughout (light colour) or showing obvious MT disorganisation in their axons (dark colour) in
1419   different *Efa6* mutant alleles (control value indicated as horizontal dashed "ctrl" line); P values
1420   from Chi^2 tests are given above the bars. For raw data see T12, which can be downloaded
1421   here: w.prokop.co.uk/Qu+al/RawData.zip.

1422
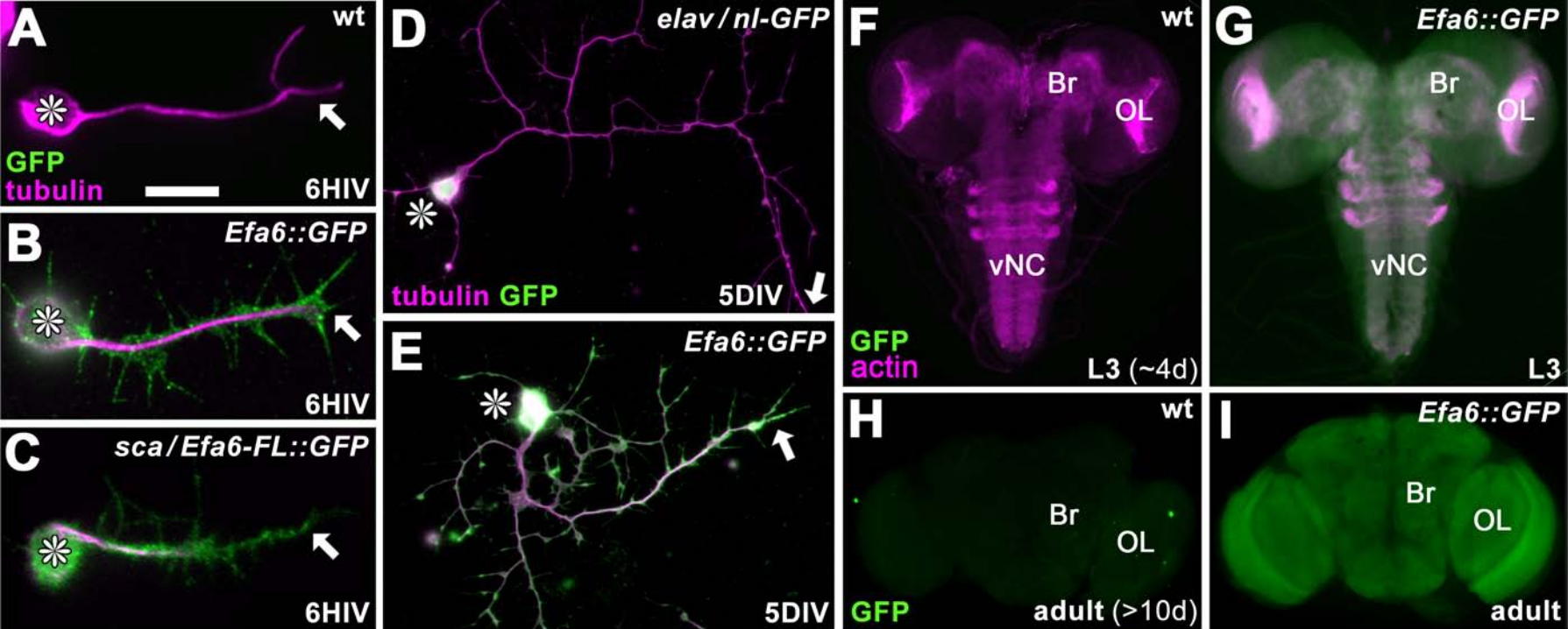
1423   **Suppl. Mov. M1**. MT behaviours in control fibroblasts transfected with GFP. Eb3 (green) was
1424   used to visualise MT polymerisation, GFP (magenta) localises throughout the cell; MTs clearly
1425   polymerise to the very fringe of the transfected cell. Movies were taken at one frame per
1426   second; the scale bar corresponds to 25μm, close ups are twofold magnified. Movie available
1427   at w.prokop.co.uk/Qu+al/SupplMov.html.

1428 **Suppl. Mov. M2**. MT behaviours in fibroblasts transfected with Efa6-FL::GFP. Eb3 (green)
1429 was used to visualise MT polymerisation, which is prevented from entering areas of high Efa6-
1430 FL::GFP (magenta), with Efa6 often accumulating where MTs enter the area. Movies were
1431 taken at one frame per second; the scale bar corresponds to 25µm, close ups are twofold
1432 magnified. Movie available at w.prokop.co.uk/Qu+al/SupplMov.html.

1433 **Suppl. Mov. M3**. Eb1::GFP in mouse NHTH3 fibroblast about 6 hours after transfection.
1434 Pictures for the movie were taken at 2 s intervals. Scale bar indicates 10 µm. Movie available
1435 at w.prokop.co.uk/Qu+al/SupplMov.html.

1436 **Suppl. Mov. M4**. MT behaviours in fibroblasts transfected with Efa6-Nterm::GFP::CAAX. Eb3
1437 (green) was used to visualise MT polymerisation, which is prevented from entering areas of
1438 high Efa6-FL::GFP (magenta), with Efa6 often accumulating where MTs enter the area.
1439 Movies were taken at one frame per second; the scale bar corresponds to 25µm, close ups
1440 are twofold magnified. Movie available at w.prokop.co.uk/Qu+al/SupplMov.html.

1441 **Suppl. Mov. M5**. Eb1::GFP in a growth cone of a wild-type *Drosophila* primary neuron at 6
1442 HIV. Arrows indicate positions where individual Eb1::GFP comets terminate. Pictures of the
1443 movie were taken at 2 s intervals. Scale bar indicates 10 µm. Movie available at
1444 w.prokop.co.uk/Qu+al/SupplMov.html.

