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Xenopus cytoplasmic linker–associated protein 1 (XCLASP1) promotes axon elongation and advance of pioneer microtubules

Astrid Marx,a,b,*, William J. Godinezc,*, Vasil Tsimashchukc, Peter Bankheadb, Karl Rohra,c, and Ulrike Engela,b

aExzellenzcluster CellNetworks, University of Heidelberg, 69120 Heidelberg, Germany; bNikon Imaging Center and Centre for Organismal Studies, University of Heidelberg BioQuant, 69120 Heidelberg, Germany; cInstitute of Pharmacy and Molecular Biotechnology, University of Heidelberg, and Department of Bioinformatics and Functional Genomics, Biomedical Computer Vision Group, Deutsche Krebsforschungszentrum, 69120 Heidelberg, Germany

ABSTRACT Dynamic microtubules (MTs) are required for neuronal guidance, in which axons extend directionally toward their target tissues. We found that depletion of the MT-binding protein Xenopus cytoplasmic linker–associated protein 1 (XCLASP1) or treatment with the MT drug Taxol reduced axon outgrowth in spinal cord neurons. To quantify the dynamic distribution of MTs in axons, we developed an automated algorithm to detect and track MT plus ends that have been fluorescently labeled by end-binding protein 3 (EB3). XCLASP1 depletion reduced MT advance rates in neuronal growth cones, very much like treatment with Taxol, demonstrating a potential link between MT dynamics in the growth cone and axon extension. Automatic tracking of EB3 comets in different compartments revealed that MTs increasingly slowed as they passed from the axon shaft into the growth cone and filopodia. We used speckle microscopy to demonstrate that MTs experience retrograde flow at the leading edge. Microtubule advance in growth cone and filopodia was strongly reduced in XCLASP1-depleted axons as compared with control axons, but actin retrograde flow remained unchanged. Instead, we found that XCLASP1-depleted growth cones lacked lamellipodial actin organization characteristic of protrusion. Lamellipodial architecture depended on XCLASP1 and its capacity to associate with MTs, highlighting the importance of XCLASP1 in actin–microtubule interactions.

INTRODUCTION

Axon guidance is essential in the formation of the nervous system, where neurons make connections over long distances. The growth cone at the tip of the axon plays a key role in this process. This is where positional cues are perceived by membrane-associated receptors and integrated to regulate cytoskeletal dynamics (reviewed by Dent and Gertler, 2003; Lee and Van Vactor, 2003). Actin polymerization in lamellipodia and filopodia drives growth cone advance, whereas microtubules (MTs) are abundant in the axon- and the organelle-rich center of the growth cone. The interactions between actin and MTs are important for the function of both networks. During axon extension, the splayed array of MTs in the growth cone needs to be bundled (engorgement; Dent and Gertler, 2003), and this requires actin contractile structures (actin arcs; Burnette et al., 2007) and MT-binding proteins (Neukirchen and Bradke, 2011). A subset of dynamic MTs, the so-called pioneer microtubules, explore the actin-rich periphery and have been studied for their role in axon guidance (Sabry et al., 1991; Tanaka and Kirschner, 1991; Tanaka et al., 1995; Tanaka and Kirschner, 1995; Dent and Kalil, 2001; Zhou and Cohan, 2004). Dynamic MTs in the periphery are required for adhesion (Suter et al., 2004), and localized stabilization of MTs can induce growth cone turning (Buck and Zheng, 2002). Plus end–binding proteins (+TIPS) are of particular interest, as they

*These authors contributed equally.

†Address correspondence to: Ulrike Engel (ulrike.engel@bioquant.uni-heidelberg.de).

Abbreviations used: EB3, end-binding protein 3; MT, microtubule; SIM, structured illumination microscopy; XCLASP1, Xenopus cytoplasmic linker–associated protein 1.

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track the growing ends of MTs and can serve as adaptors for actin-binding proteins (reviewed by Akhmanova and Steinmetz, 2008; van der Vaart et al., 2009).

Orbit/MAST, one of the +TIPS originally identified by its mitotic phenotype (Inoue et al., 2000; Lemos et al., 2000), is involved in axon guidance in Drosophila (Lee et al., 2004). Orbit’s homologues in vertebrates are the cytoplasmic linker protein-associated proteins 1 and 2 (CLASP1/2; Akhmanova et al., 2001), which have been shown to regulate MT dynamics selectively at the cell periphery of HeLa cells (Mimori-Kiyosue et al., 2005) and stabilize MTs close to focal adhesions (Lansbergen et al., 2006). In neurons, a recent study highlights a dual role for CLASP in promoting or inhibiting axon extension depending on the levels of GSK-3 (Hur et al., 2011). Whereas in other vertebrates the redundancy between CLASP1 and 2 complicates analysis, in Xenopus there is only one CLASP, which is a homologue of vertebrate CLASP1 (Hannak and Heald, 2006), referred to in this article as Xenopus cytoplasmic linker-associated protein 1 (XCLASP1).

For many MT-binding proteins a role in axon outgrowth has been established; however, our understanding of the precise role of MT dynamics in this process is limited. This is partly due to the lack of methods to quantify MT dynamics in neurons. To overcome this, we developed automated tracking of MT plus ends in growth cones. When MTs are labeled by fluorescent tubulin, analysis of MT dynamics is limited to regions of low MT density (e.g., the periphery) and relies on manual tracking (Tanaka and Kirschner, 1991; Tanaka et al., 1995; Tanaka and Kirschner, 1995; Dent and Kalil, 2001; Komarova et al., 2002). Detection of +TIPS to highlight growing MTs has been successfully used in neurons (Stepanova et al., 2003; Lee et al., 2004; Ma et al., 2004).

The need for automated tracking approaches has been widely recognized, and automated tracking programs for +TIPS have been developed and used in epithelial cells (Matov et al., 2010; Applegate et al., 2011). Automation is required because of the stochastic nature of MT dynamics (Mitchison and Kirschner, 1984), which demands the analysis of many tracks to analyze knockdown phenotypes (Nishimura et al., 2012). In neurons, semiautomated tracking of end-binding protein 3 (EB3) comets along neurites based on kymographs was used by Stepanova et al. (2010). This approach was successful for the axon shaft, where the comets follow a predetermined direction. In growth cones, dynamic MTs follow individual, curved tracks, and an alternative solution is therefore required.

We developed automated tracking of MT plus ends in growth cones to examine CLASP’s role in MT dynamics and axon outgrowth in embryonic spinal cords of Xenopus laevis. We used low-level expression of fluorescent +TIP EB3 (Stepanova et al., 2003, 2010) and low–temporal resolution time lapse compatible with extended imaging of neurons. We found that XCLASP1 promotes axon extension and is a positive modulator of MT plus ends in the growth cone. Similar to XCLASP1 depletion, Taxol reduced EB3 comet velocities, underlining the importance of MT dynamics for growth cone advance. In addition to its role in the regulation of pioneer MTs, we found XCLASP1 to be important for lamellipodial actin architecture.

**RESULTS**

**XCLASP1 is up-regulated during axon outgrowth and promotes axon extension**

In situ hybridization revealed that the mRNA expression of XCLASP1 in the nervous system was developmentally regulated. In stage 28 embryos, XCLASP1 mRNA was strongly enriched in spinal cord, spinal cord motor neurons, and cranial nerves (Figure 1A; Park et al., 2012). In the fully motile tadpoles of stage 41, XCLASP1 mRNA could no longer be detected by in situ hybridization (Figure 1, compare B and C). This pattern suggests that XCLASP1 is up-regulated in spinal cord and other motor nerves during their development and decreases dramatically after axon outgrowth is completed. An antibody against XCLASP1 (Xorbit antibody; Hannak and Heald, 2006) recognizes XCLASP1 in growth cones of stage 28 embryos, where it localizes to MT tips and only to a lesser extent along the MT shaft (Figure 1D). This distribution is mimicked by enhanced green fluorescent protein (EGFP)–XCLASP1ΔN (Supplemental Movie S1) and resembles the localization of human EGFP-CLASP2y (Figure 2A; Lee et al., 2004).

