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1 # The model of local axon homeostasis - explaining the role and regulation of microtubule
2 bundles in axon maintenance and pathology

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19 ## Abstract

20 Axons are the slender, cable-like, up to meter-long projections of neurons that electrically wire
21 our brain and body. In spite of their challenging morphology, they usually need to be maintained
22 for an organism's lifetime. This makes them key lesion sites in pathological processes of ageing,
23 injury and neurodegeneration. The morphology and physiology of axons crucially depends on
24 the parallel bundles of microtubules (MTs), running all along to form their structural backbones
25 and highways for life-sustaining cargo transport and organelle dynamics. Understanding how
26 these bundles are formed and then maintained will provide important explanations for axon
27 biology and pathology. Currently, much is known about MTs and the proteins that bind and
28 regulate them, but very little about how they functionally integrate to regulate axons. As an
29 attempt to bridge this important knowledge gap, we explain here the model of local axon
30 homeostasis, based on our own experiments and published data. (1) As the default, we observe
31 that axonal MTs have a strong bias to become disorganised, likely caused by the physical
32 forces imposed by motor proteins and their life-sustaining functions during intra-axonal transport
33 and dynamics. (2) Preventing MT disorganisation and promoting their bundled conformation,
34 requires complex machinery involving most or even all major classes of MT-binding and -
35 regulating proteins. As will be discussed, this model offers new explanations for axonopathies,
36 in particular those linking to MT-regulating proteins and motors; it will hopefully motivate more
37 researchers to study MTs, and help to decipher the complex regulatory networks that can
38 explain axon biology and pathology.

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39 Introduction

40 Axons are the slender, cable-like extensions of nerve cells which form the nerves and nerve
41 tracts that wire our brain and body, sending neuronal messages in highly regulated manners.
42 With diameters of only 0.1-15µm (Hoffman, 1995), they extend over distances of up to a meter
43 in humans. To adopt such a unique morphology and physiology, axons display many
44 specialised features (Fig.1).

45 Axons are indispensable for nervous system function, as illustrated by paralysis in spinal cord
46 injury caused by the interruption of ascending and descending axon tracts (Bichenback, 2013;
47 Tedeschi and Bradke, 2016). Axons are key lesion sites in injury-induced trauma and coma
48 (Gaetz, 2004; Medana and Esiri, 2003; Smith et al., 2000; Tang-Schomer et al., 2012), and
49 axon decay is believed to be an important trigger for neuronal loss in ageing and many
50 neurodegenerative disorders (Adalbert and Coleman, 2012; Salvadores et al., 2017). Notably,
51 most neurons cannot be replaced, and compensation of lost axons through collateral branching
52 of intact neighbouring axons has obvious limitations (Adalbert and Coleman, 2012; Sturrock,
53 1987).

54 This means that most axons have to be maintained for an organism's life time, i.e. up to a
55 century in humans; unsurprisingly, mammals tend to lose almost half their axon mass towards
56 high age (Calkins, 2013; Marner et al., 2003). This trend is severely enhanced in
57 neurodegenerative disorders, as illustrated by gradually increasing paralysis in spastic
58 paraplegia or motorneuron disease (Blackstone et al., 2011; Riancho et al., 2019).

59 Research into neurodegenerative disorders typically approaches the problem by describing
60 observed phenotypes and unravelling the molecular mechanisms performed by proteins linked
61 to the disease. However, this approach rarely leads to satisfactory explanations of the pathology
62 (Aguzzi, 2019). We believe that more profound understanding will arise when widening the
63 scope from molecular to cellular mechanisms, by studying how proteins work within regulatory
64 networks to underpin observable processes of axon biology - thus performing investigations at
65 the same level of complexity at which pathology becomes manifest. Here we will illustrate this
66 approach by focussing on the axonal cytoskeleton.

67

68 The importance of microtubule bundles for axon biology

69 As illustrated in Fig. 1, the cytoskeleton of the axon shaft consists of straight parallel bundles of
70 MTs, which are interspersed with intermediate filaments (not shown) and longitudinal actin
71 fibres called 'actin trails' - all running through evenly spaced periodic rings, proposed to consist
72 either of short and adducin-capped actin filaments (Qu et al., 2017; Xu et al., 2013) or of two
73 long intertwined actin filaments (Vassilopoulos et al., 2019) - future will show. Significant
74 deviations from this organisation that will not be considered in this review, exist at axon initial
75 segments (not shown in Fig.1), growth cones and synapses (Dent et al., 2011; Leterrier, 2018;
76 Leterrier et al., 2017; Prokop, 2013).