1445 **Suppl. Mov. M6**. Eb1::GFP in a growth cone of a wild-type *Drosophila* primary neuron at 6
1446 HIV. Arrows indicate positions where individual Eb1::GFP comets terminate. Pictures of the
1447 movie were taken at 2 s intervals. Scale bar indicates 10 µm. Movie available at
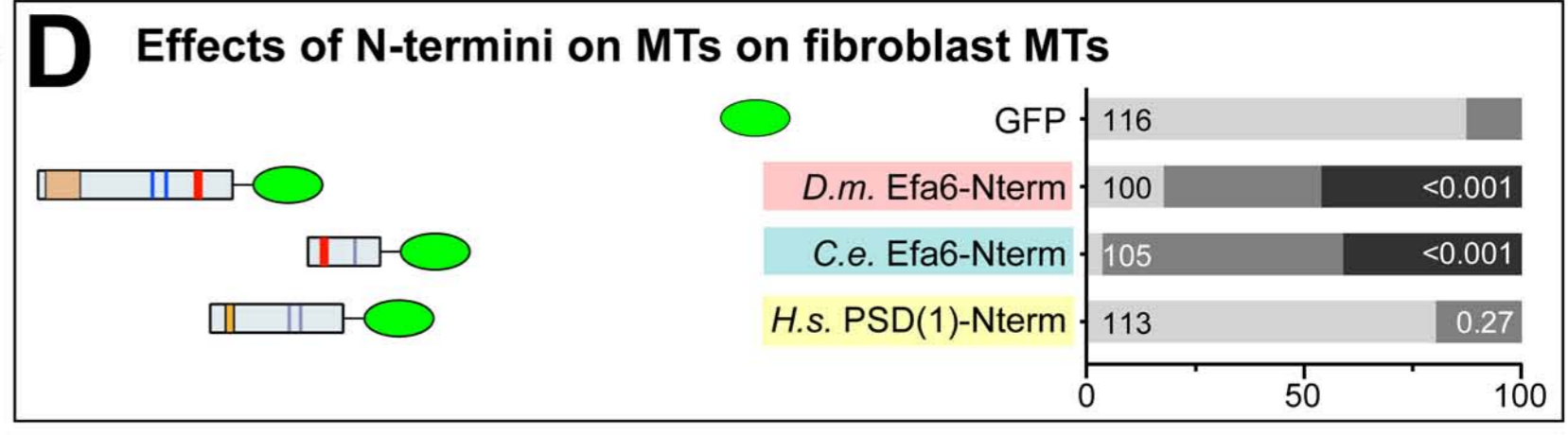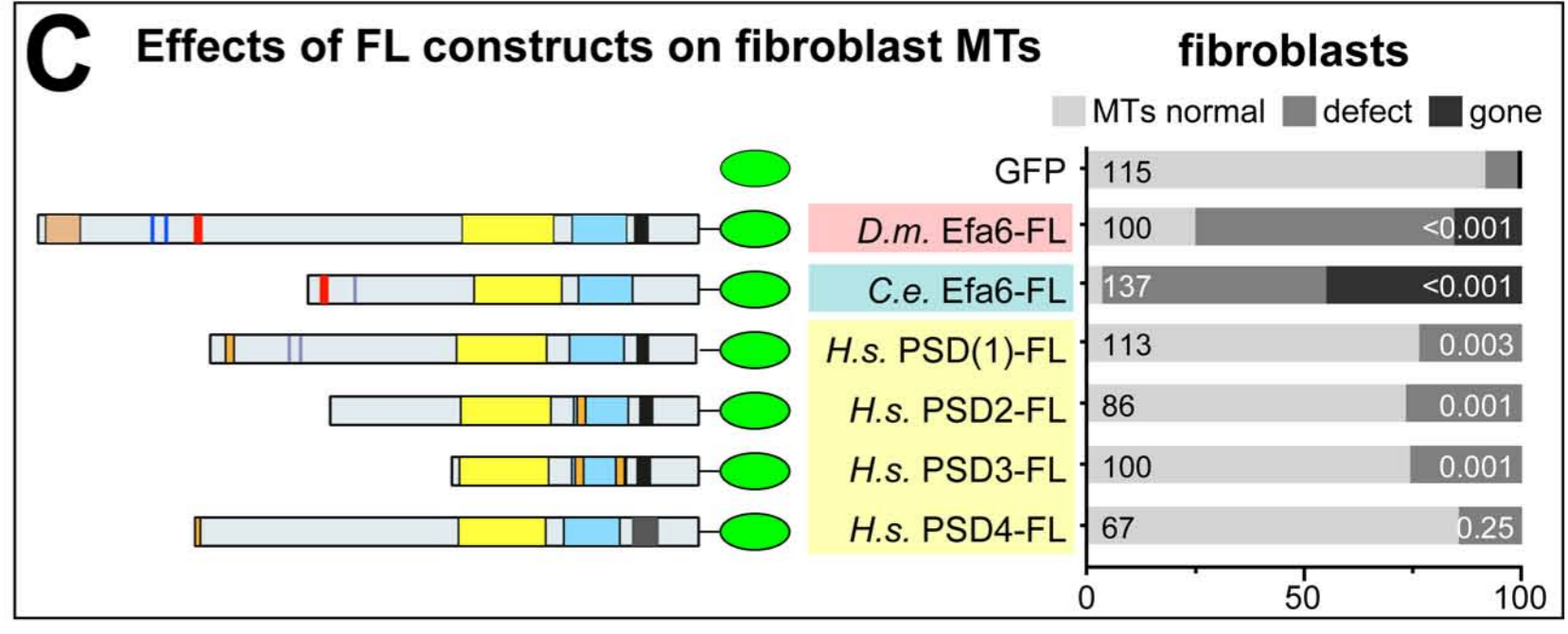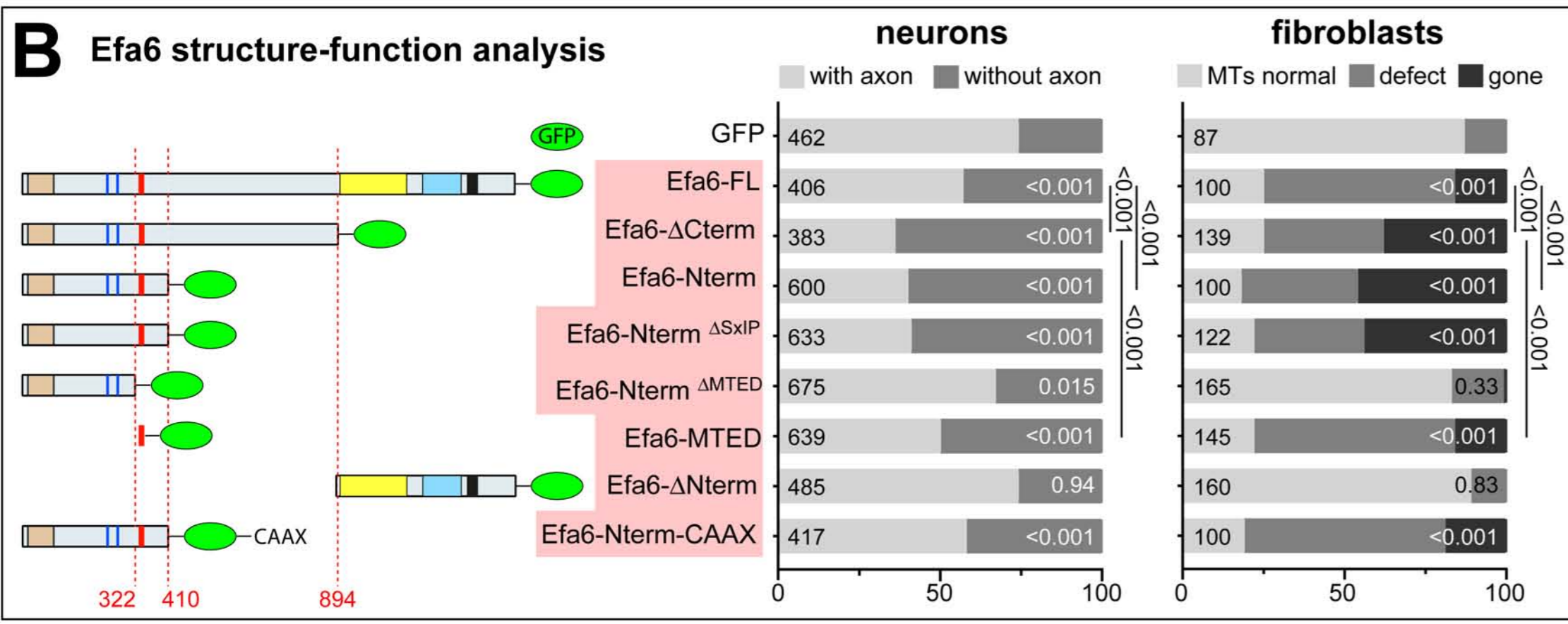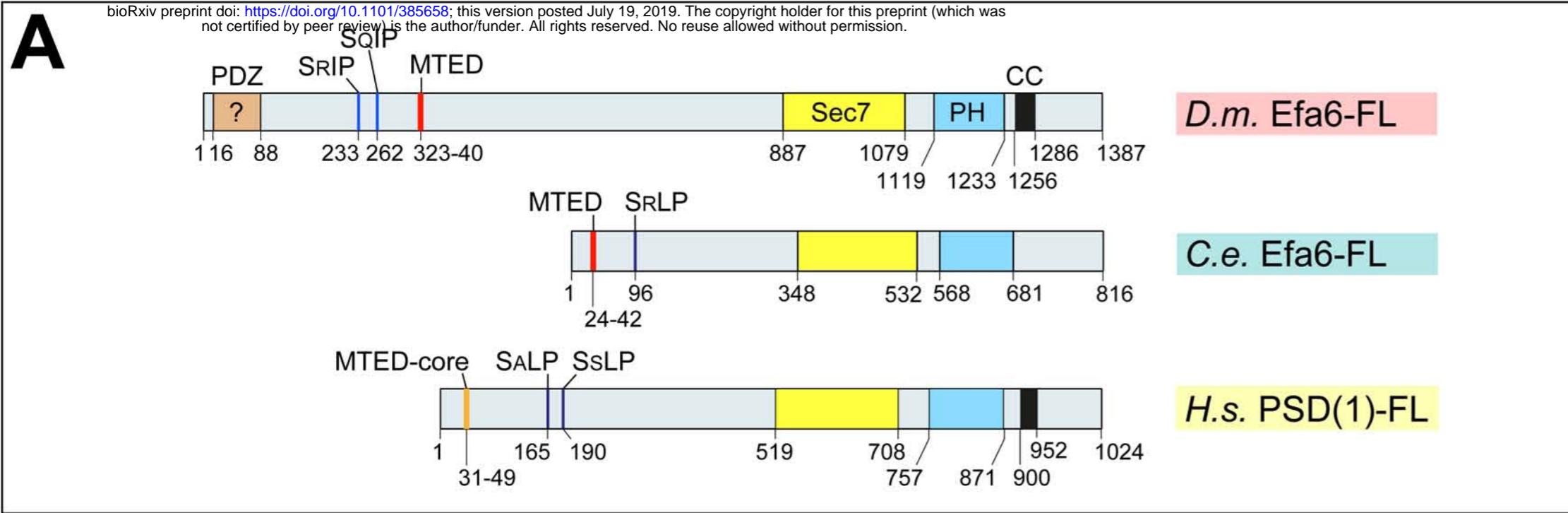1448 w.prokop.co.uk/Qu+al/SupplMov.html.

1449 **Suppl. Mov. M7.** Eb1::GFP in a growth cone of an *Efa6* $^{GX6[w-]}$ mutant *Drosophila* primary
1450 neuron at 6 HIV. The arrow heads follow individual Eb1::GFP comets illustrating either their
1451 trajectories adjacent to the membrane or prolonged dwell time at filopodial tips. Pictures for
1452 the movie were taken at 2 s intervals. Scale bar indicates 10 µm. Movie available at
1453 w.prokop.co.uk/Qu+al/SupplMov.html.

1454 **Suppl. Mov. M8.** Eb1::GFP in a growth cone of an *Efa6* $^{GX6[w-]}$ mutant *Drosophila* primary
1455 neuron at 6 HIV. The arrow heads follow individual Eb1::GFP comets illustrating either their
1456 trajectories adjacent to the membrane or prolonged dwell time at filopodial tips. Pictures for
1457 the movie were taken at 2 s intervals. Scale bar indicates 10 µm. Movie available at
1458 w.prokop.co.uk/Qu+al/SupplMov.html.

Fig.1 Qu et al.

A wt · 6 HIV

B *Efa6⁻/⁻*

C Efa6-FL

actin
tubulin

D Relative axon length (6 HIV)

Fig. 2 Qu et al.

Fig. 3 Qu et al.

A — control (no peptide) | 1:1 peptide:tubulin | 10:1 peptide:tubulin

B — tubulin input | with peptide | no peptide

Fig. 4 Qu et al.

Fig. 5 Qu et al.