To investigate the role of XCLASP1 in axon outgrowth, we used a loss-of-function approach. We depleted XCLASP1 selectively in the nervous system by injecting a morpholino oligonucleotide (XCLASP1 MO) into the early embryo (see Materials and Methods). Efficiency of depletion was confirmed by immunocytochemistry in neurons (Supplemental Figure S1A) and by Western analysis in Xenopus epithelial cells (Supplemental Figure S1B). Neuronal outgrowth rates were significantly reduced in XCLASP1 knockdown cultures (Figure 1E). Of interest, when comparing the growth path to the direct distance from origin to end of the track, we detected increased curved outgrowth for XCLASP1-depleted axons (see later discussion of Figure 6F). Low concentrations of Taxol likewise decreased axon extension and resulted in a loss of growth directionality (Figure 1, G and H). Because both depletion of the +TIP CLASP and the microtubule drug Taxol resulted in attenuated axon extension, we wanted to investigate how MT dynamics and growth cone advance are related.

**Automated MT plus-end tracking in neuronal growth cones allows analysis of large data sets**

To analyze MT dynamics in growth cones, we used EB3-EGFP or EB3-mCherry to visualize growing MT plus ends in X. laevis spinal cord growth cones (Figure 2, A and C). EB1 and EB3 are well-known markers for growing MTs (Mimori-Kiyosue et al., 2000; Stepanova et al., 2003; Morrison, 2007). To gauge expression levels, we compared EB3-mCherry signal (Figure 2C) to the endogenous pattern of EB1 as detected by a commercial antibody (Figure 2B). The expression resulting from injection of EB3-EGFP or EB3-mCherry capped mRNA (see Materials and Methods) resulted in the same pattern as observed for the endogenous EB1. We found that EB3 fused to fluorescent proteins localized on MT plus ends, where it localized in a comet-like manner. This is in contrast to EGFP-CLASP2y expressed in X. laevis neurons, which we found to localize also to the MT lattice (Figure 2A; Lee et al., 2004). EB3-mCherry localized to plus ends in growth cone and shaft (Figure 2A), as did EGFP-CLIP-170, whereas CLASP2y showed a preference for growth cone MTs (Figure 2A; Lee et al., 2004). We therefore used EB3-mCherry at low expression for MT plus-end visualization (Figure 2C and Supplemental Movie S2). EB3-mCherry comets can be clearly separated from each other, and single MT plus ends can be followed over many frames (Figure 2D, arrow, and Supplemental Movie S2).

To quantify the MT dynamics in wide-field fluorescence time-lapse sequences, we developed a probabilistic approach for tracking EB3 comets in movies with very moderate temporal resolution (one image every 3 s). To localize comets within an image, we used an approach based on two-dimensional (2D) Gaussian fitting (Wörz et al., 2010). The positions over time of the individual comets were estimated using an approach based on Kalman filters (Godinez et al., 2009; see Materials and Methods for detailed description).

When we compared all the tracks visible in the image sequence to the tracks that were automatically recognized (Figure 2E, green lines) it became evident that the majority of tracks were recognized,
were almost identical (6.98 ± 2.34 μm/min in automated tracking and 6.99 ± 2.37 μm/min in kymograph; Figure 3A). Finally, when we verified our tracking on a wider range of image sequences, we compared the mean velocities of 244 tracks obtained in 37 sequences with the results obtained by kymograph analysis in the same sequences. Comparing results, we did not find a significant difference (Figure 3B), demonstrating that our automated tracking of EB3-EGFP comets is a reliable method to assess MT dynamics.

Our mean MT advance rates are lower than reported earlier for Xenopus growth cones (Lee et al., 2004). In three independent experiments in this study, mean plus-end velocities differed by around 2 μm/min (Table 1). Frog cultures are kept and imaged at room temperature, which is not tightly controlled and can change MT dynamics. For statistical analysis data from different days was therefore normalized to the wild-type values of a given day.

We also wondered whether short pauses contributed to lower average velocities. When we analyzed instantaneous velocities, these rarely dropped to <1 μm/min within a track (nine events in 560 tracks).

Low Taxol concentration and XCLASP1 depletion reduce MT dynamics and axon outgrowth

Taxol-treated neurons showed decreased axon extension similar to XCLASP-depleted neurons. We therefore sought to learn how
MT advance in Xenopus spinal cord neurons.

When we analyzed MT plus-end dynamics by automated tracking in XCLASP1-depleted growth cones, we detected that MTs in XCLASP1 knockdown growth cones had reduced velocities compared with control morpholino and wild type (Figure 4A). This was due to a significant decrease in track lengths (Figure 4B), whereas comet lifetimes were even slightly increased (Figure 4C). In an experiment that directly compared morpholino-injected neurons to those with Taxol treatment, the plus-end velocity was shifted to 5 μm/min for both Taxol treatment and XCLASP1 depletion, whereas MT velocities of control morpholino and untreated neurons in the growth cone peaked around 6–7 μm/min (Figure 4D). Although we see differences in the way Taxol affects lifetimes, Taxol and XCLASP1 both reduce pioneer MT advance into the growth cone periphery, and this is correlated with decreased axon extension (Figure 1, E and G). The sustained lifetime in XCLASP1-depleted cells points to fewer transitions to catastrophe or pause. In the context of growth cone morphology, this suggests that in XCLASP1-depleted cells, most plus ends travel to the growth cone periphery before catastrophe occurs but need more time to reach it. In addition to reduced growth, MT plus ends followed more curved routes in XCLASP1-depleted growth cones, resulting in less directional advance of MTs into the periphery (Figure 4E).

MT dynamics differs in axon shaft, growth cone, and filopodia

In the axon, MTs are aligned parallel to one another and polymerization occurs mainly in the anterograde direction (Stepanova et al., 2003, 2010). MTs in the axonal shaft therefore grow predominantly in direction of the growth cone neck, where they splay out and grow at different angles toward the periphery (Supplemental Movie S2). We sought to investigate the difference in dynamics of MTs in the axon and subcompartments of the growth cone. On the basis of the stereotypical behavior of MT ends advancing from the growth cone center to the periphery (see tracks in Figure 2C and Supplemental Movie S2), we first used a very simple method to obtain information about MT behavior in the periphery. We analyzed the life histories of tracks. Starting from the end of the track (catastrophe or pause), we compared the final five frames with the five frames before them. The velocity of the five last frames was on average 12% lower than that of the previous frames (Supplemental Table 1), indicating that in the growth cone transition to catastrophe or pause is preceded by a reduction in advance rate. In the axon shaft, the same analysis showed that MTs slowed by only 5% or less in their final five frames before disappearance of axonal outgrowth was connected to MT dynamics. In Taxol-treated spinal cord cultures, we detected a dose-dependent reduction of plus-end advance in the growth cone (Figure 3, C and D). Motile EB3 comets are visible with up to 10 nM Taxol (Supplemental Movie S3), demonstrating that polymer is still added at this concentration. Using automatic tracking of plus ends, we observed that 2.2 nM Taxol was the concentration that had a half-maximal effect (IC50) on advance of growth cone MTs (Figure 3D). Track length was approximately halved (Figure 3E), whereas track lifetime was unaffected by concentrations up to 3.3 nM and only decreased marginally at high concentrations (Figure 3F). In summary, Taxol at <10 nM concentration reduced axon outgrowth and MT advance in Xenopus spinal cord neurons.
To learn how MT behavior correlated with MT-actin interaction, we imaged EB3-mCherry together with Lifeact-EGFP (Figure 5 and Supplemental Movie S4), where Lifeact visualizes F-actin (Riedl et al., 2008). Actin structures localize predominantly to filopodia and lamellipodia in the periphery. (Figure 5, A and B, schematic). A typical example of an EB3 track passing from a region of low actin density into the actin-rich periphery is shown in detail in Figure 5D. After 18 s of growth, the track reaches a strong actin bundle at an angle of nearly 90°. The MT stops growing and intermittently loses the EB3-mCherry signal in the next frame. The effect of coupling to actin is visible in the velocity profile (Figure 5F): on contact with the bundle, there is a marked decrease in advance rates in the following time frames.