77 Of the three cytoskeleton classes, intermediate filaments were suggested by anatomical,
78 developmental and genetic studies to regulate axon diameter, and their axonal aggregation is a
79 hallmark of many neurodegenerative diseases (Friede and Samorajski, 1970; Hoffman, 1995;
80 Perrot et al., 2008; Rao et al., 2003; Sakaguchi et al., 1993). However, intermediate filament
81 accumulations are not necessarily the cause, but can be the consequence of axon decay (Eyer
82 et al., 1998; Nguyen et al., 2000; Perrot et al., 2008). Notably, *Neurofilament-H-lacZ* mutant
83 mice or *Quiver* mutant quail completely lack axonal intermediate filaments, but develop and

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84 breed fairly normally (Eyer and Peterson, 1994; Yamasaki et al., 1991). Furthermore, various
85 arthropods form axons of defined diameters in the absence of any axonal intermediate filaments
86 (Allen et al., 2006; Hirokawa, 1986; Voelzmann et al., 2016a). In contrast to the moderate roles
87 of intermediate filaments, actin and microtubules (MT) are essential for all stages of neuronal
88 development and maintenance (Sakakibara et al., 2013; Tas and Kapitein, 2018; Voelzmann et
89 al., 2016a); this review will be dedicated to the role and regulation of MTs.

Box 1 The roles of axonal MT bundles

(1) Axonal MT bundles serve as structural backbones, comparable to the vertebral column of a snake; since MTs in these bundles are discontinuous and expected to be interlinked via flexible connections (see section on cross-linkers), they are ideally suited to respond to longitudinal stretch and compression (similar to a half-extended telescope ladder), but also to torsion and flexure (Fig.2).

(2) Axonal MT bundles provide the highways for life-sustaining axonal transport between cell bodies and the axonal compartment. This transport is driven anterogradely by kinesins and retrogradely by the dynein/Dynactin complex; the cargoes include mRNAs, cytoplasmic proteins including signalling factors, vesicles delivering synaptic proteins, transmembrane proteins, neuropeptides and/or membrane lipids, as well as entire organelles including mitochondria (Fig. 3A-D; Goldstein et al., 2008; Gondre-Lewis et al., 2012; Gonzalez and Couve, 2014; Hirokawa et al., 2010; Pfenninger, 2009). Furthermore, local dynamics of organelles, such as fission or fusion of mitochondria, can be expected to require forces generated by MT-associated motor proteins (Fig. 3E; Saxton and Hollenbeck, 2012).

(3) Axonal MT bundles provide a source for readily available MTs that can be used for other purposes (curved arrows in Fig.1); for example, splaying MTs can trigger axon extension processes in growth cones (Dent et al., 2011; Prokop et al., 2013; Miller and Suter, 2018), induce branching through growth cone splitting (Acebes and Ferrus, 2000) or collateral branch formation along the axon shaft (Kalil and Dent, 2014; Tint et al., 2009; Tymanskyj et al., 2017), as well as support physiological changes at synapses (Bodaleo and Gonzalez-Billault, 2016).

90

91 Axons contain bundles of MTs that run along the entire length of their shafts (Fig.1); these
92 bundles are essential for axon biology in at least three ways: as structural backbones, as
93 highways for axonal transport and organelle dynamics, and as source for splaying MTs that can
94 contribute to axon morphogenesis or physiology (details in Box 1). Maintaining MT bundles is
95 therefore crucial for axon longevity. Accordingly, there are prominent and numerous genetic
96 links from MT regulators to hereditary neurodegenerative disorders (Suppl. Mat. in Prokop et al.,
97 2013), and axon decay is a frequent side effect of MT-targeting chemotherapies (Prior et al.,
98 2017; Wozniak et al., 2018; Wu et al., 2014). Of particular interest for this review are reports of
99 pathological axon swellings where MT bundles have disintegrated into loops or waves (bottom
100 of Fig.3), occurring in ageing, after injury and in certain axonopathies (Adalbert et al., 2009;
101 Bernier and Kothary, 1998; Dalpe et al., 1998; Denton et al., 2014; Fassier et al., 2013; Havlicek
102 et al., 2014; Sorbara et al., 2014; Tang-Schomer et al., 2012; Tarrade et al., 2006; Yamasaki et
103 al., 1991; Yin et al., 2016). Notably, one study suggests that MT aberration upon ageing could
104 cause swellings that trap and damage mitochondria, thus triggering axon degeneration (Fiala et
105 al., 2007). However, in the existing literature too little emphasis is given to MTs and there are
106 simply not enough data to deduce meaningful correlations between axon degeneration and MT
107 bundle decay.

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108 Even if there were a close correlation, this still does not exclude that, depending on the
109 pathological condition, MT bundle deterioration may be a mere consequence rather than cause
110 of axon decay (details in Fig.4). Ultimate clarification will only arise from developing a better
111 understanding of MT bundle-forming and -maintaining machinery. Here we propose a
112 conceptual framework that may facilitate such developments.

113

114 From work in *Drosophila* to the integrated model of local axon homeostasis

115 The foundations for this conceptual framework were laid when we took the decision to use the
116 fruit fly *Drosophila melanogaster* as a means to study how cytoskeletal regulators collaborate in
117 orchestrating the morphogenetic changes that drive axon growth (Sánchez-Soriano et al.,
118 2007). *Drosophila* is not a miniature human, but it has many advantages and provides powerful
119 means to uncover the regulatory concepts behind the roles and regulations of axonal MTs,
120 which then often apply to higher organisms (Box 2; Aguzzi, 2019; Bellen et al., 2010; Elden et
121 al., 2010; Prokop, 2018). Through using *Drosophila* neurons as a consistent standardised cell
122 system, our group alone performed functional analyses of over 50 actin- and/or MT-binding or -
123 associating regulators (Prokop et al., 2013); these studies form an unprecedented pool of data
124 on the basis of which to develop novel concepts (Alves-Silva et al., 2012; Beaven et al., 2015;
125 Gonçalves-Pimentel et al., 2011; Qu et al., 2018; Qu et al., 2017; Voelzmann et al., 2016b).