A EB1 comet number in axon shafts/length
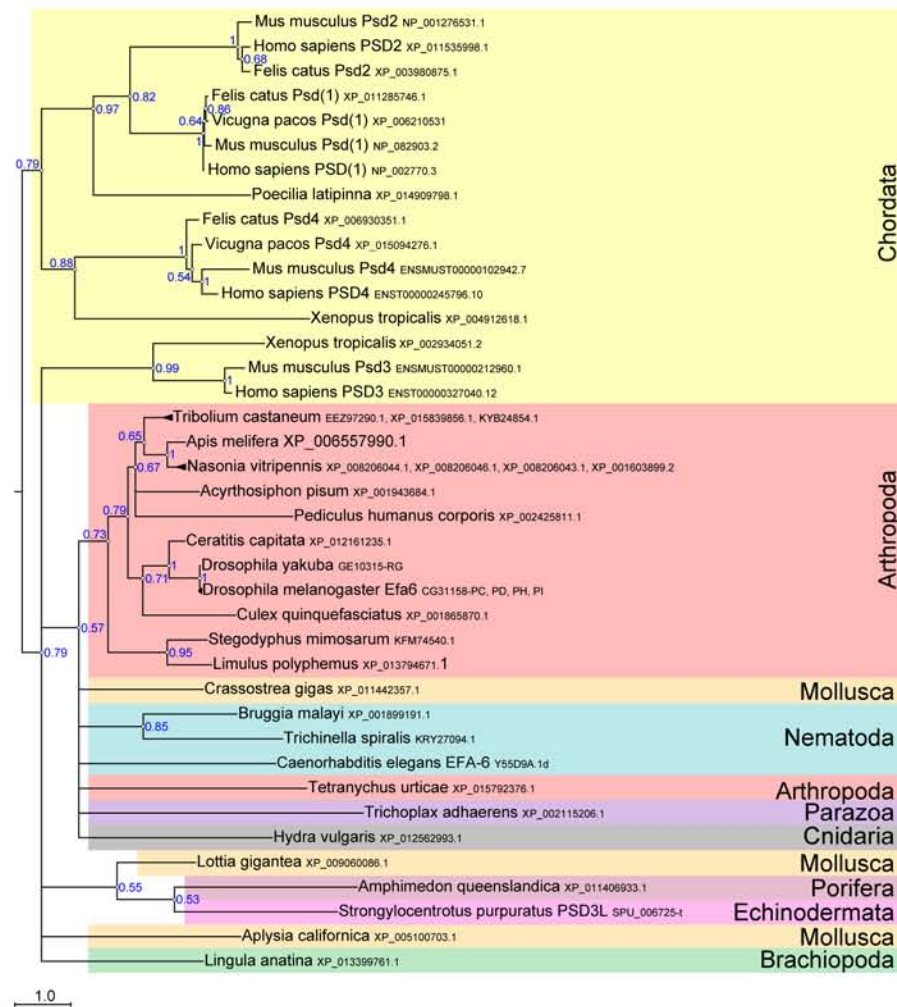
live Eb1::GFP

B EB1 comet lifetime

live Eb1::GFP

C EB1 comet velocity

live Eb1::GFP

D EB1 comet direction in axon shaft

live Eb1::GFP
light: retro- dark: anterograde

E Neurons with Eb1 comets in shaft filopodia

light: w/ comets dark: w/o comets

F Neurons with tubulin-stained shaft filopodia

light: with MTs dark: no MTs

G Number of shaft filopodia with tubulin staining/length

Fig. 6 Qu et al.

A wt   5 DIV

B *Efa6⁻/⁻*

actin
tubulin

C Efa6-FL

D Efa6-Nterm-CAAX

E Branch number relative to axon length (5 DIV)

	0.002	0.08	<0.001

rel. fold increase

ctrl

wt   *Efa6^{KO#1}*   Efa6-FL   Efa6-NtermCAAX

65   78   107   94

control   ***Efa6-RNAi***   **Efa6-FL::GFP**

F young (2-5 d)   ato / myr-tdTomato

G

H

I old (15-18 d)

J

K

L No. of primary branches

rel. fold increase

<0.001   <0.001   <0.001   <0.001

ctrl

wt   *Efa6RNAi*   Efa6-FL   wt   *Efa6RNAi*   Efa6-FL

26/254   31/284   39/118   40/363   21/194   44/385

young   old

M Normalised no. of medullas with axons

light: with axons   dark: no axons

0.75   <0.001   >0.99   0.65

wt   *Efa6RNAi*   Efa6-FL   wt   *Efa6RNAi*   Efa6-FL

59   37   67   94   30   57

young   old

Fig. 7 Qu et al.

Fig. 8 Qu et al.

Fig. 9 Qu et al.

A

actin

Efa6

Shot

②

MTs

③

④

①

B wt

axon shaft

filopodium

growth cone

— MT
— linker
● vulnerable + end
● extending + end
⋯ cortical actin
— actin filament
— Efa6
— Shot

C *Efa6*

growth / split

branching

D *shot*

E *Efa6 shot*
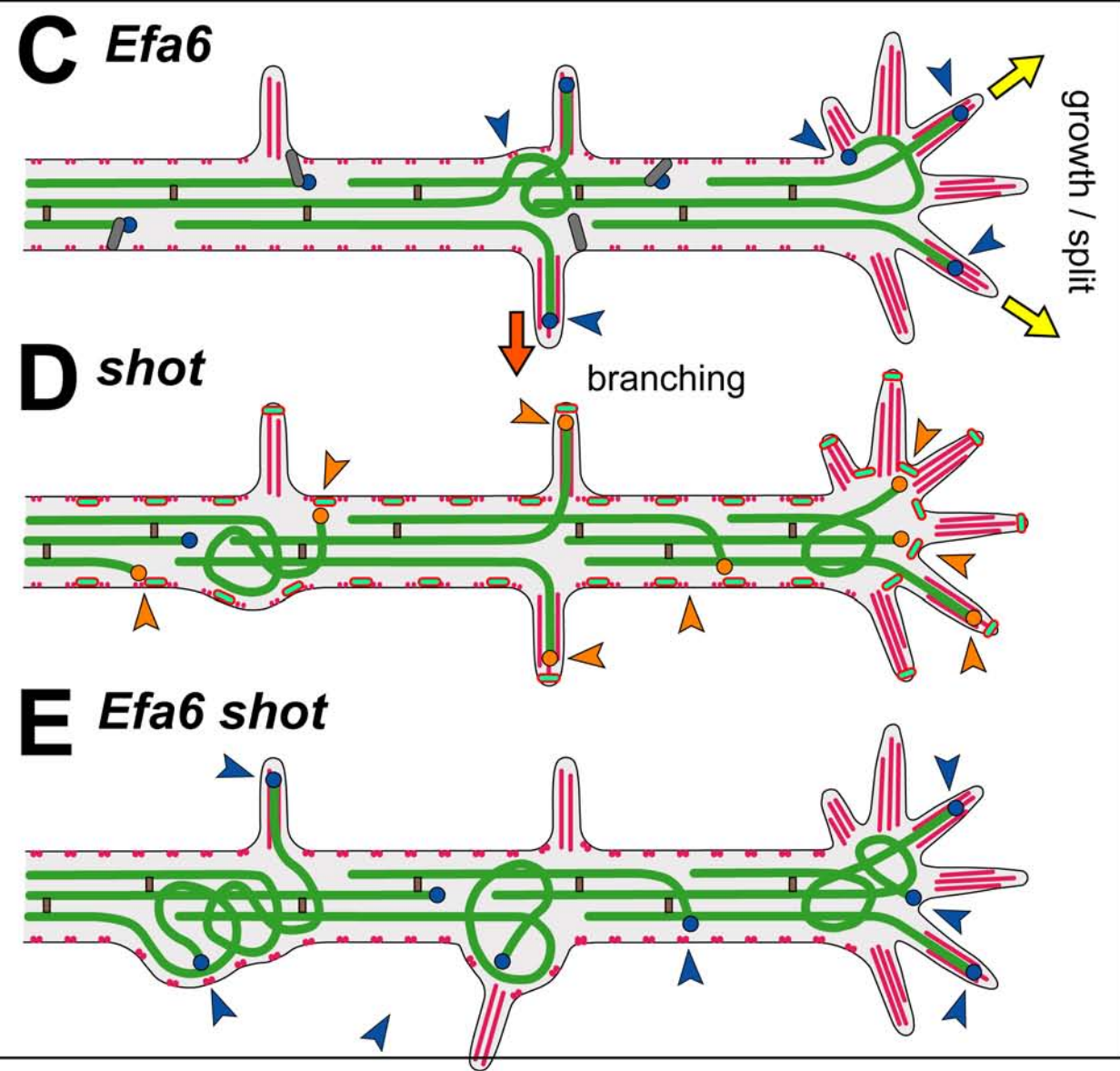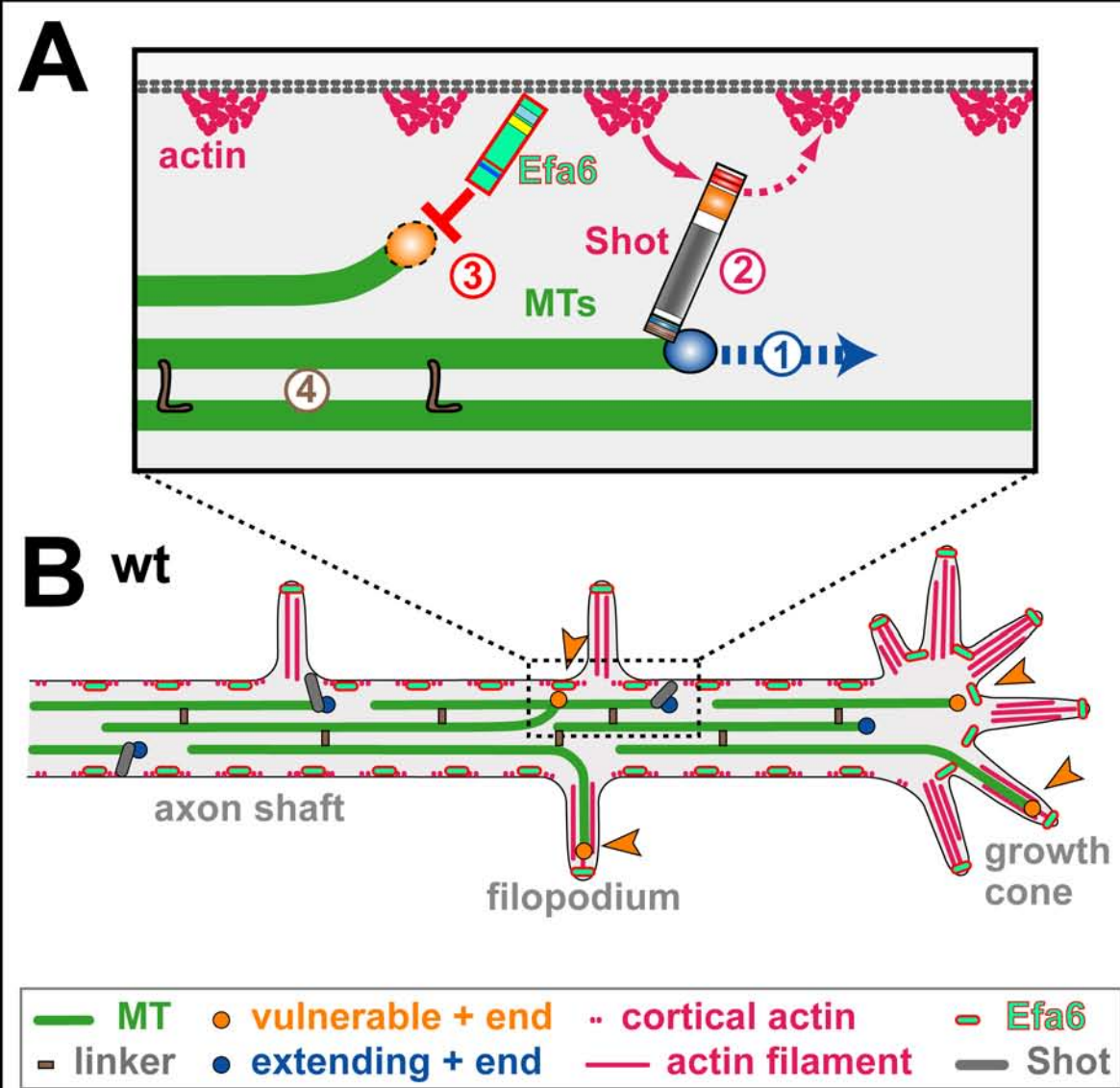
Fig. 10 Qu et al.