MT plus ends often enter the base of filopodia (Figure 5E; quantitation in Supplemental Table 2; Lee et al., 2004); however, when they do so, we always observed a decrease in advance rate (Figure 5G). To quantify MT growth rate changes induced by filopodia interactions, we developed an algorithm that automatically recognizes filopodia based on segmenting them as thin lines (Figure 5C; see Materials and Methods). This dynamic segmentation was then combined with our tracking software to divide tracks into two parts: the part where the EB3-EGFP tracks grow inside the central region, and the part after they enter filopodia. Our analysis in wild-type neurons revealed that MTs in the axonal shaft of wild-type neurons exhibit the highest growth velocity, with a mean of 10.2 μm/min, which decreased to 8.9 μm/min in the growth cone. When the plus ends entered filopodia they further slowed to 8.0 μm/min (Figure 5H). These changes in velocity were visualized by assigning a color code to individual tracks (Figure 5I; see Supplemental Movie S5). In XCLASP1-depleted neurons, comet velocities were significantly decreased compared with wild type and control morpholino in all compartments; however, the difference was most pronounced in the

### Table 1: Variation of comet velocities in growth cones.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
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<tbody>
<tr>
<td></td>
<td>wt XCLASP1 MO</td>
<td>wt XCLASP1 MO</td>
<td>wt XCLASP1 MO</td>
</tr>
<tr>
<td>Number of tracks analyzed</td>
<td>269 385</td>
<td>560 728</td>
<td>647 532</td>
</tr>
<tr>
<td>Comet velocity in growth cone (μm/min ± SEM)</td>
<td>7.2 ± 0.17 5.4 ± 0.14</td>
<td>10.5 ± 0.17 6.1 ± 0.10</td>
<td>9.6 ± 0.15 8.1 ± 0.15</td>
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growth cone and less pronounced in the shaft. The analyzed shaft in these experiments comprised only the first 50 μm closest to the growth cone because our field of view was limited by the objective and camera we used for EB3 imaging. To overcome this restriction, we conducted an additional study with a large camera sensor, which allowed us to image up to 150 μm of the shaft in addition to the growth cone (Figure 5J and Supplemental Movie S6). Indeed, when we compared the closest 40 μm of shaft (proximal) with the more distant part (distal; Figure 5J) we observed that plus-end tracks picked up speed as they approached the growth cone and then slowed strongly in the periphery of the growth cone (Figure 5K). In XCLASP1-depleted axons, this behavior differed, as plus-end velocities changed less as plus ends passed from the distal part of the axon to the growth cone and then slowed further in filopodia (Figure 5K). This suggests that XCLASP1 regulates MT dynamics in and near the growth cone but not in the axon closer to the cell body.

MTs undergo anterograde movement in the shaft and can couple to retrograde flow in the periphery

The reduced displacement rates of EB3 in the growth cone periphery and in filopodia could be caused by changed regulation of polymerization or physical coupling with actin retrograde flow. Association of EB3 with slow-growing plus ends argues for the MT to be still polymerizing; however it is impossible to decide whether decreased advance is caused by reduced polymerization or backward movement of the polymer.

To gain insight into whether MT polymer transport was involved, we injected embryos with low concentrations of Hilitye488-tubulin or EGFP-tubulin and used the uneven incorporation of fluorescent tubulin to visualize speckles of labeled tubulin in the polymer (speckle microscopy; Waterman-Storer and Salmon, 1998). This analysis was easiest to perform in the actin shaft because the abundance of polymer there provides good signal even at low tubulin concentrations. Of 24 examined spinal cord axons, 15 showed anterograde polymer transport up to 3 μm/min, whereas nine showed no net transport. The only instance in which we observed retrograde transport in the shaft was when a growth cone collapsed and the axon retracted. In the shaft both speckle movement and plus-end movement could be measured by kymograph analysis (Figure 6, C and D). By concurrent analysis of EB3 comet displacement and MT flow in the shaft of 10 axons, we detected a correlation between speckle advance and MT displacement (Figure 6E). Forward movement of MT polymer was described for Xenopus axons before and linked to active forward movement of the growth cone (Chang et al. 1998, 1999).

Assessment of polymer transport in the growth cone proved much more difficult because MTs undergo individual fates, and the outline of MTs is hard to identify with low label incorporation. In most growth cones, anterograde transport of MTs seemed to continue in the central domain of the growth cone (Figure 6D and Supplemental Figure S3B). In growth cones we observed MTs at the periphery that exhibited retrograde transport of several micrometers/minute (Figure 6, H′ and J′). Some of these MTs were polymerizing while being transported backward, as revealed by EB3 comets in the same sequences (Figure 6, I′). It became clear that stationary EB3 comets at the periphery (Figure 6I) were in a tug of war between retrograde transport and forward movement by polymerization (Figure 6, J, J′, and J′′). We measured rates of MT backward flow up to 5–6 μm/min (Figure 6, H′ and J′; see also Supplemental Figure S3, D and E) with a sharp transition in the central domain, where retrograde flow was almost completely attenuated (0.8 μm/min; Figure 6H). In the case of the EB3 comet that stayed for 12 s at the periphery (Figure 6I, d), the retrograde flow visible on the MT was around 5 μm/min (Figure 6J′) indicating that what looks like pausing is the net outcome of tubulin addition and polymer movement in the opposite direction. In these experiments we could not introduce an additional label to trace actin retrograde flow, but our measurement of actin retrograde flow in the growth cone periphery in parallel experiments show rates that are in agreement with the observed MT transport (4.2 ± 2.8 μm/min; Figure 6K). Our results indicate that MT coupling to actin in the periphery may contribute to slowed advance of MTs in the periphery.

When we quantified actin retrograde flow in XCLASP1-depleted growth cones, there was little difference between XCLASP1-depleted growth cones and control (Figure 6K), and the difference was not sufficient to explain the decreased advance of pioneer MTs
FIGURE 5: Advance rates of MT plus ends are reduced in the actin-rich leading edge of the growth cone. (A) Xenopus growth cone expressing EB3-mCherry (red) and Lifeact-EGFP (green) imaged at 3 s/frame. (B) Schematic view of localization of MTs, +TIPs (green), and actin in the growth cone. (C) Dynamic filopodia segmentation based on actin signal in A. (D) Detail (upper boxed region in A) of time lapse where an EB3 comet (position marked by arrowheads) advances toward the growth cone periphery and enters a region of high actin density. The yellow line marks the result of the automated tracking, and an arrow in the merged channel marks the initial contact between EB3-mCherry and actin. (E) Detail (lower boxed region in A) showing an EB3 comet (position marked by arrowheads) growing from the growth cone interior into a filopodium (entry point marked by arrow). (F, G) Velocity profiles of tracks in D and E.
CLASP regulates MT advance in axons

in XCLASP1 morpholino–injected neurons in comparison with controls (Figure 5, H and K). Of interest, our results on actin retrograde flow differ from the findings in HeLa cells, where CLASP1/2 depletion reduced retrograde flow by almost 50% (Mimori-Kiyosue et al., 2005).