Box 2 Why use *Drosophila*?

The use of *Drosophila* neurons to study the neuronal cytoskeleton has a number of advantages that were detailed elsewhere (Prokop et al., 2013). Key aspects are the high degree of conservation of cytoskeletal proteins, regulators and dynamics, the experimental amenability of neurons in primary cell culture and *in vivo* (Prokop et al., 2013; Prokop et al., 2012; Sánchez-Soriano et al., 2010), and the relative ease of genetic manipulation based on available resources and efficient combinatorial genetics (Hahn et al., 2016). The power of combinatorial genetics is rooted in the relative ease, speed and cost effectiveness with which genes can be manipulated and functionally analysed, facilitating also combined analyses of multiple factors in the same animals or cells (Prokop, 2018; Prokop et al., 2013; Roote and Prokop, 2013). Combinatorial genetics has been extremely successful in overcoming problems of redundancy, and generating new conceptual understanding of co-operative networks of MT regulation (see main text). This can hardly be achieved through isolated work on individual factors.

126

127 Our loss-of-function analyses of 24 MT-binding or -associating (2nd order) proteins, revealed that
128 more than half displayed significant MT disorganisation. Interestingly, the MT disorganisation
129 found in these various conditions appears to display certain common characteristics: axons
130 display areas in which their bundles are dissolved into chaotic, intertwined, crisscrossing
131 arrangements of curled MTs (see examples in Fig.5). These phenotypes were surprising when
132 considering that MTs usually behave like rigid rods (Fletcher and Mullins, 2010; Hawkins et al.,
133 2010; Howard, 2001). Notably, when using some of the same genetic conditions *in vivo*,
134 comparable phenotypes were observed in the fly brain (Qu et al., 2018). Such *in vivo*
135 phenotypes in the fly remind of the curled MT conformations in pathological axon swellings of
136 mammalian models mentioned in the previous section. Potential evolutionary conservation of
137 this phenomenon is further supported by the occurrence of similar MT curling and
138 disorganisation in mouse and rat primary neurons (Ahmad et al., 2006; Sánchez-Soriano et al.,
139 2009) - and more reports will emerge once researchers consider MT disorganisation a

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140 phenotype worth quantifying, not just an artefact.

141 As an attempt to explain the occurrence of this unusual phenotype across mutant conditions
142 and animal groups, we developed the model of '*local axon homeostasis*' (Prokop, 2016;
143 Voelzmann et al., 2016a), based on two fundamental elements:

144 (1) The model proposes that MTs in axons show a strong bias to become disorganised, most
145 likely because they are challenged to buckle ('d' in Fig.3) and/or curl up by the narrow
146 axonal environment enriched in MTs, force generating motor proteins and physical
147 obstacles posed by organelles and protein complexes (Fig.1, 'A-E' in Fig.3). Once MT
148 disorganisation occurs, it can develop into pathological axon swellings.

149 (2) The model proposes that this risk is contained through the actions of different classes of
150 MT-associating and -regulating proteins, which co-operate and complement each other to
151 form robust machinery that 'tames' MTs into bundles ('1-16' in Fig.3).

152 In this model, each axon segment uses local action of MT regulators to maintain its bundled MT
153 organisation (hence 'local axon homeostasis'). Hereditary or acquired loss of single regulators
154 would be expected to weaken this machinery and increase the statistical risk of MT
155 disorganisation. Such heightened probability might explain why many axonopathies affect
156 primarily long axons (Prior et al., 2017), and why certain disorders linked to MT regulators
157 display late onset of axon decay (Voelzmann et al., 2017).

158 In the next two sections, we discuss potential causes explaining the bias of axonal MTs to
159 become disorganised. We will then summarise experimentally demonstrated MT bundle-
160 maintaining mechanisms, and speculate about further mechanisms based on existing
161 knowledge of known classes of axonal MT-regulating proteins.