A Full length EFA6 orthologs
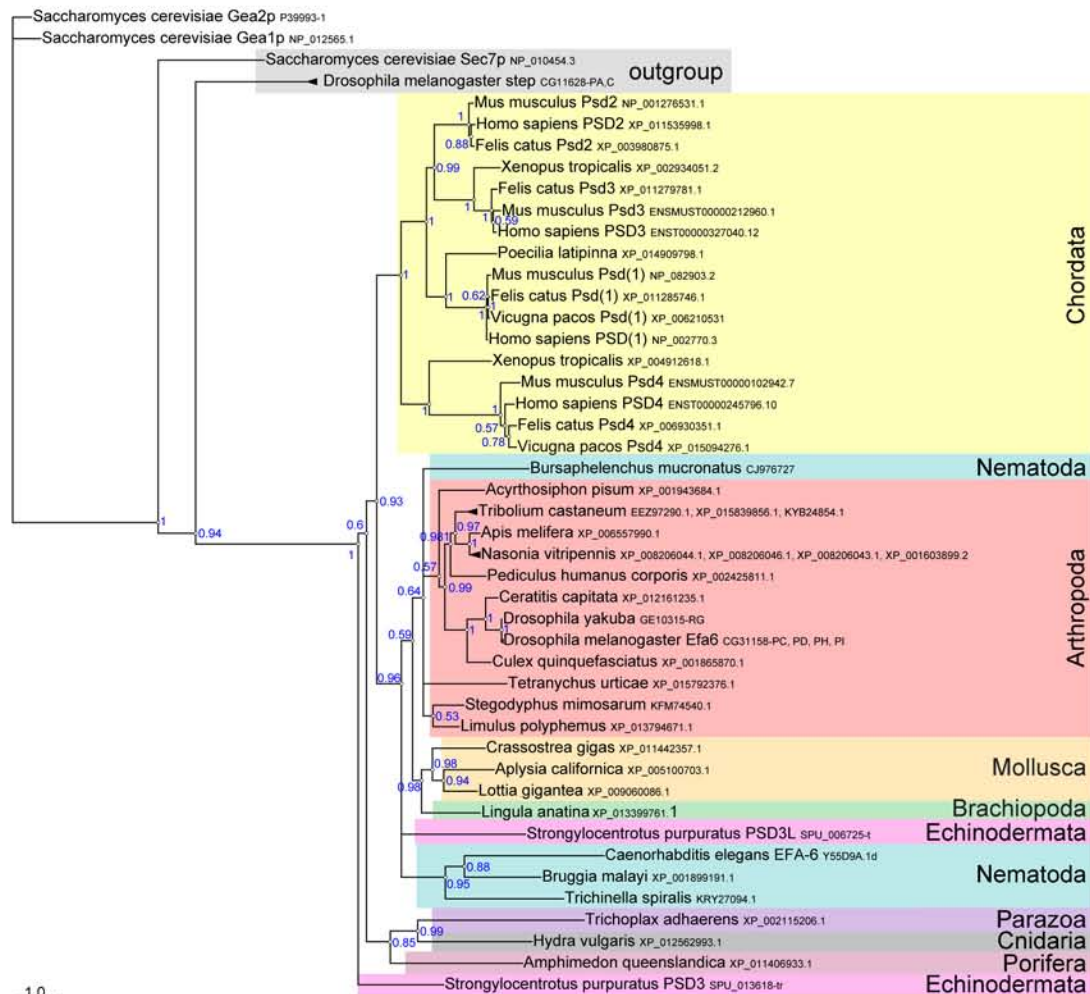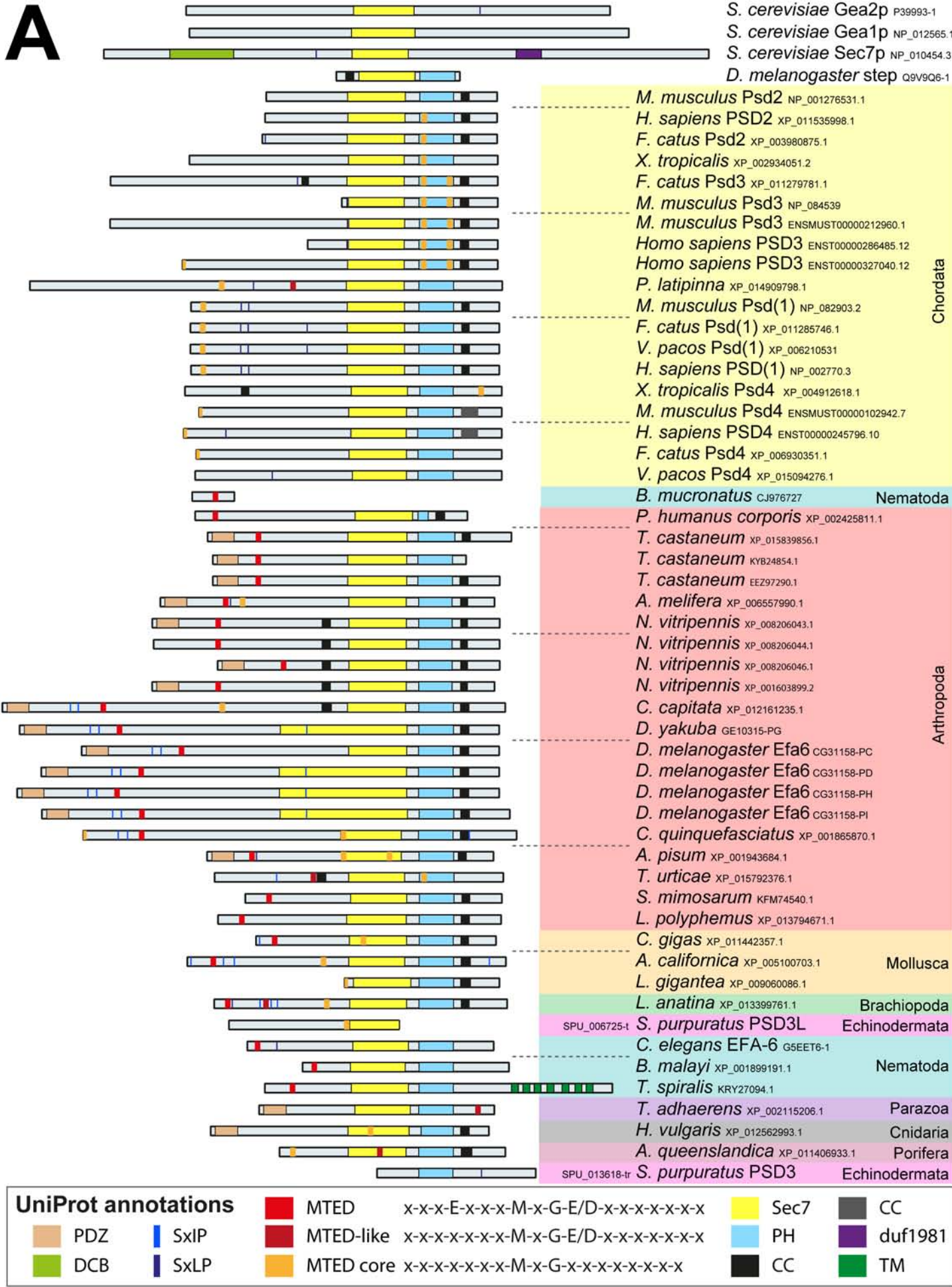
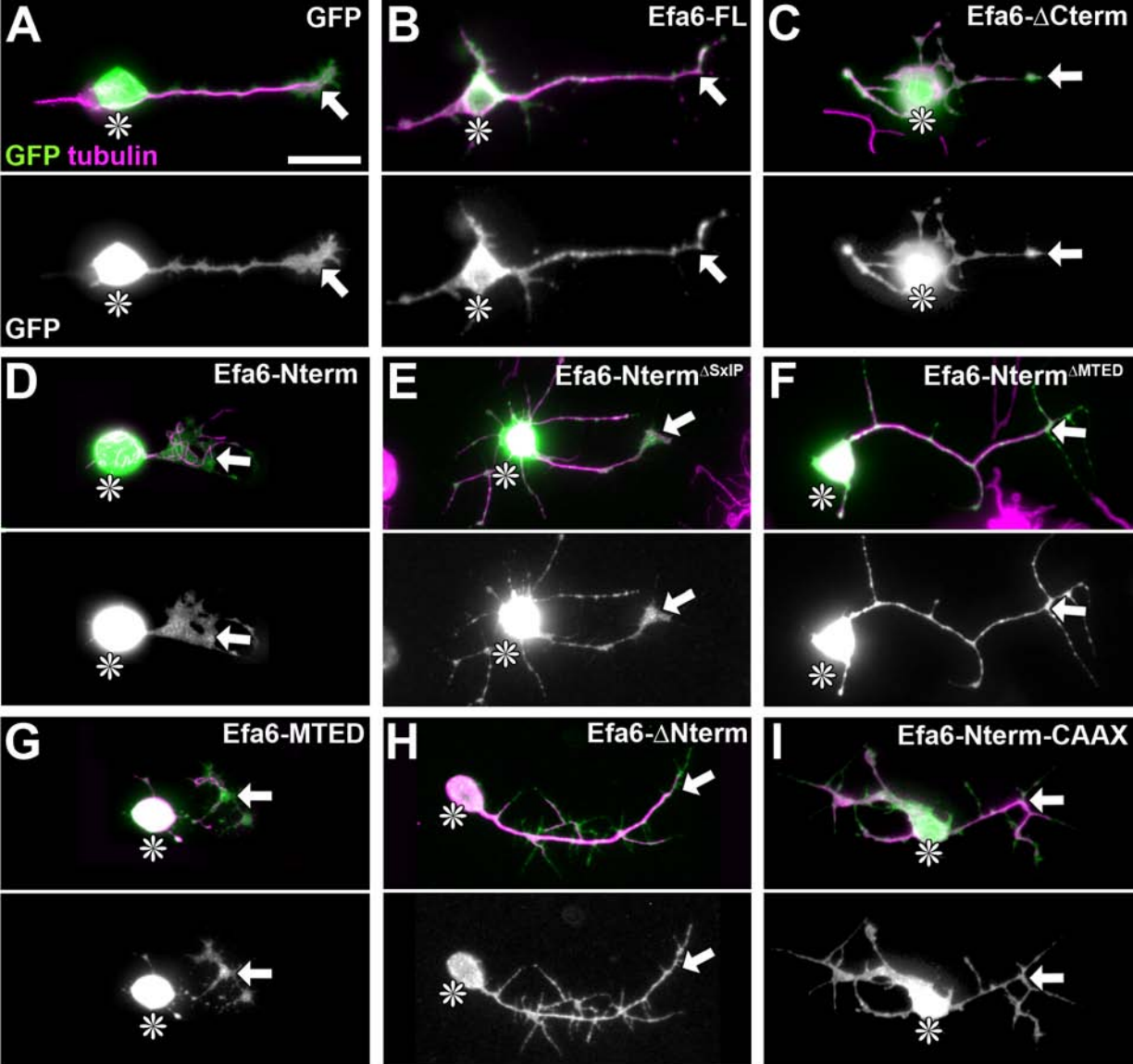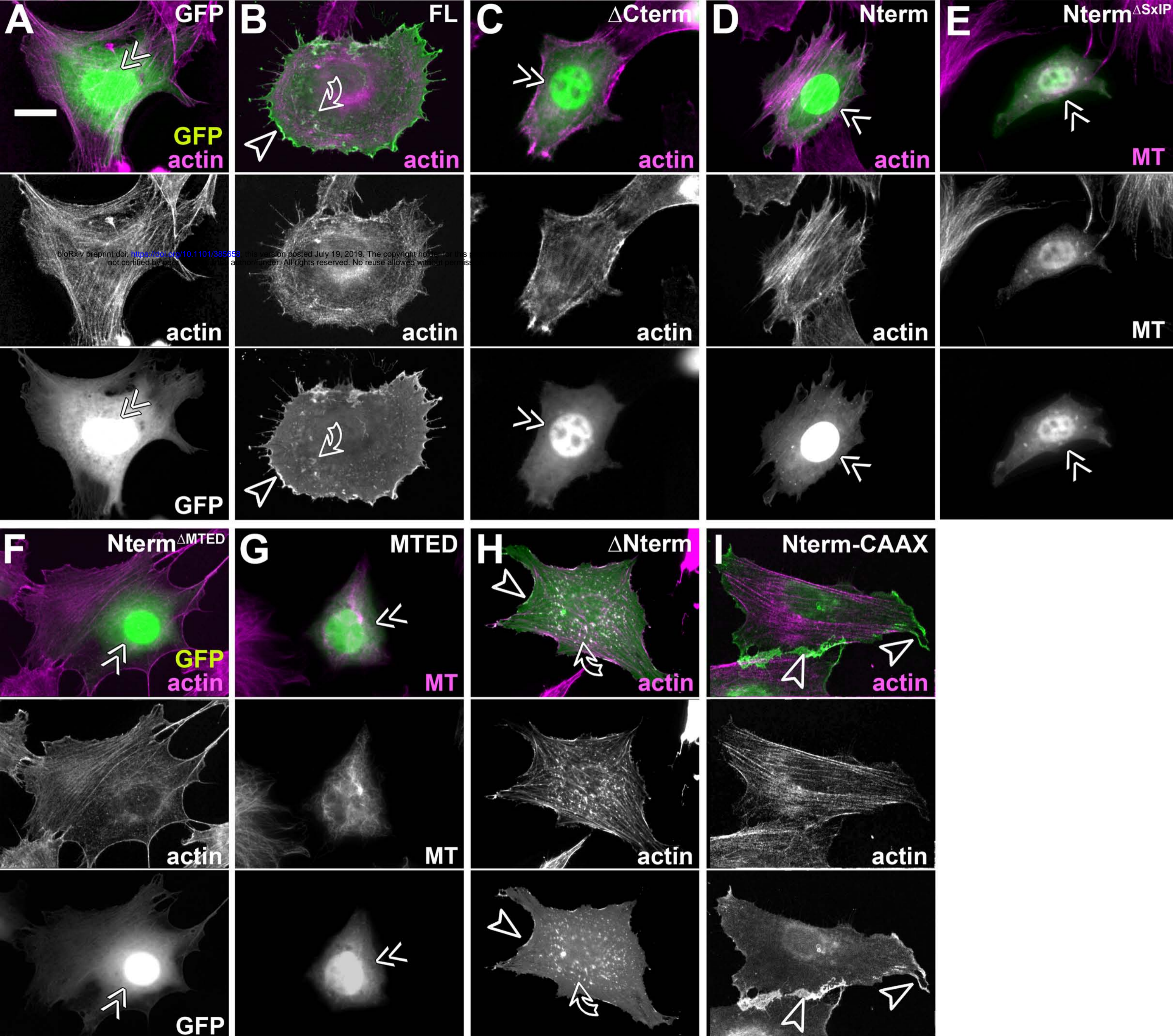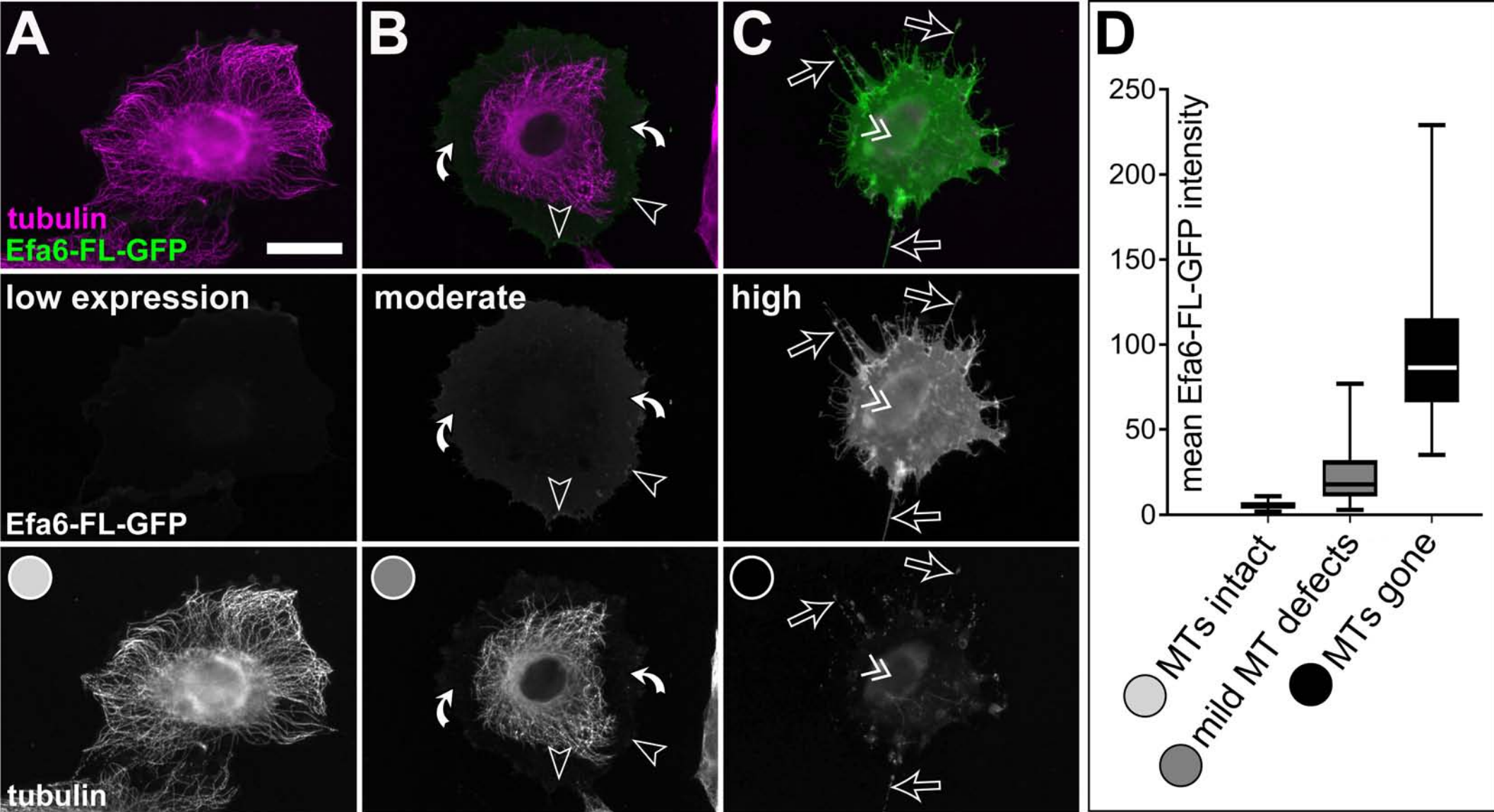**B** N-termini of EFA6 orthologs