XCLASP1 promotes lamellipodial actin meshworks in growth cone veils

Spinal cord neurons in Xenopus are very heterogeneous in morphology (Tanaka and Kirschner, 1991), and we could not find strong differences in MT arrangement upon XCLASP1 depletion. However, when we investigated actin morphology, we found that with lowered XCLASP1 levels, an increased proportion of neurons showed scarce actin in the growth cone. In Xenopus neuronal growth cones, peripheral actin forms prominent filopodia, which extend rearward into the growth cone (intrapodia). Filopodia are interconnected by lamellipodial veils, which show a dense meshwork a few micrometers into the growth cone (Strasser et al., 2004; Korobova and Svitkina, 2008; Figure 7, A and B). The prevalence of growth cones that lack cortical meshwork and filopodia anchorage (Figure 7C) was around 20% in control and uninjected neurons but much higher in XCLASP1-depleted spinal cord cultures (Figure 7D, compare III with I and II). These growth cones showed inward bending of veils

FIGURE 6: Microtubule growth is counteracted by retrograde flow at the leading edge. In spinal cord neurons, EB3-mCherry (A–C, F–J) was used for measurements of plus-end advance in combination with tubulin speckle microscopy to assess polymer movement (D, G', H'). Fluorescence of EB3-mCherry and tubulin speckles is shown intensity inverted in all panels where the background is bright. (A) Plus-end labeling by EB3-mCherry in spinal cord neuron at start of time lapse. (B) Time-lapse sequence in maximum projection (39 frames, 190 s) shows straight EB3 tracks in shaft and curved tracks in the growth cone periphery. (C) Region of interest (see A) and kymograph, where the slope (α) of EB3-positive traces is a measure of plus-end advance rate. (D) Same region with kymograph of tubulin speckles. The angle (β) of speckle traces is a measure of the polymer bulk transport rate. (E) Linear correlation between tubulin bulk transport and plus-end advance rates in the axon shaft. Quantitation of rates as in C and D (10 axons). (F) Growth cone at beginning of time lapse with regions used for kymograph analysis. (G) Kymograph of shaft with EB3 positive plus ends; green line indicates slope (velocity) of moving plus ends. (G') Kymograph of tubulin speckles that display lack of anterograde polymer transport. (H) Kymograph of region b, where EB3 traces indicate plus-end movement (green line), and “hooks” at the end of the traces near the growth cone periphery indicate plus-end stalling or retrograde transport (cyan arrowheads). (H') Matching kymograph of tubulin speckles, which shows retrograde tubulin polymer transport of 5–6 μm/min. (I) Time lapse of detail of region c, where plus ends approach the growth cone margin and then stall while remaining positive for EB3. Phases of plus-end stalling at the periphery are indicated by brackets (d, e, and f). (J) Kymograph analysis of region c. EB3-positive plus ends first advance into the periphery and while still EB3 positive are transported backward, resulting in hooked traces (cyan arrowheads). (J') Kymograph of tubulin speckles, which shows retrograde movement of speckles (red line), which can be attributed to MTs marked in I and J (d and f). (J'') Overlay of both kymographs; speckle movement–inverted gray scale, EB3-mCherry signal red. (K) Actin retrograde flow in the periphery of wild-type, control, and XCLASP1-depleted neurons. Number of traces analyzed is indicated. Error bars, SEM; **p <0.01. Scale bar where not labeled, 5 μm.
FIGURE 7: XCLASP1 promotes lamellipodial actin architecture. Classification of F-actin morphology in fixed growth cones. Staining of F-actin imaged by confocal microscopy is shown intensity inverted (A–C) or in green (A′–C′); the growth cone outline is highlighted in red (A′–C′).

(A) Well-spread growth cone with many filopodia, where actin bundles extend into the interior of the growth cone (arrows). Lamellipodia bulge out as convex protrusions (arrowheads) between filopodia. (B, B′) A strongly polarized growth cone with strong actin bundles (arrows) and a big lamellipodium (arrowhead). (C, C′) Growth cone devoid of lamellipodia; the membrane between filopodia is predominantly concave (open arrowheads), and no strong actin bundles are found inside the growth cone. Images in A, B, and C were taken from wt, control MO–injected neurons, and XCLASP1–MO–injected neurons, respectively. (D) Classification of actin morphology. Proportion of growth cones with strong actin fibers in the interior and/or lamellipodial actin networks as in A and B (dark gray) vs. proportion of growth cones lacking lamellipodia and strong actin bundles in the growth cone as in C (colored according to morpholino). Light gray represents growth cones that fit neither category. Experiments were as follows: I, n = 20; II; n = 25; III, n = 20; IV, n = 3; V, n = 6; VI, n = 4; VII, n = 4. Numbers of cells analyzed per treatment are indicated. Values in wild-type neurons (I) were significantly different from those for all other treatments except where indicated (n.s.). Error bars, SEM; ***p < 0.0001, **p < 0.01.

On the basis of our finding that XCLASP1 is important for MT dynamics, we sought to investigate whether the MT-binding domains in XCLASP1 are important for actin morphology. We constructed a series of XCLASP1 truncations (Figure 8A) and a construct identical to XCLASP1ΔN with two point mutations (XCLASP1ΔN-2SD; Figure 8B) near the only SXIP motif of XCLASP1 (Kumar et al., 2012). EGFP-XCLASP1ΔN-2SD showed reduced MT plus-end binding (Figure 8C and Supplemental Figure S1, C–F). EGFP-XCLASP1ΔN-2SD failed to efficiently rescue the actin phenotype (Figure 7D, compare IV and V). In addition, the C-terminus of XCLASP1 (XCLASP1-Cterm), responsible for interaction with CLIP-170 (Akhmanova et al., 2001; Figure 8A), showed no rescue of the actin phenotype. This construct only showed cytoplasmic localization in growth cones and binding to the microtubule-organizing center in the cell body (Figure 8C, last two panels). The middle part of XCLASP1, XCLASP1ΔNAC, showed incomplete rescue. We cannot conclude about the contribution of the C-terminus to actin integrity, as the rescue was not significantly different from either XCLASP1 morpholino (III) or control MO (II). In summary, our data suggest that the capacity of XCLASP1 to associate with plus ends through the SXIP domain is essential for convex lamellipodia and intrapodia.