162

163 Understanding the unusual curling behaviours of MTs in axons

164 Although curvature is a key driver of MT plus end dynamics during de-/polymerisation (Brouhard
165 and Rice, 2018; van Haren and Wittmann, 2019), MT lattices *in vitro* usually behave as rigid
166 rods with a persistence length of 1-10 mm (as compared to ~12 µm measured for actin
167 filaments; Fletcher and Mullins, 2010; Hawkins et al., 2010; Howard, 2001). MTs are polar
168 polymers composed of α/ß-tubulin heterodimers which are arranged in a head-to-tail fashion
169 into linear protofilaments; usually 13 of these protofilaments are laterally aligned forming a
170 straight tube of roughly 25 nm diameter (Fig.6A, C). But MTs can deviate from this norm, and
171 this may introduce an intrinsic element of disorder into MT bundles: for example, axonal MTs
172 were reported to contain 13 protofilaments in frog olfactory or goldfish brain axons, but 11 or 15
173 in *C. elegans*, and 12 in *Drosophila*, crayfish and lobster (Benshalom and Reese, 1985; Burton
174 et al., 1975; Savage et al., 1989). Deviation from the straight 13 protofilament conformation
175 appears to equip MTs with distinct, functionally relevant physical properties (Chaaban and
176 Brouhard, 2017; Chalfie and Thomson, 1982). But it also introduces a skew into the MT
177 structure, which causes a supertwist of the tubule (Fig.6D; Chrétien and Fuller, 2000; Chrétien
178 et al., 1996; Chrétien and Wade, 1991); this supertwist forces motor proteins to rotate around
179 MTs (Ray et al., 1993) and is the likely explanation for supercoil of entire axons observed upon
180 MT bundle destabilisation (Krieg et al., 2017; Shaw and Bray, 1977).

181 Furthermore, MTs are structurally active: their physical properties can change when proteins
182 bind to them (e.g. kinesins, see below) or when the 'tubulin code' is altered. The tubulin code is
183 determined by the incorporation of different existing isotypes of α- and ß-tubulin into the MT
184 lattice, and the addition of a range of distinct post-translational modifications (Fig.6B; Janke and

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Kneussel, 2010; Park and Roll-Mecak, 2018; Ti et al., 2018; Vemu et al., 2017). Some modifications influence the interaction with MT-binding proteins (e.g. poly-glutamylation attracts spastin; Valenstein and Roll-Mecak, 2016), others are believed to structurally protect MTs from damage or depolymerisation, such as poly-aminations on various residues (Song et al., 2013), or acetylation of luminal lysine 40 which has been suggested to make MTs more flexible and break-resistant (Fig.6B; Baas et al., 2016; Howes et al., 2014; Portran et al., 2017; Soppina et al., 2012; Xu et al., 2017). Notably, site-directed mutation of lysine 40 in *Drosophila* α1-tubulin could demonstrate that intraluminal MT acetylation is physiologically relevant (Jenkins et al., 2017; Yan et al., 2018). In addition, the MT lumen may contain MIPs (MT inner proteins) that likely also modify MT stability (Ichikawa and Bui, 2018).

These intrinsic or acquired physical properties are likely to determine how MTs respond to external forces - and we can expect such forces to be highly enriched in axons (see next section). Some ideas about how forces may impact on axonal MTs can be derived from *in vitro* experiments. For example, MTs in flow chambers that are anchored at one end, will bend when applying flow and rapidly return to straight confirmation thereafter; when bent repeatedly in this way, MTs experience structural damage that triggers subsequent repair responses (Akhmanova, 2018; Schaedel et al., 2015; Triclin et al., 2018); when certain shaft-binding proteins (e.g. doublecortin or non-motile kinesin-1) are added, MTs become locked in bent conformation and fail to re-straighten (Bechstedt et al., 2014; Ettinger et al., 2016; Peet et al., 2018).

Another example is provided by so-called *in vitro* gliding assays, where MTs are moved around on carpets of active motor proteins. On carpets of (axonemal) dynein, MTs move plus-end-first; they undergo collisions at high frequency, but seem to stay fairly straight and form vortices at the millimetre scale (Sumino et al., 2012). In contrast, if similarly prepared MTs are on kinesin carpets, they move minus-end-first and undergo fewer collisions because they can pass over one another, likely owed to kinesin-1's adaptable length (Kerssemakers et al., 2006; Palacci et al., 2016; Sumino et al., 2012); however, if they collide or become pinned to the substrate (e.g. by dead kinesins) they frequently undergo dramatic shape changes at the micron-scale, including fishtailing and arc or loop formation (Amos and Amos, 1991; Lam et al., 2016; Weiss et al., 1991). The smallest diameters of curvature observed are similar to those of curled MTs in axons with values as low as 1-3 µm (Tab.1, Fig.5; Ahmad et al., 2006; Sánchez-Soriano et al., 2009) - and below 1µm, MTs are believed to break (Odde et al., 1999; Waterman-Storer and Salmon, 1997).

If MTs on kinesin carpets are reversibly cross-linked with biotin-streptavidin, they coalesce into bundles containing dozens of MTs which frequently curl up into spools with inner diameters similar to those of loops. Spools can take on similar appearances as looped MT bundles observed in growth cones of fly or mammalian neurons (Dent and Kalil, 2001; Hess et al., 2005; Sánchez-Soriano et al., 2010). Furthermore, single MTs can escape from spools which may trigger spool disassembly (Hess et al., 2005; Liu et al., 2008; VanDelinder et al., 2016b), bearing some resemblance with off-track MTs in axons.