Fig. S1 Qu et al.
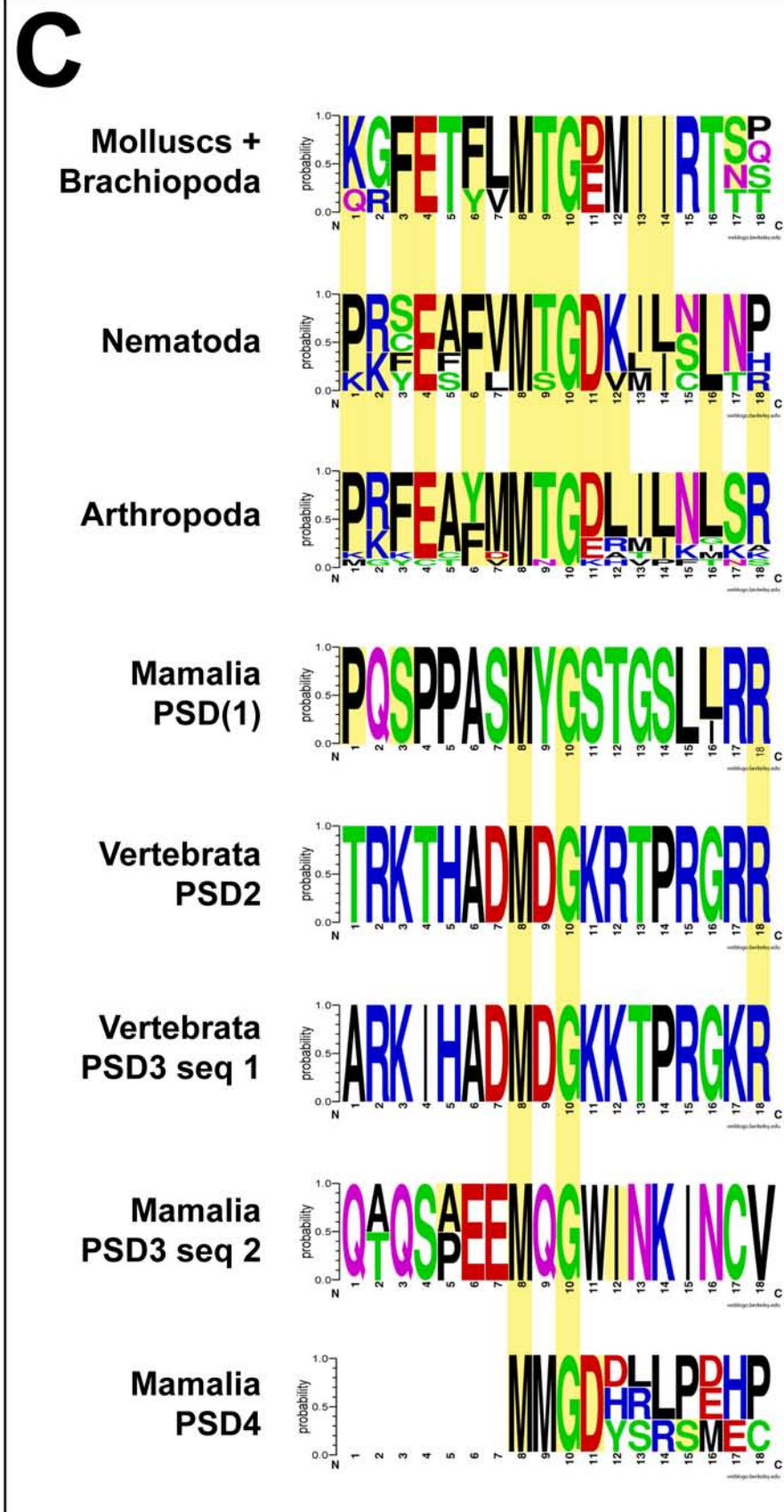
Fig. S2 Qu et al.