A previous electron microscopy study of protruding and retracting actin veils in Xenopus neurons highlighted that protrusion was always associated with a dense actin meshwork behind the membrane and retraction with scarce actin, often forming parallel bundles to the membrane (Mongiu et al., 2007). To analyze the fine structure of actin, we used structured illumination microscopy (SIM). In growth cones typical for control and wild-type neurons, we could clearly identify filopodia, which were embedded as actin bundles in lamellipodia (intrapodia; Figure 9, A′ and B′, arrows in insets), and thinner actin filaments with varying angles that are typical for lamellipodia (Figure 9, A′ and B′, arrows in insets; Korobova and Svitkina, 2008). In contrast, the growth cone periphery in XCLASP1-depleted neurons lacked dense actin meshwork (Figure 9, C–D′), only a few actin bundles were visible, and they were associated with neither intrapodia nor lamellipodia. The membrane between filopodia was concave and resembled very much the rear-oriented veils of other growth cones (Figure 9B′, open arrowhead). In summary, XCLASP1-depleted neurons had an increased number of growth cones that show an actin distribution in veils reminiscent of retracting rather than protruding periphery as described by Mongiu et al. (2007). This hints to disturbed lamellipodial protrusion in XCLASP1-depleted neurons. We also analyzed XCLASP1-depleted neurons by live imaging using Lifeact-GFP. The resolution of actin in these images is far inferior to that in fixed samples; however XCLASP1-depleted growth cones show reduced lamellipodial activity compared with controls (see Supplemental Movies S7–S9).

DISCUSSION
Automated tracking of MTs reveals differential regulation of MT advance in shaft, growth cone, and filopodia
We devised a method of analyzing MT dynamics in neurons. On the basis of low expression levels of the +TIP EB3 to track plus ends
For the first time, we can compare MT dynamics in different compartments of the vertebrate axon (Figure 5). Previously, MTs growing along the axonal shaft were shown to progress without being influenced by their position (e.g., branch point; Stepanova et al., 2010).

In Xenopus spinal cord neurons used here, plus-end velocities depend on location: MT advance is highest in the axon shaft close to (Stepanova et al., 2003, 2010) and wide-field fluorescence time-lapse microscopy, we developed automated tracking to determine the velocity, track length, and lifetime of MT tracks. Our algorithm can operate on sequences acquired at low frame rates to minimize the exposure of the cells, which is extremely important for neurons, as they are very sensitive to phototoxicity.

For the first time, we can compare MT dynamics in different compartments of the vertebrate axon (Figure 5). Previously, MTs growing along the axonal shaft were shown to progress without being influenced by their position (e.g., branch point; Stepanova et al., 2010). In Xenopus spinal cord neurons used here, plus-end velocities depend on location: MT advance is highest in the axon shaft close to...
FIGURE 9: Actin morphology is altered in XCLASP1-depleted growth cones. SIM of Xenopus growth cones, where F-actin stained by phalloidin is shown in green and MTs in red (A–C). For clarity phalloidin staining is shown intensity inverted (A′–D′ and insets). (A–B′) Examples of actin morphology that are characteristic for most growth cones in wild-type or control MO–injected neurons. Filopodia are anchored in the growth cone with actin bundles that are anchored deep inside the growth cone (arrows). These prominent actin bundles are embedded in a dense lamellipodial actin meshwork (arrowheads). (C–D′) In XCLASP1-depleted growth cones (XCLASP1 MO) filopodia do not extend rearward into the growth cone interior and lamellipodia are missing. Instead, the outline between filopodia is bent inward (open arrowheads). Scale bar, 5 μm; insert size, 10 × 10 μm.

the growth cone (Figure 5K) and decreases as MT ends reach the growth cone periphery (Figure 5, H and K). These differences are reminiscent of the graded MT dynamics in epithelial cells, which help MTs to explore the periphery (Komarova et al., 2002). The periphery of U2OS cells in wound healing also showed reduced MT advance rates compared with the cell interior (Nishimura et al., 2012).
XCLASP1 promotes microtubule advance in the growth cones but not in the shaft

Dynamic MTs in neuronal growth cones are necessary for directed outgrowth (Sabry et al., 1991; Tanaka and Kirschner, 1991; Tanaka et al., 1995; Tanaka and Kirschner, 1995; Dent and Kalil, 2001; Zhou and Cohen, 2004). Stabilization of MTs by Taxol is sufficient to induce growth cone turning (Buck and Zheng, 2002), and MT-stabilizing proteins like MAP1B have been shown to influence guidance (Mack et al., 2000; Hahn et al., 2005). Many +TIPs show high expression in the developing nervous system and especially in outgrowing nerves (Park et al., 2012). Indeed we found that XCLASP1 mRNA localization in spinal cord nerves was high during axon outgrowth but disappeared in later stages (Figure 1, A–C). In a genetic screen in Drosophila, we previously identified the CLASP homologue Orbit (Lee et al., 2004) downstream of Slit repulsion. With a loss-of-function approach, we depleted XCLASP1 and found that MT advance in the growth cone is reduced (Figure 4 and Table 1). Of interest, XCLASP1 regulated MT dynamics only in the growth cone and the most proximal shaft. In a segment >40 μm away from the growth cone, XCLASP1 depletion showed no effect on comet velocities.

A study by Hur et al. (2011) proposed a dual role for CLASPs in axon outgrowth. In cortical neurons where GSK-3 activity is low, MT binding of CLASP2 through its lattice binding (Kumar et al., 2009) inhibited outgrowth. In contrast, depletion of CLASP1 and 2 in dorsal root ganglia led to decreased outgrowth, pointing to a role of CLASPs in axon elongation (Hur et al., 2011). This latter effect is consistent with our results on XCLASP1 in outgrowing Xenopus spinal cord neurons.

Reduced MT advance in the growth cone correlates with dampened axon extension

Several studies pharmacologically interfered with pioneer MTs and found a link between MT dynamics and axon behavior. In cortical neurons, both Taxol (10 nM) and nocodazole (33 nM) reduced axon extension and led to curved axon trajectories (Dent and Kalil, 2001). Using automated tracking, we were able to quantify the effects of Taxol on plus-end advance. Taxol at <10 nM reduced plus-end velocities in the growth cone while still allowing for polymerization, as indicated by EB3-positive comets (Figure 3, C and D, and Supplemental Movie 3). Low Taxol concentrations and XCLASP1 depletion resulted in subtle interference with +TIP advance (Figure 4D), and both interfered with axon extension and directionality (Figure 1, E–H). Microtubule drugs applied in a bath application necessarily affect the whole axon. However, XCLASP depletion affected dynamics only in and near the growth cone, suggesting that it is the changed MT dynamics in the growth cone that affects axon outgrowth.

Mechanistically it is hard to conceive how axon elongation and MT advance into the periphery of the growth cone might be linked directly, for example, by MTs pushing the growth cone forward, as axon elongation is on average 10 times lower than MT polymerization (Lee et al., 2004). An attractive model is that dynamic MTs deliver signaling factors that influence actin polymerization, retrograde flow, or substrate coupling (Suter et al., 2004). This is also compatible with cell type-specific effects of altered pioneer MT dynamics. For example, Taxol at the same concentrations as used here promoted outgrowth in regenerating sensory neurons in vivo (Hellal et al., 2011).

MTs at the leading edge are affected by actin-mediated retrograde flow

Neuronal growth cones are very dynamic, and although advance rates depend on the substrate (Lee et al., 2004; Jacques-Fricke et al., 2006), 2 μm/min is common for Xenopus spinal cord neurons plated on laminin (Tanaka et al., 1995). Tubulin speckle microscopy demonstrated forward transport of MT polymer in the shaft and central domain at rates up to 3 μm/min (Figure 6), consistent with earlier measurements in Xenopus neurons, where forward transport was attributed to pulling tension generated by the growth cone (Chang et al., 1998). We found that in the shaft there is a clear correlation between MT bulk transport and plus-end advance, where anterograde transport positively affects plus-end translocation (Figure 6E).