Loops and spools *in vitro* might therefore be an experimental proxy for curling MTs or bundled loops in axons, and gliding assays might provide mechanistic insights into these MT behaviours (details in Tab.1): for example, loop formation is favoured by high density of MTs and/or kinesins (Lam et al., 2014; Liu et al., 2011), and both are clearly given in axons; kinesins directly impact on MTs (see below), but they can also cause pinning events in gliding assays, which could be seen as a potential proxy for the abundant obstacles in the narrow axons. Furthermore, the diameters of curls and spools in gliding assays increase with the degree of MT

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232 rigidity (Wada et al., 2015), and their clockwise versus counter-clockwise directionality of circle
233 formation is a function of the right- versus left-handed supertwist of the MTs involved - all
234 properties that could potentially be tested in axons (Fig.6D; Kawamura et al., 2008; Liu et al.,
235 2008). Furthermore, exposure to non-polar interfaces (e.g. n-heptane or air bubbles) induces
236 strong curling (Rashedul Kabir et al., 2012), and this may be relevant in axons: in ageing or
237 degenerative disease, changes in physical and chemical parameters of neurons affect liquid-
238 liquid phase separation (Alberti and Hyman, 2016); liquid compartments likely are of low polarity
239 (Nakashima et al., 2019) and might therefore influence the curling bias of MTs.

240 MT loops in gliding assays can be surprisingly stable (frequently >5 mins, as reported in Liu et
241 al., 2011). To explain this, it has been proposed that tubulin-heterodimers on the concave side
242 of the tube take on a shorter conformation than those on the convex site, and that this
243 asymmetric distribution can be maintained as an energetically favoured state (Fig.6F, bottom
244 right; Ziebert et al., 2015). In support of this model, tubulins in non-hydrolysed GMPCPP-MTs
245 were shown to be 1-3% longer than hydrolysed GDP-tubulin, and taxol added after (but not
246 during) polymerisation achieves a similar elongation (Fig.6F; Alushin et al., 2014; Amos and
247 Löwe, 1999; Arnal and Wade, 1995; Castle et al., 2017; Hyman et al., 1995). Notably, this
248 conformational length change seems physiologically relevant, as its suppression by the T238A
249 mutation in yeast ß-tubulin stabilises MTs *in vivo* and causes mitotic defects (Geyer et al., 2015;
250 Machin et al., 1995).

251 Such intrinsic properties of MTs may contribute to MT curling in axons, but we also need to
252 consider the presence of MT lattice-associating proteins, such as tau, doublecortin or kinesin-1
253 which were reported to bind differently to straight and curved MTs (Balabanian et al., 2017;
254 Bechstedt et al., 2014; Ettinger et al., 2016; Peet et al., 2018; Samsonov et al., 2004). In
255 particular kinesins-1 was shown to stabilise MT curvature by extending their lattice to similar
256 degrees as taxol (Peet et al., 2018), involving local compaction of tubulin that goes beyond
257 taxol- or GMPCPP-induced effects (Krebs et al., 2004; Morikawa et al., 2015). Since kinesin-1
258 has a preference for convex MT surfaces and was reported to undergo cooperative binding, this
259 may lead to a curvature-enhancing and -stabilising snowball effect with an estimated diameter
260 of curvature of 3.2 µm (Cross, 2019; Muto et al., 2005; Peet et al., 2018). Mathematical
261 modelling suggests that the kinesin carpet in gliding assays might induce stable yet reversible
262 curling in this way (Fig.6F, top right; Pearce et al., 2018).

263 Naturally, current models are in their infancy and further findings need to be incorporated. For
264 example, MTs behave as elastic cylinders (comparable to a garden hose) and can undergo
265 softening through cross-sectional flattening when strongly bent (Fig.6E; Kononova et al., 2014;
266 Memet et al., 2018). In this same vein, conformational changes of MTs upon kinesin-1 binding
267 were reported to soften MTs locally (Kabir et al., 2014). If confirmed, this would have important
268 implications for any existing models; together with the kinesin-induced tubulin compaction
269 (yellow asterisks in Fig.6F), it might be a mechanism to absorb energy and reduce the shear
270 force load on MTs. Notably, softening of MTs is also observed upon taxol application (usually
271 used in gliding assays; Tab.1; Castle et al., 2017) or MT acetylation (abundant in axons; Portran
272 et al., 2017; Xu et al., 2017), and might be a common prerequisite for curling behaviours.

273 Loop and spool formation in gliding assays are considered processes of 'active self-
274 organisation' (Lam et al., 2016); given the above listed similarities, the same might be true for
275 the formation of MT disorganisation in axons. Any *in vitro* studies addressing MT bending can
276 provide potential mechanisms that could underlie MT curling in axons; gliding and flow chamber
277 assays both suggest motor proteins, in particular kinesins, as key factors. In the next section we
278 will therefore summarise roles of axonal MT-associated motors during axon pathology.

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279

280 The intricate relationship between MTs and their associated motor proteins

281 MT-associated motors comprise the minus end-directed dynein/Dynactin complex and the
282 mostly plus-end directed proteins of the kinesin family (Hirokawa et al., 2010). Several kinesins
283 display direct roles in MT regulation (Sturgill and Ohi, 2013). These include active MT
284 depolymerisation (kinesin-8, -13; Walczak et al., 2013), MT polymerisation (kinesin-2, -5; Chen
285 and Hancock, 2015; Gumy et al., 2013; Guzik-Lendrum et al., 2017), MT-cross-linkage (kinesin-
286 5, -6, -12; see section on bundling), and roles in promoting MT orientation as a feature of
287 neuronal polarity (Tas et al., 2017; Zheng et al., 2008).