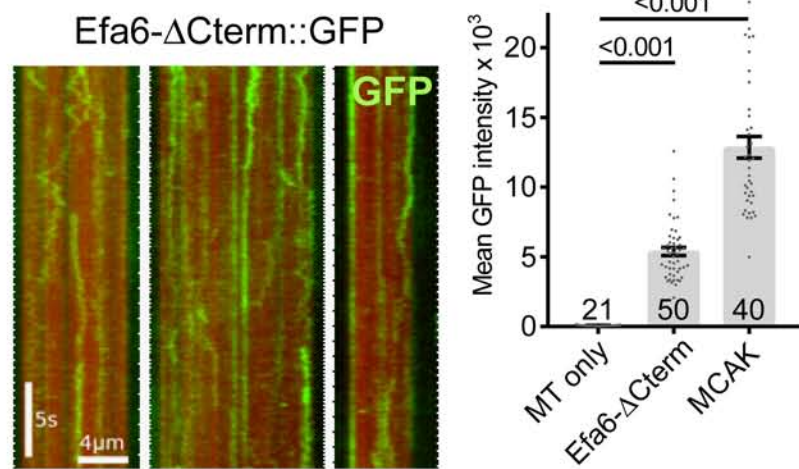Fig. S3 Qu et al.

A GFP — GFP / actin — actin — GFP

B FL — actin — actin

C ΔCterm — actin — actin

D Nterm — actin — actin

E Nterm^ΔSxIP — MT — MT

F Nterm^ΔMTED — GFP / actin — actin — GFP

G MTED — MT — MT

H ΔNterm — actin — actin

I Nterm-CAAX — actin — actin
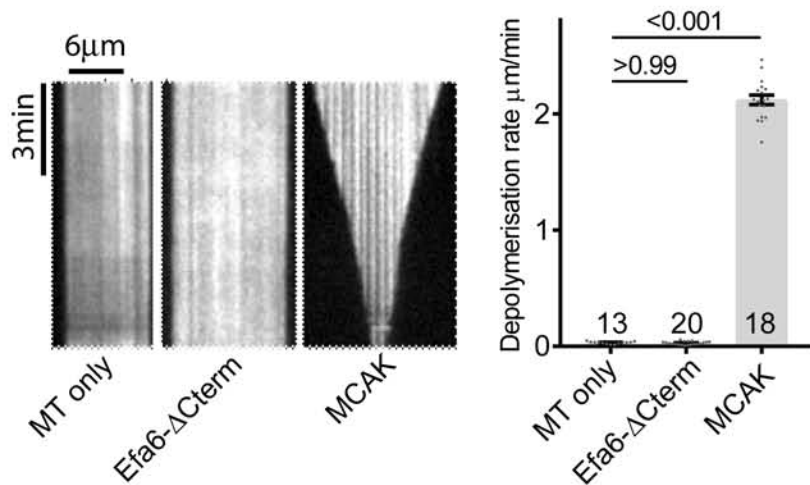
Fig. S4 Qu et al.

Fig. S5 Qu et al.

Fig. S6 Qu et al.