In contrast to the shaft, MTs in the growth cone periphery are transported backward (Figure 6, H–J), and the rates at which they are transported are similar to retrograde flow rates of actin (Figure 6K). We observed MTs at the cortex that seemingly paused but were actually in a tug of war between polymerization and retrograde movement of the tubulin polymer (Figure 6, H–J). This suggests that in vertebrate growth cones MT retrograde transport cooperates with catastrophe in removing MTs from the periphery, very much like what was demonstrated for invertebrate neurons (Medeiros et al., 2006; Burnette et al., 2007).

XCLASP1 is necessary to maintain protrusive actin morphology

CLASPs have a role as rescue factors that mediate rapid transition from depolymerization to polymerization (Al-Bassam and Chang, 2011), but in mammalian cells CLASPs seems to promote not only rescue but also catastrophe to keep plus ends near to the periphery (Mimori-Kiyosue et al., 2005). Similarly, CLASP was shown to maintain the MT array at the cortex of Drosophila cells, but by keeping MTs in a paused state (Sousa et al., 2007). In accordance with these observations, we found that XCLASP1 is important for maintaining pioneer MTs in the growth cone periphery. Our data suggest that transitions from the growing state in XCLASP1-depleted growth cones are not altered, since track lifetimes are even slightly increased (Figure 4C). However, we are limited in our analysis because we cannot discriminate between transitions to catastrophe and pause, nor can we identify rescue events. The reduced plus-end velocities we measured do not necessarily signify reduced polymerization, because MTs are subject to considerable translocation movements (Figure 6). To disentangle these processes in the XCLASP1-depleted neuron, automated quantitative speckle microscopy would be required in addition to plus-end tracking.

Previously, CLASPs were shown to affect retrograde flow (Mimori-Kiyosue et al., 2005). Because retrograde flow would also change pioneer MT advance, we looked at actin in XCLASP1-depleted neurons. We found that retrograde flow was hardly changed (Figure 6K) but were intrigued that actin architecture in the growth cone periphery changed when XCLASP1 was depleted. Our morphological analysis indicates that XCLASP1 promotes lamellipodial actin meshwork in growth cone veils. A significant number of XCLASP1-depleted growth cones show concave veils poor in actin filaments (Figure 9C′ and inset), and our high-resolution images with SIM closely resemble electron micrographs of retracting actin veils (Mongiu et al., 2007). In addition, live imaging supports the interpretation that XCLASP1 function is necessary for lamellipodial protrusion (Supplemental Movie S9).

It is unclear whether XCLASP1 is important for formation or maintenance of lamellipodial networks, and we can only speculate on the mechanism. XCLASP1 might bind actin indirectly, such as through cross-linker short stop (Rogers et al., 2004), and it has also been reported to bind actin filaments directly (Tsvekov and Popov, 2007). The actin morphology in XCLASP1-depleted neurons resembles to a certain degree the phenotype in rac-inhibited Xenopus
neurons (Rajnicek et al., 2006), which might suggest that pioneer MTs are important to deliver signaling molecules. Indeed, we showed that the capacity XCLASP1 to bind to MT plus ends is important for actin architecture. We could compensate for the effect of the XCLASP1 morpholino by concomitant expression of GFPXCLASP1ΔN but not by a mutated form that displayed reduced plus-end binding. This suggests that XCLASP1 plays a role in coordination of actin and microtubules in growth cone protrusion.

MATERIALS AND METHODS

Constructs

Murine EB3-2FLAGi-mCherry (Straube and Merdes, 2007) was cloned into pCS2 vector to result in EB3-mCherry pCS2 or EB3-EGFP pCS2. Similarly, Lifeact-EGFP was cloned into pCS2 for in vitro transcription of capped mRNA with an SP6 polymerase. Xenopus CLASP 1 cDNA (National Center for Biotechnology Information gene ID 494817) was obtained in pCMV-Sport6 from Source BioScience (Nottingham, United Kingdom) and used as a template for the design of morpholino oligonucleotides and in situ probes. An N-terminal deletion of XCLASP1 comprising amino acids 524–1468 (XCLASP1ΔN) was inserted into pCS2-EGFP, and point mutations in S726 and S730 to glutamate resulted in XCLASP1AN-S2/D. Truncations XCLASP1ΔNΔC and XCLASP1-Cterm were inserted in pCS2-EGFP for in vitro mRNA synthesis and are described in Figure 8.

Injection in X. laevis embryos

Fertilized eggs of X. laevis were injected at the four-cell stage into dorsal blastomeres with 5 nl of solution in vitro–transcribed capped mRNA (mMessage mMachine, Ambion, Austin, TX) at different concentrations (0.019 μg/μl EB3-EGFP, 0.028 μg/μl EB3-mCherry, 0.06 μg/μl Lifeact-EGFP, 0.1 μg/μl EGFP tubulin). For speckle microscopy HiLyte488 porcine tubulin (Cytoskeleton, Denver, CO) was injected together with mRNA at concentration of 0.2–1 μg/ml. EGFP-XCLASP1ΔN, EGFP-XCLASP1ΔNΔC, and EGFP-XCLASP1-Cterm mRNA were injected at 0.15, 0.1, and 0.7 μg/ml, respectively.

At stage 28, embryos were dissected as described (Tanaka and Kirschner, 1991). Pieces of spinal cord were plated on glass-bottomed dishes coated with laminin (20 μg/ml; Sigma-Aldrich, St. Louis, MO) in culture medium (Ming et al., 1997) supplemented with 100 ng/ml brain-derived neurotrophic factor (Sigma-Aldrich). Outgrowing neurons were imaged or fixed for immunocytochemistry 14–18 h after plating. Taxol (Sigma-Aldrich) was added in conditioned medium in concentrations indicated 1 h before imaging.

For knockdown experiments 0.4 pmol of XCLASP1 morpholinol 5′-ATGGAACAGGGATGACTACTGGC-3′ or control morpholinol 5′-CCTTTACCTAAGTCATTTATA-3′ (Gene Tools, Philomath, OR) was injected at the four-cell stage together with mRNA or 1.5 μg/μl fixable fluorescent dextran (Invitrogen, Carlsbad, CA).

Time-lapse microscopy

Image sequences for automated tracking were recorded on an inverted Nikon TiE microscope equipped with a 60 or 100× numerical aperture (NA) 1.4 Planapo VC objective and a Nikon Intensilight (Nikon, Melville, NY) as light source with a frame rate of 3 s/frame. Cameras used were either the charged-coupled device (CCD) ORCA (Hamamatsu, Hamamatsu, Japan) or the Xion DU-897 electron multiplier CCD (Andor, Belfast, United Kingdom), resulting in a pixel size of 107 or 160 nm, respectively. For EB3 tracking in distal and proximal shaft, an sNEO cMOS (Andor) was used in combination with a 60×/NA 1.4 objective, resulting in a field of view of 200 × 270 μm and a pixel size of 108 nm.

For analysis of axon outgrowth and the collapse assay, bright-field images were recorded with 3-min intervals for 30 min using a Nikon 20×/NA 0.75 Planfluor objective. Injected neurons were identified by EGFP fluorescence recorded at the beginning and end of the sequence. The path of the growth cone was tracked manually.