288 However, most attention is given to the active cargo and organelle transport and dynamics in
289 axons (Fig.3A-E; see section on axonal cytoskeleton), which is driven retrogradely by
290 dynein/Dynactin (Allan, 2011) and anterogradely by kinesins (primarily kinesin-1, -2, and -3;
291 Hirokawa et al., 2010). The forces imposed by these dynamics and/or the size of cargoes
292 moved, poses an obvious challenge to MT bundles (Appert-Rolland et al., 2015) and might be
293 an important factor leading to MT disorganisation.

294 Clearly, there is an intricate mutual regulatory relationship and finely tuned balance between the
295 amount of transport, and the structural properties of the transport highways (Appert-Rolland et
296 al., 2015; Prokop, 2013). On the one hand, MTs influence transport: for example, MT density is
297 higher in small calibre axons than in large axons (~15 *versus* ~150 MTs/μm^2), and mathematical
298 modelling suggests that this is required to achieve the same transport efficiency as in large
299 axons (Wortman et al., 2014; and references within); furthermore, the tubulin isotype
300 composition of MTs, their posttranslational modifications, and the physical presence of other
301 MT-binding proteins influence motor protein dynamics ('a' in Fig.3; Balabanian et al., 2017;
302 Monroy et al., 2018; Sirajuddin et al., 2014; Subramaniyan Parimalam et al., 2016). *Vice versa*,
303 transport affects MT bundles: for example, kinesin binding changes the physical properties of
304 MTs (see previous section), and motor proteins cause damage to the MTs they walk on,
305 triggering maintenance responses including MT repair or potentially even replacement ('14' in
306 Fig.3; Akhmanova, 2018; Dumont et al., 2015; Peet et al., 2018; Triclin et al., 2018; VanDelinder
307 et al., 2016a).

308 Tipping the balance in this mutual relationship can easily be imagined to cause reciprocal
309 deficiencies in transport rate and MT bundle organisation. For example, disorganisation or
310 partial breakage of MTs has been reported to cause pathological transport deficits (option '1' in
311 Fig.4; Fiala et al., 2007; Tang-Schomer et al., 2012). *Vice versa*, immunological lesioning
312 experiments to induce demyelination (Abdul-Majid et al., 2000; Baker et al., 1990), initially
313 caused transport defects, which were then followed by MT disorganisation ('2' in Fig.4; Sorbara
314 et al., 2014). Analogously, we observe severe MT disorganisation in *Drosophila* primary
315 neurons upon loss of kinesin-1 or -3 ('2' in Fig.4; unpublished; kinesin-1 shown in Fig.5E).

316 How loss of these kinesins may cause MT disorganisation can currently only be hypothesised.
317 For example, it has been reported for dendrites that kinesin-1 migrates on acetylated and
318 kinesin-3 on tyrosinated MTs (Tas et al., 2017). Provided the same is true in axons, the loss of
319 kinesin-1 would relieve acetylated MTs, but tyrosinated MTs would still bear their full transport
320 load - and *vice versa*. Such imbalances in transport distribution within MT bundles could lead to
321 shear forces that buckle MTs and seed MT disorganisation. In the same vein, MT
322 disorganisation was reported to be triggered by directional changes in motor traffic upon
323 deficiency of the dynein regulator NDEL1 at the axon initial segment (Kuijpers et al., 2016).
324 Furthermore, the movement of large cargoes likely induces dynamic and transient

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325 rearrangements of local MT-MT crosslinking networks (see section on cross-linkage) to make
326 the necessary space; in this scenario, violating the balanced proportion between cross-linkers
327 and transport may be a path to bundle aberration.

Box 3. The intricate relationship between MTs and axonal organelles

Mitochondria are the main source for ATP (Sheng, 2017), required to fuel multiple processes relevant for MT regulation (red arrows in Fig.3); these include actin assembly and dynamics relevant for MT regulation (Krendel and Mooseker, 2005; Skruber et al., 2018), protein phosphorylation for example of MT regulators (Bogoyevitch and Fairlie, 2007), GTP production required for MT polymerisation and signalling (Berg et al., 2002; Hall and Lalli, 2010; Voelzmann et al., 2016a), MT severing (McNally and Roll-Mecak, 2018), and MT-motor dynamics (Hirokawa et al., 2010; but note that vesicular transport uses local glycolysis to generate its own ATP; yellow star in Fig.3A; Hinckelmann et al., 2016; Zala et al., 2013). Secondly, the mitochondrial surface is an important signalling platform potentially required to orchestrate MT regulation locally (not shown in Fig.3; McBride et al., 2006). Thirdly, mitochondria cooperate with ER in the regulation of intracellular free calcium (yellow cloud in Fig.3; Rieusset, 2017; Wu et al., 2017) which has direct impact on MT regulators (e.g. spectraplakins, tau, kinesins; Kapur et al., 2012; McVicker et al., 2015) or even on MTs themselves (O'Brien et al., 1997). Fourthly, mitochondria collaborate with peroxisomes in the regulation of reactive oxygen species ('ROS' in Fig.3; Fransen et al., 2017; Pascual-Ahuir et al., 2017), which have known effects on MT regulation (Wilson and Gonzalez-Billault, 2015). If excessive amounts of the wrong ROS species are produced upon transport-induced mitochondrial damage or dysregulation of the mitochondria-peroxisome system, this causes oxidative stress as a major path to axon pathology (Fiala et al., 2007; Liu et al., 2017; Pascual-Ahuir et al., 2017).