A — GFP	B — FL	C — ΔCterm
D — Nterm	E — Nterm-CAAX	F — Nterm^ΔSxIP
G — Nterm^ΔMTED	H — MTED	I — ΔNterm

☐ no MT defects   ◼ mild MT defects   ■ MTs gone
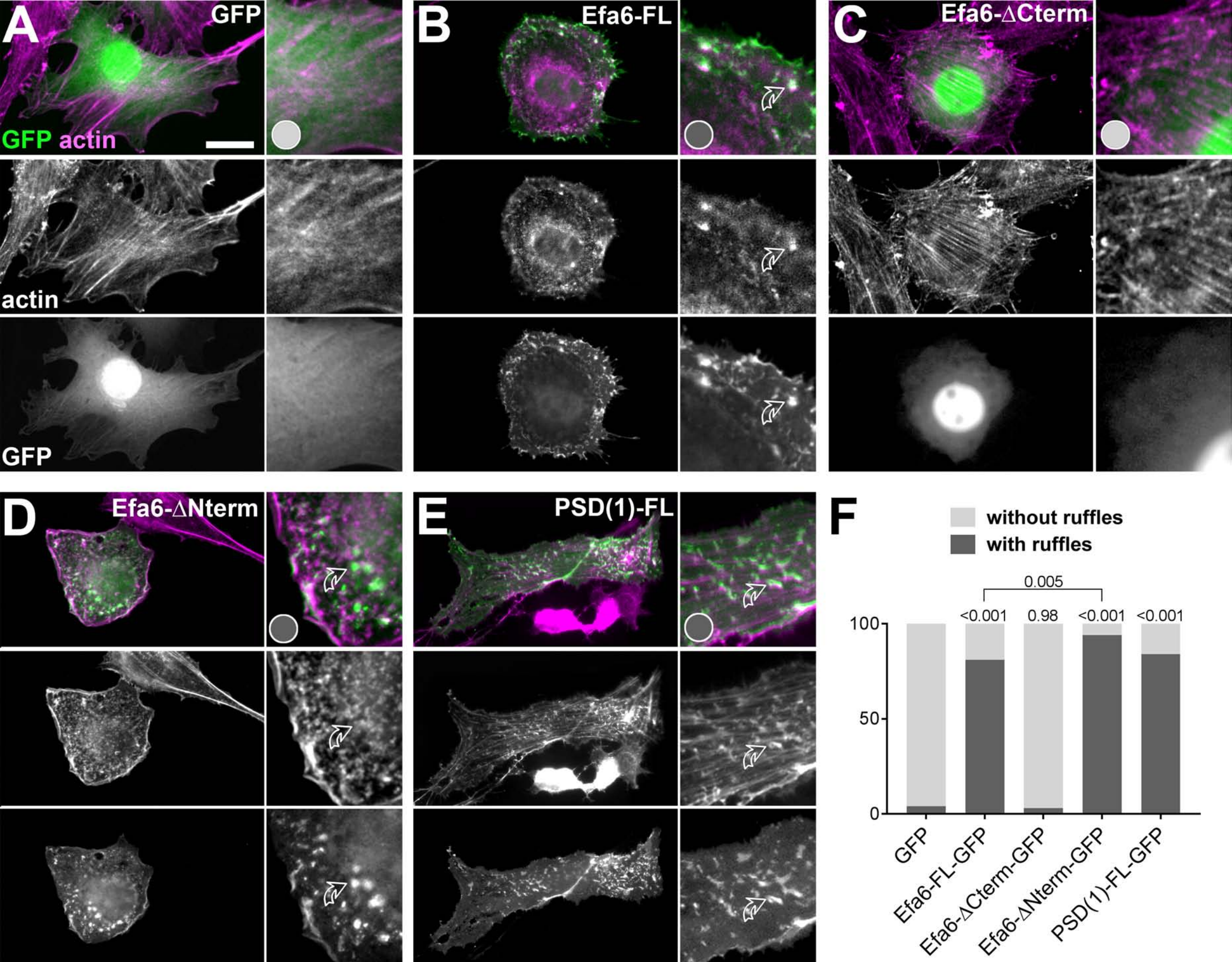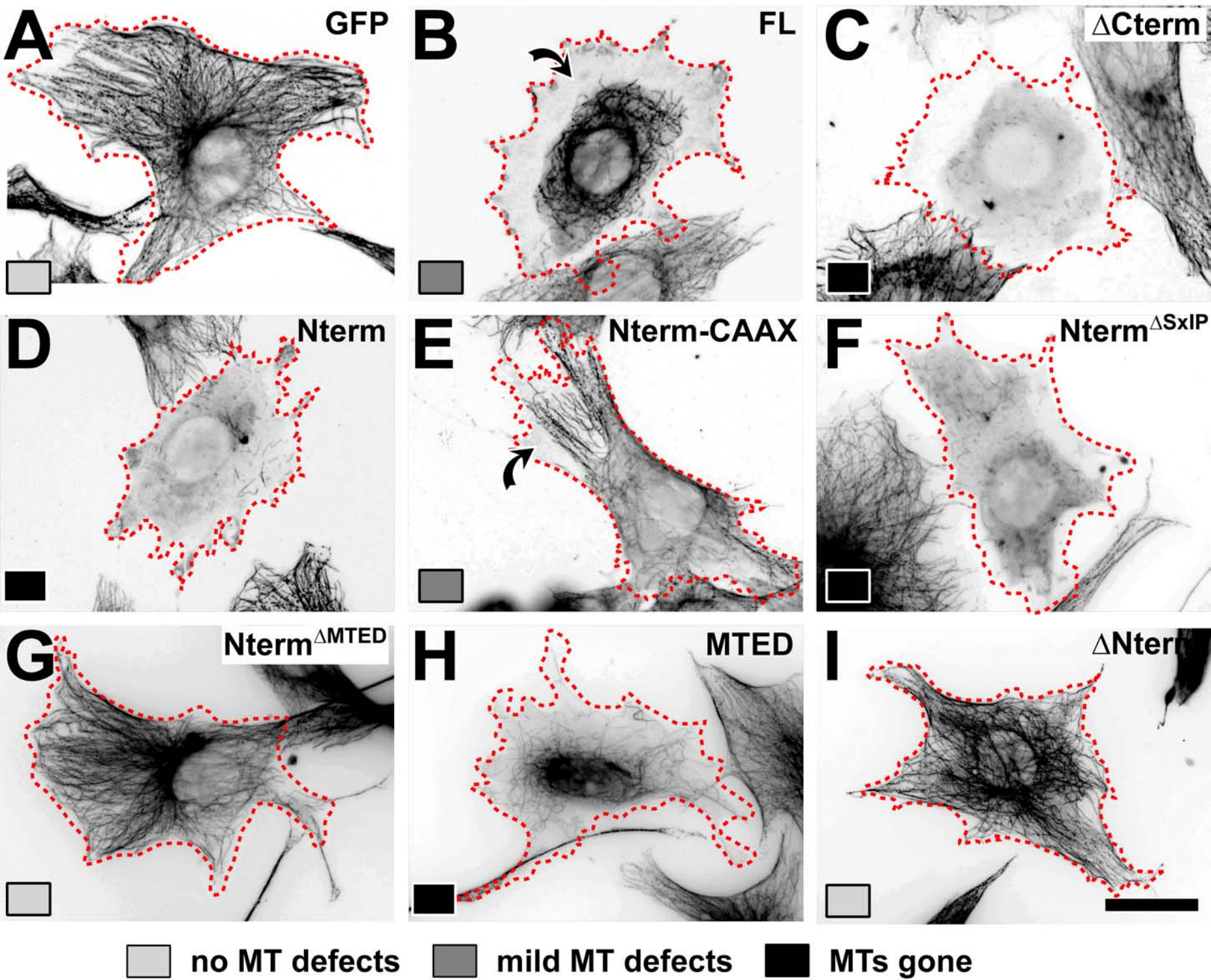
Fig. S7 Qu et al.

A *In vitro* MT binding assay (TIRF)

Efa6-ΔCterm::GFP

GFP

5s

4μm

Mean GFP intensity x 10^3

<0.001

<0.001

MT only (21) | Efa6-ΔCterm (50) | MCAK (40)

B *In vitro* MT depolymerisation assay

6μm

3min

MT only | Efa6-ΔCterm | MCAK

Depolymerisation rate μm/min

<0.001

>0.99

MT only (13) | Efa6-ΔCterm (20) | MCAK (18)

C MT depolymerisation assay on *Xenopus* extract

before wash | 10s after wash | 120s after wash

MCAK

Efa6-ΔC

D control

normal light

fluorescence

E Efa6-Nterm

normal light

fluorescence

Fig. S8 Qu et al.