Automated +TIP tracking and statistical analysis

We developed a probabilistic approach for tracking EB3 comets in fluorescence microscopy image sequences. In our approach, each EB3 comet is represented via a 2D anisotropic Gaussian function that is parameterized by the position of the comet (x, y), its peak intensity I_m, and the standard deviations σx and σy. We assumed that the orientation of the comet agrees with the orientation of its velocity vector (x′, y′). At each time point, our approach carries out two steps: 1) detection and localization of comets, and 2) estimation of the position of the individual comets. To detect and localize comets, we first used a Gaussian filter to reduce the image noise. The values for the SD σ of the filter were set based on the size of the comets (typically, we used a value of σ = 3). To suppress the background, intensity values below a threshold I_{clip} were removed. The clipping threshold I_{clip} was computed as the mean intensity of the image plus a factor c times the SD of the image intensities. Image regions corresponding to EB3 comets were detected by performing a search for local intensity maxima. To localize comets, we fitted a 2D Gaussian function to each candidate image region.

To estimate the position of the tracked comets, we predicted the position of each comet using a Kalman filter with a constant-velocity motion model. Initial estimates for the velocity components were obtained by computing the optical flow between two consecutive images. To find the correspondences between the positions predicted by the Kalman filter and the positions computed by the 2D Gaussian fitting scheme, we used a global nearest-neighbor approach (Balzarini and Koumoutsakos, 2005). Using the predicted position, as well as the position from 2D Gaussian fitting, the Kalman filter calculated the final position estimate for each comet.

Track data were saved as text files and visualized by a custom-designed track visualization plug-in for ImageJ (National Institutes of Health, Bethesda, MD; Abramoff et al., 2004). This allowed tracks to be sorted, exported, and viewed as overlays on top of the corresponding image sequences. Tracks with durations shorter than three frames (15 s) were discarded. Kymograph analysis of EB3 comets was performed by drawing manually segmented lines defining the image track and creating kymographs with the ImageJ plug-in bundle Multiple Kymograph by J. Rietdorf and A. Seitz (European Molecular Biology Laboratory, Heidelberg, Germany).

Statistical analysis and graphs were made using Prism 5 (GraphPad Software, La Jolla, CA). Mann–Whitney tests were used to test for statistical significance in data of non-Gaussian distributions.

Dynamic segmentation of filopodia

For segmentation of the filopodia, first, thin structures in the original actin image were enhanced based on a method previously used to detect blood vessels (Frangi et al., 1998). To detect image regions corresponding to thin structures, we then applied an automatic thresholding technique (Zack et al., 1977) to the enhanced image. With this technique, also known as the triangle method, the histogram is normalized, and then the line between the maximum and minimum value of the histogram is determined. Afterward, the distances between this line and all values of the histogram are computed, and the intensity value for which the distance is maximal is taken as the threshold.
To exclude those regions that corresponded to the inner part of the growth cone, we segmented the central part of the growth cone and excluded it from the segmentation result for filopodia. For segmentation of the central part of the growth cone, we applied Otsu’s method (Otsu, 1979), as well as morphological operations (opening and closing). The method of Otsu subdivides an image into pixels of two classes so that their intraclass variance is minimal. To determine moving comets along filopodia, we considered only those parts of a trajectory from tracked comets that are located within the segmented filopodia and used this information to subdivide tracks for analysis.

Analysis of tubulin speckles

Using ImageJ, we enhanced time-lapse sequences with tubulin speckles by a difference-of-Gaussians method in which the image was first smoothed by a small Gaussian filter to reduce noise (σ₁ = 1), and a more highly filtered duplicate of the image (σ₂ = 4) was then subtracted. The resulting difference image showed the particles more clearly. To analyze flow, rectangular regions of interest were cropped and the dimensions of the image reordered to convert an x–y–time stack into an x–time–y stack. A kymograph was then created using a maximum projection along the y-axis. By manually identifying the angles of speckle traces, we calculated their velocities along the x-axis. Additional methods are described in the Supplemental Data.

ACKNOWLEDGMENTS

We thank Rebecca Heald for Xorbit antibody, Michael Siki for Life-act-EGFP, and Anne Straube for EB3-mCherry constructs. We thank Lars Kaderali for advice in statistics. We are extremely grateful to Christoph Niehr’s lab and especially to Ya-Lin Huang for access to frogs and Doris Wedlich for providing XLKE cells. We are grateful to Nikon GmbH for continued support of the Nikon Imaging Center at the University of Heidelberg. A.M. was supported by the Exzellenzcluster CellNetworks Heidelberg and P.B. by Heidelberg Molecular Life Science. K.R. gratefully acknowledges support of the Bundesministerium für Bildung und Forschung project ViroQuant FORSYS, Research Units for Systems Biology.

REFERENCES


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Supplemental data Marx et al.

Table 1: Plus-end velocities decrease prior to transition to pause or catastrophe in the growth cone.

<table>
<thead>
<tr>
<th></th>
<th>&gt;30 sec before catastrophe</th>
<th>30-15 sec before catastrophe</th>
<th>last 15 sec before catastrophe</th>
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</thead>
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<tr>
<td><strong>Number of tracks in</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>growth cone</strong> (%</td>
<td>240</td>
<td>390</td>
<td>390</td>
</tr>
<tr>
<td>Comet velocity in growth cone (%)</td>
<td>97.9 ±2.4</td>
<td>100.0 ±2.1</td>
<td>88.7±2.2</td>
</tr>
<tr>
<td><strong>Number of tracks in shaft</strong></td>
<td>101</td>
<td>155</td>
<td>155</td>
</tr>
<tr>
<td>Comet velocity in shaft (%)</td>
<td>99.0 ± 3.3</td>
<td>100.0 ± 3.8</td>
<td>96.2 ±3.1</td>
</tr>
</tbody>
</table>

Table 1: Relative comet velocities of tracks divided into different phases. EB3 comet velocities were normalized to the average velocities of all tracks in growth cone or shaft of the period 30-15 seconds before catastrophe and are shown with relative s.e.m. calculated from all tracks analyzed.

Table 2: Decreased pioneer microtubules enter filopdia in XCLASP1-depleted neurons.

<table>
<thead>
<tr>
<th></th>
<th>Tracks in filopodia</th>
<th>Tracks in growth cone*</th>
<th>Proportion of tracks in filopodia per growth cone ± s.e.m</th>
<th>Number of growth cones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-injected</td>
<td>131</td>
<td>1184</td>
<td>0.14 ±0.052</td>
<td>21</td>
</tr>
<tr>
<td>Control MO</td>
<td>192</td>
<td>853</td>
<td>0.18 ±0.098</td>
<td>17</td>
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<tr>
<td>XCLASP1 MO</td>
<td>91</td>
<td>1084</td>
<td>0.16±0.045</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 2. Automatic separation of tracks in filopodial versus growth cone interior tracks.