Such causative relationships between MTs and oxidative stress can be experimentally demonstrated: for example the MT-stabilising drug epothilone B rescues oxidative stress caused by peroxisome transport deficiencies in a human iPSC model of SPG4 (spastin-linked spastic paraplegia 4; Wali et al., 2016), suggesting that MTs are the cause for the transport deficit in the first place.

Similar interdependencies would apply to other important organelles or membrane compartments that likewise depend on MT-binding motor proteins to undergo meaningful dynamics (Fig.3D); of particular importance are the ER with its multiple roles in calcium homeostasis, protein synthesis and lipidogenesis (Gonzalez and Couve, 2014), or the endolysosomal system required for proteostasis (Winckler et al., 2018). Also drug-induced inhibition of the proteasome-ubiquitination system has been shown to induce alteration in MTs and axonal transport (Poruchynsky et al., 2008; Staff et al., 2013).

328

329 Alternatively, transport defects might affect MTs through biochemical routes, simply caused by
330 the fact that the bundle-maintaining machinery runs out of supply and/or regulators. One would
331 expect deficient supply (a) of tubulin heterodimers as building blocks, (b) of the proteins
332 required to execute MT bundle maintenance work ('b' in Fig.3), and an absence or wrong
333 distribution (c) of organelles which are expected to play major roles in MT bundle maintenance
334 (see Box 3 for details).

335 Functional interdependencies between transport, organelle dynamics and MTs provide potential
336 explanations for a number of observations. For example, they may explain why axonal swellings

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337 induced by senile plaques in the *Tg-swAPP^{Prp}* mouse (overexpressing an amyloid precursor
338 protein carrying a familial Alzheimer's disease-linked mutation; Stokin et al., 2008) were strongly
339 enhanced when removing one copy of the KLC1 gene (a linker required for kinesin-1 mediated
340 transport) - and this effect is conserved in *Drosophila* (Stokin et al., 2005). They may explain
341 why different types of Charcot-Marie-Tooth disease or hereditary spastic paraplegias can be
342 caused through motor proteins as well as regulators of membranous compartments
343 (Blackstone, 2018; Bucci et al., 2012). They may also explain why MT stabilising drugs can be
344 beneficial in animal models of neurodegeneration as diverse as SPG4 (Box 3) and Alzheimer's
345 disease (Brunden et al., 2014).

346 Naturally, the argumentative framework presented here is highly speculative, given the
347 enormous complexity of the relationships between MT bundle organisation, motor protein
348 activity and systemic factors. But we hope that these reflections will motivate experimenters to
349 have a closer look at MTs in future studies of axon biology and pathology, and include
350 statements in their reports as to whether MTs are affected. More data are urgently needed,
351 which does often not require more than analysing neuronal morphology with antisera against
352 MTs (rather than restricting to intermediate filaments), or increasing the magnification in
353 ultrastructural studies to have a closer look at MTs. In the following sections we will explore the
354 mechanisms that are potentially used to form and maintain MT bundles against the odds of
355 motor-induced aberration or damage.

356

357 MT polymerisation as a fundamental requirement for bundle maintenance

358 The *de novo* formation of MT bundles during developmental, plastic or regenerative axon
359 growth ('8' in Fig.3) requires MT polymerisation. At later stages, MTs continue to undergo
360 polymerisation {Kleele et al., 2014; Voelzmann et al., 2016a}, likely to maintain a steady state
361 and prevent MT senescence through polymerisation-dependent MT repair and/or turn-over ('14'
362 in Fig.1; Akhmanova, 2018; Triclin et al., 2018). A well-regulated machinery of MT
363 polymerisation and disassembly (blue stippled arrows in Fig.3) is therefore needed to keep the
364 numbers of axonal MTs in balance with the transport load (see previous section; Wortman et al.,
365 2014).

366 As we detailed in a previous review (Voelzmann et al., 2016a), the machinery of MT de-
367 /polymerisation requires three sub-machineries: (1) dynamic protein complexes at the MT plus
368 end (blue balls, 'Eb1' in Fig.3); (2) the supply of α/β-tubulin heterodimers as building blocks
369 which occurs through a complex regulatory network in close co-regulation with MT dynamics ('c'
370 in Fig.3; Al-Bassam, 2017; Gasic and Mitchison, 2018; Preitner et al., 2014); (3) proteins which
371 bind or post-translationally modify the MT lattice with impact on plus end dynamics, for example
372 by stabilising MTs against depolymerisation ('7' in 'Fig.3).