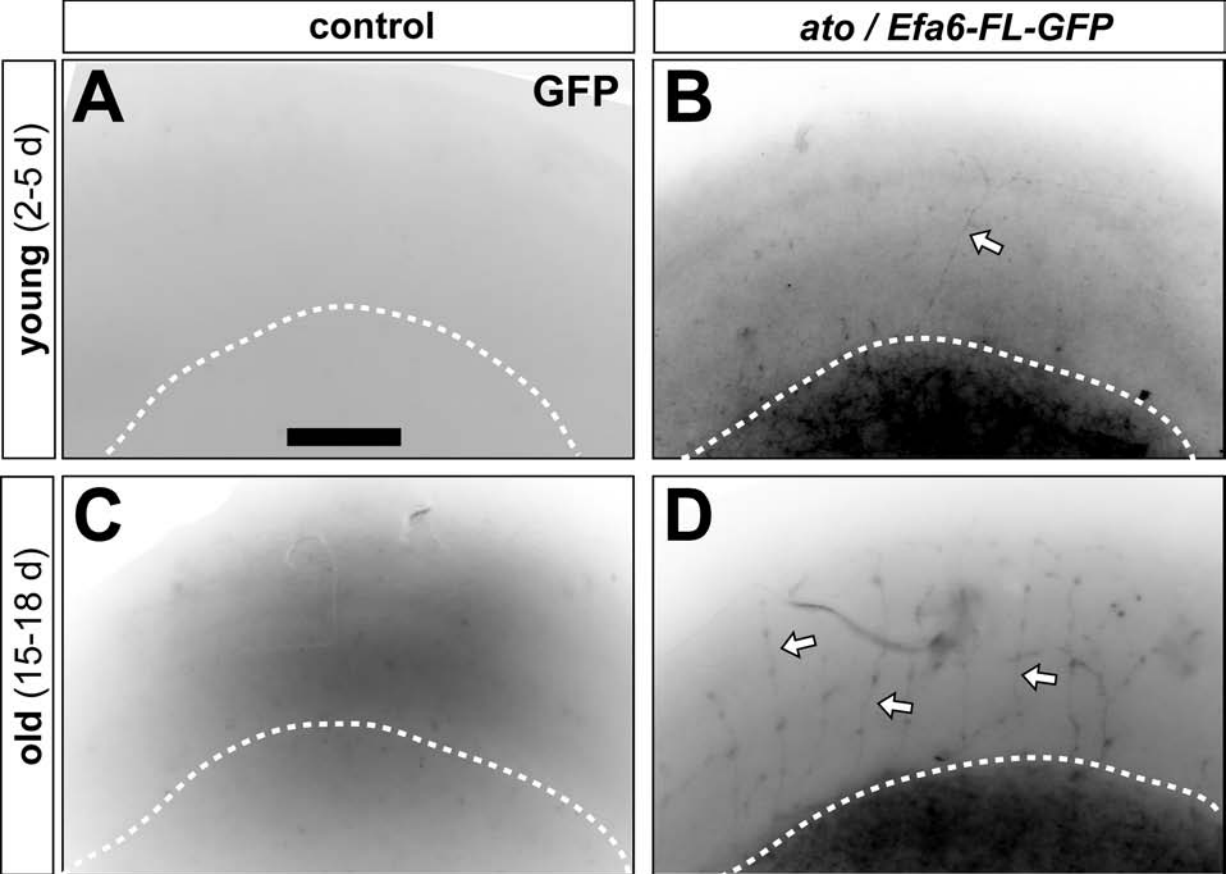|	control	*ato / Efa6-FL-GFP*
young (2-5 d)	**A** GFP	**B**
old (15-18 d)	**C**	**D**
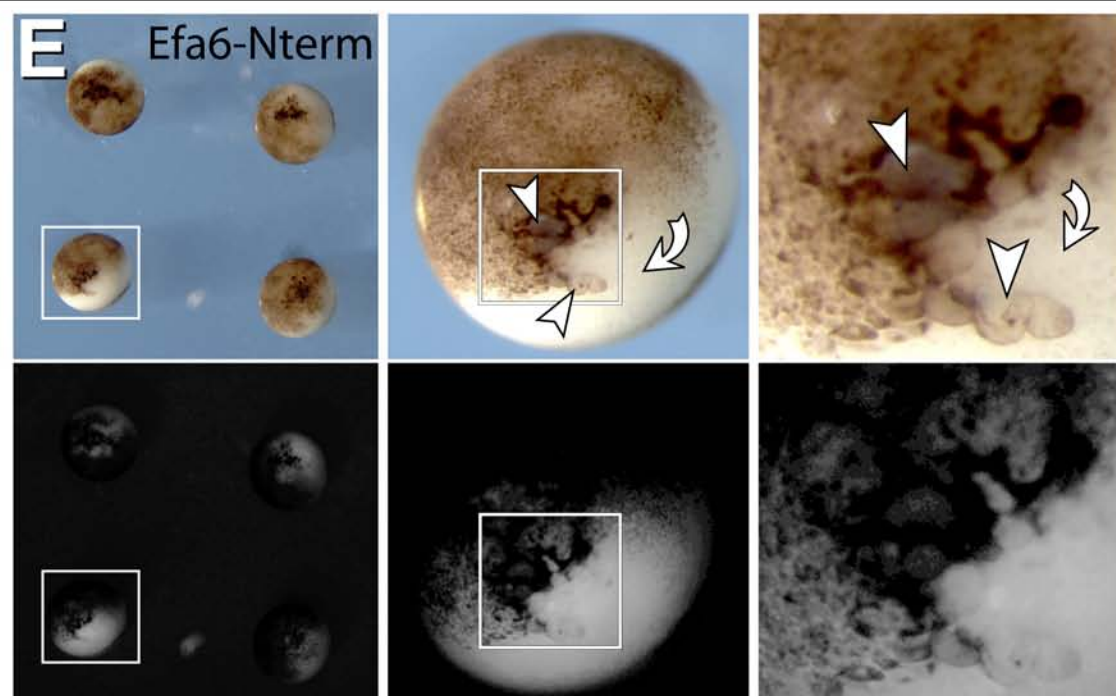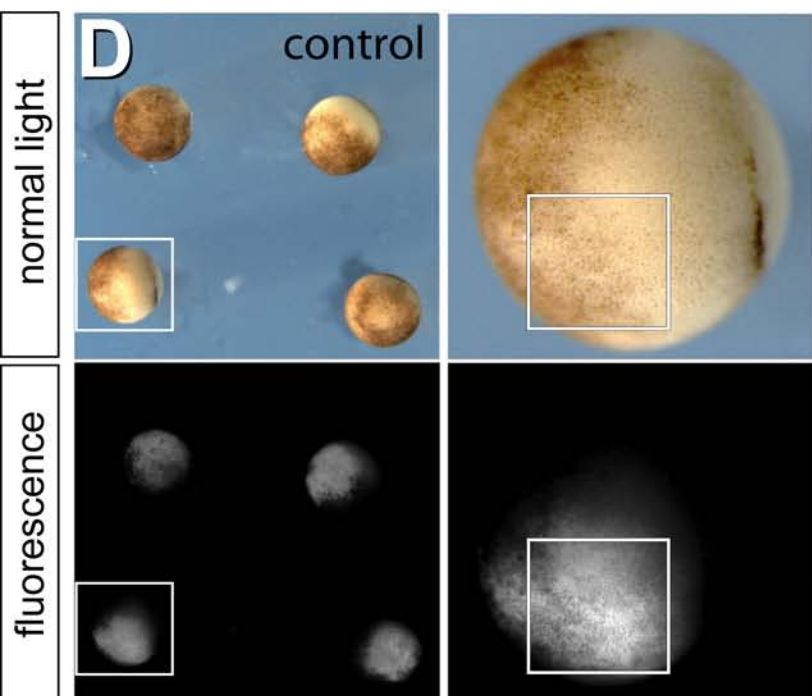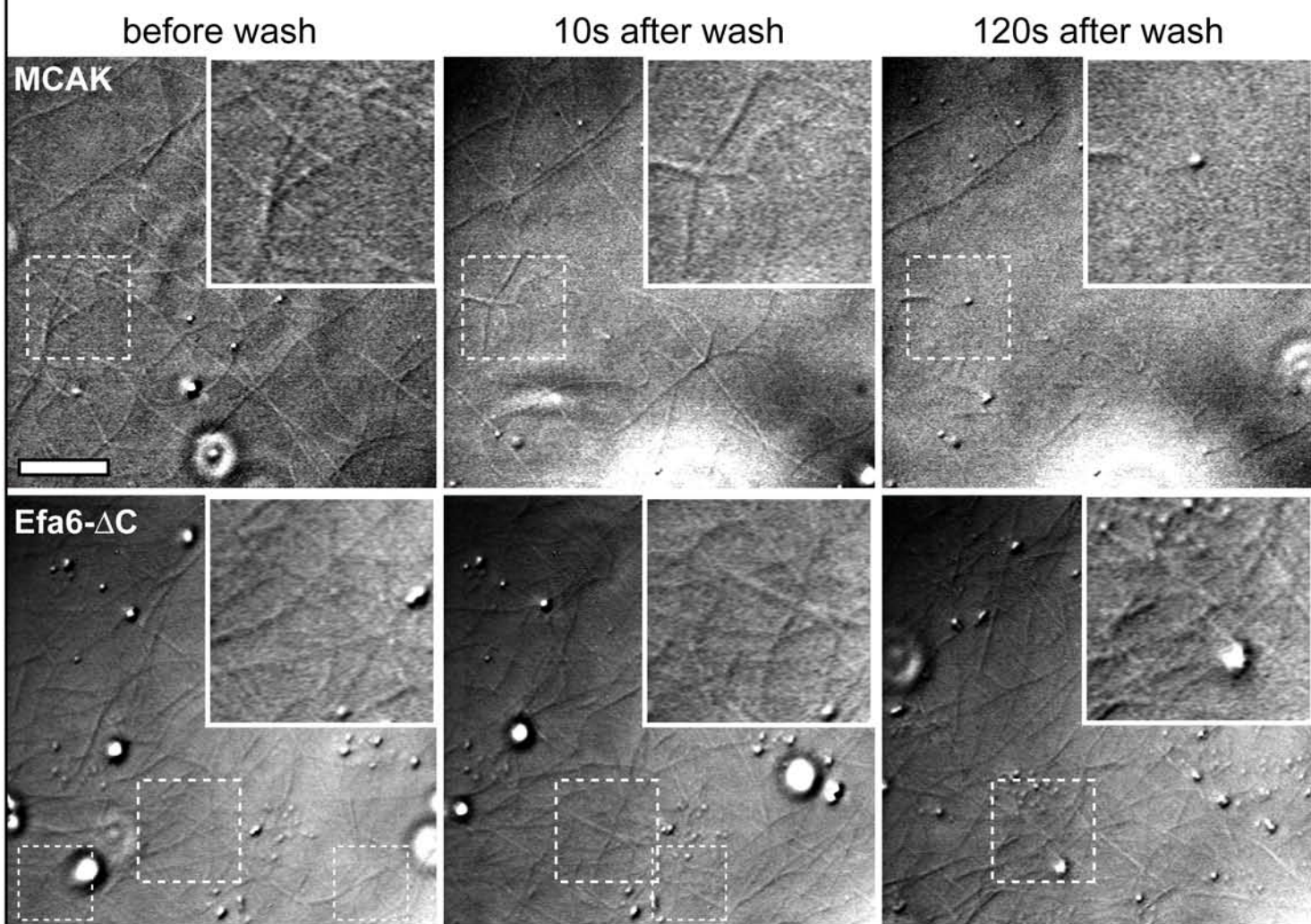
Fig. S9 Qu et al.

MT disorganisation in *Efa6* mutants (binary data)

light: all MTs bundled
dark: with disorganised MTs

Fig. S10 Qu et al.