*tracks in growth cone but not in filopdia
**Supplemental Figures**

**Supplemental Figure 1.** XCLASP1 depletion in *Xenopus* and analysis of MT-binding. (A) Quantification of XCLASP1 depletion by XCLASP1 MO in *Xenopus leavis* growth cones by immunocytochemistry using an affinity purified XCLASP1 antibody (Xorbit antibody, Hannak and Heald, 2006). The average intensity in the growth cone of control MO was normalized to 100%. (B) Morpholino based XCLASP1 knockdown in *Xenopus laevis* kidney epithelial cell line analyzed by Western analysis with XCLASP1 antibody. 1: wt; 2: control MO; 3: XCLASP1 MO. (C-E) Analysis of plus-end binding of XCLASP with spinning disk confocal microscopy in growth cones. EGFP-XCLASP△N shows plus-end tracking (C) while EGFP-XCLASP△N-2SD shows only very reduced plus-end tracking and more cytoplasmic localization. (E) Quantitation of plus-end binding as ratio of plus-end to cytoplasmic intensity in XCLASP△N and XCLASP△N-2SD expressing growth cones. Error bars represent standard deviation.
Supplemental Figure 2. Comparison of kymograph analysis with automated tracking of EB3 comets. (A) Automated tracking of EB3-EGFP. The trajectory (red) of the growing microtubule plus-end is shown superimposed with the original image sequence. (B) Maximum intensity projection of frames shown in a. (C) Superimposition of manually tracking of trajectory (yellow). Scale bar: 5 μm. (D) Kymograph along trajectory shown in (C). (E) Plot of instantaneous velocities over time reveals decrease of velocity as the MT plus-end advances into periphery. (F) Relation between lifetime (frames) and instantaneous velocity of wt, XCLASP1 MO injected neurons and neurons treated with 7 nM taxol. Frame rate was one image every 3 seconds.
Supplemental Figure 3. MTs in the growth cone undergo positive and negative transport. (A-E) Tubulin speckle microscopy in spinal cord growth cone. Speckles of HiLyte488-tubulin are shown intensity-inverted. (A) MT polymer labeled with low concentrations of conjugated tubulin shows vague outline of MTs (approximate growth cone outline indicated by broken yellow line) (B) Kymograph analysis in region a (see A). The almost horizontal lines indicate very weak anterograde bulk transport. (C) Kymograph analysis in region b (see A) in a growing filopodium with anterograde tubulin flow (red line). (D) Kymograph analysis in region c, where a single MT advances and then retracts. The green line shows plus-end advance, the red line retrograde movement of the MT. (E) Kymograph analysis in region d in a filopodial branch that retracts. The red lines indicate different phases of MT polymer transport in the direction of the axonal shaft.
Supplemental Material and Methods

Immunofluorescence and in-situ hybridization

Neuronal cultures were fixed with icecold Methanol, 1 mM EGTA for 20 min at -20°C, then blocked 30 min with 5% normal goat serum (SIGMA-Aldrich) in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl$_2$, pH 6.9) at room temperature. Primary antibodies were incubated 2 hours at room temperature in 5% goat serum in PHEM. Washing was performed 3 times with PHEM followed by incubation of secondary antibodies 1 hour at room temperature in 5% normal goat serum in PHEM. After washing 3 times with PHEM, images were recorded. Antibody combinations were Primary antibodies were mouse antibody anti-EB1 (1:200, BD Biosciences) and monoclonal rat anti-tyrosine-tubulin YL 1/2 (1:500, Millipore) detected with the secondary antibodies preabsorbed goat anti-mouse Cy3 and goat anti-rat Cy5 (Jackson ImmunoResearch Laboratories Inc., 1:300), Xorbit antibody 1:1000, detected by anti-rabbit Alexa-Fluor 488 1: 400 (Invitrogen). For detection of f-actin with phallloidin-Atto565 (SIGMA), growth cones were fixed in 4% paraformaldehyde in Krebs buffer (Dent and Kalil, 2001) for 10 minutes. After washing with PHEM phalloidin was added directly, or samples were incubated in 0.5% Triton X in PHEM for 30 minutes and immuno-stained with DM1α (SIGMA, 1:500), with the phalloidin added with the secondary antibody (anti-rabbit Alexa-Fluor 488, 1:500).

Stage 28 embryos were fixed in 1x MEMFA containing 3.7% formaldehyde for in-situ hybridization. The XCLASP1 in-situ probes were transcribed with T7 (anti-sense) and Sp6 (sense) on pCMV-SPORT6 after linearization of the plasmid with EcoRI (anti-sense) and XhoI (sense). Whole mount hybridization was performed as described in (Gawantka et al., 1995).

Microscopy of fixed specimen:

Immunofluorescence was evaluated on a Nikon TiE equipped with a 60x TIRF Apo NA 1.49 objective using a Orca AG CCD camera (Hamamatsu) or with a Perkin Elmer spinning disk confocal system with a Yokogawa CSU-22 and a Nikon 100x PlanApo NA 1.4 objective (Figure 8A-C). For super-resolution imaging (Figure 9) a Nikon N-SIM with a 100x TIRF Apo NA 1.49 with a 3-dimensional patterned illumination was used. To reconstruct super-resolution images, 5 phases and 3 orientations were collected in a single plane onto a Andor Xion DU-897 Electron Multiplier CCD charged coupled device (EM-CCD). A 2.5x magnification was mounted in front of the camera to achieve sampling at 64 nm pixel size. Control MO and XCLASP1 MO positive neurons were identified by dextran Alexa Fluor 647 (Invitrogen).

Western analysis

Proteins were immobilized from SDS Page gel on nitrocellulose membrane by Western blotting. The membrane was then blocked with 5 % milk powder, 0.1 % Tween 20 in PBS and then incubated with rabbit-XCLASP1 antibody (1:200), monoclonal anti-tubulin DM1A (1: 1000, Sigma-Aldrich) and monoclonal GAPDH 6C5 antibody (1:1000, Merck). The membrane was washed with 0.1 % Tween 20 in PBS and the secondary antibodies were added at 1:5000 dilution (goat anti-rabbit horseradish peroxidase and goat anti-mouse horseradish peroxidase, Jackson ImmunoResearch Laboratories Inc.). Then the membrane
was washed again and incubated with ECL Western Blot solution before the signal was detected with a light sensitive film.

**XCLASP1 knock down in Xenopus cell culture**

XCLASP1 knock down was performed in XLKE cells (*Xenopus laevis* kidney epithelial cells) growing in medium containing 60% DMEM 4.5 g/l Glucose (PAA Laboratories GmbH), 10% FCS (PAA Laboratories GmbH), 1% penicillin/streptomycin, 29% H2O at 25°C and 37% CO2. At 90% confluency, 3 µM morpholino oligonucleotide and 6 µM Endo-Porter (Gene Tools LLC) were added. Cells were incubated for two days before cells were washed once with PBS, before being lysed with NP-40 buffer (40 mM Tris pH 7.5, 5 mM EDTA, 0.5% NP-40, 150 mM NaCl and protease cocktail). The solution was incubated 30 min on ice before being centrifuged. After measuring protein concentration, the supernatant was mixed with SDS loading dye and heated before being analyzed on SDS Page gel. For each lane 10.6 µg protein was loaded.

**Supplemental references**


**Movies:**

**Movie 1:** Timelapse sequence of EGFP-XCLASP1ΔN in *Xenopus* spinal cord growth cone.

**Movie 2:** EB3-mCherry comet imaging in *Xenopus* spinal cord neurons for tracking of MT ends. Analysis of this time lapse sequence is shown in Figure 2 and Figure 5.

**Movie 3:** EB3-EGFP comet movement in *Xenopus* spinal cord neurons treated with 3.3 nM Taxol. Microtubule dynamics are attenuated, but EB3 still binds to plus-ends.

**Movie 4:** EB3-EGFP and Liveact-EGFP in *Xenopus* spinal cord neurons for simultaneous tracking of MT ends and F-actin. Analysis of this time-lapse sequence is shown Figure 5.

**Movie 5:** Visualization of MT plus end velocities in movie 2. The color added at each frame relates to the average velocity of neighboring frames.

**Movie 6:** EB3-mCherry comet imaging in *Xenopus* spinal cord neurons for tracking of MT ends in proximal and distal shaft. Analysis of this timelapse sequence is shown in figure 5, K and J.

**Movie 7:** Wild type neuron expressing Lifeact-EGFP (shown intensity inverted).

**Movie 8:** Control MO-injected neuron expressing Lifeact-EGFP (shown intensity inverted).

**Movie 9:** XCLASP1 MO-injected neuron expressing Lifeact-EGFP (shown intensity inverted).