373 The fine-tuning of net MT polymerisation appears to depend on complex regulatory networks.
374 This is illustrated by our recent work in *Drosophila* neurons, showing that loss of cortical actin
375 rings in the axon shaft (Fig.1) causes a reduction in MT polymerisation speed, eventually
376 affecting MT bundle integrity; simultaneous application of MT-destabilising drugs or removal of
377 the MT-stabilising spectraplakin Short stop (Shot) exacerbated these effects, frequently even
378 eliminating entire axons (Qu et al., 2017). Similar dependencies of MT polymerisation on actin
379 networks are suggested by other reports: (1) parallel loss of spectrin and tau causes axonal MT
380 loss in *C. elegans* (Krieg et al., 2017); (2) axon-shortening induced by the MT-stabiliser taxol
381 can be ameliorated through co-application of actin-destabilising drugs (in both chick and
382 *Drosophila* neurons; Letourneau et al., 1987; Sánchez-Soriano et al., 2010); (3) application of

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383 actin-destabilising drugs changes the tubulin-to-microtubule ratio in PC12 cells (Dennerll et al.,
384 1988) and causes axon retraction in chick dorsal root ganglia neurons (Datar et al., 2019; see
385 also Box 4). The best explanations for the mechanistic links from actin networks to net MT
386 polymerisation are currently provided by biomechanical models (see Box 4).

Box 4. Biomechanical models of axon growth

The regulation of axonal growth dynamics has been explained in terms of balance of forces between the microtubule and actin cytoskeleton (de Rooij et al., 2018; Fan et al., 2017; Miller and Suter, 2018). In axons, "*actin is under tension supported in part by microtubules under compression*" (Dennerll et al., 1988; Heidemann and Buxbaum, 1990). Tension is provided by the pull of the growth cone (Chan and Odde, 2008; Koch et al., 2012; Lamoureux et al., 1989) and the active contraction of acto-myosin along the axon shaft (Fan et al., 2017; O'Toole et al., 2015; Fig.1); the stiff nature of cross-linked MT bundles is well suited to oppose compressive forces up to a certain threshold (Buxbaum and Heidemann, 1992; de Rooij et al., 2018; Fig.2).

In such a balanced system, manipulations such as externally imposed pulling forces (Bray, 1984; Lamoureux et al., 2010; Pfister et al., 2004; Zheng et al., 1991) or genetic/pharmacological de-/stabilisation of acto-myosin (Ahmad et al., 2000; Datar et al., 2019; Dennerll et al., 1988; Ketschek et al., 2007; Turney et al., 2016; Wylie and Chantler, 2008) clearly modulate axon length or growth. Part of this response is expected to be due to changes in MT assembly, as was found when applying external forces to non-neuronal cells (Kaverina et al., 2002). But MTs themselves are not only responders in this context: the dis-/assembly or motor-based sliding of MTs can actively contribute by generating forces (Ahmad et al., 2000; Brouhard and Rice, 2018; Roossien et al., 2014; Winding et al., 2016).

How forces are sensed and translated into compensatory force generation and/or changes in axonal length or growth, remains an important question (see also the last section on cortical anchorage). Potential mechanisms might involve mechanically induced conformational changes of MTs (single MTs polymerise faster when being pulled on *in vitro*) or responses of polymerases such as XMap215 (Brouhard and Rice, 2018); but especially mechano-sensitive calcium channels in the axonal membrane (Franze et al., 2009; He et al., 2019; Heidemann and Buxbaum, 1990; Song et al., 2019) are promising candidates to orchestrate local responses that can even go beyond mere changes in MT polymerisation.

387

388

389 Maintaining MTs bundles through cortical guidance and elimination of polymerising MTs

390 Whilst MT polymerisation is a requirement for axon formation and maintenance, it also poses a
391 risk: for example, extending MTs may be obstructed by the abundant organelles or protein
392 complexes in axons, thus causing accidental 'off-track' MTs that project out of the bundle
393 towards the cortex ('4' in Fig.3). Apart from MT buckling, off-track MTs may be a second cause
394 for axonal MT disorganisation.

395 A key factor preventing this from happening is Eb1 (Alves-Silva et al., 2012; Figs.3 and 5B).
396 Eb1 directly binds at extending MT plus ends where it promotes polymerisation (Zanic et al.,
397 2013) and serves as a scaffold for many other proteins (Gupta et al., 2014). In the absence of
398 Eb1, MTs are severely disorganised, indicating important roles in MT maintenance (Alves-Silva
399 et al., 2012). One underlying mechanism is the guidance of polymerising MTs through binding
400 of Eb1 to Short stop (Shot); Shot is a well-conserved spectraplakin, able to cross-link cortical

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401 actin, MTs and Eb1 ('5' in Fig.3), thus guiding polymerising MTs in parallel to the axonal surface
402 and laying them out into parallel bundles (Alves-Silva et al., 2012). Accordingly, also loss of
403 Shot causes severe MT disorganisation in axons - and the same is true for its two mammalian
404 homologues ACF7 and dystonin (Bernier and Kothary, 1998; Dalpe et al., 1998; Sánchez-
405 Soriano et al., 2009; Voelzmann et al., 2017) - of which the latter links to the axonopathy
406 HSAN6 (type 6 hereditary sensory and autonomic neuropathy; Edvardson et al., 2012).

407 Such cortical guidance is complemented by at least one control mechanism: if MTs
408 (accidentally) leave their bundled arrangements and extend towards the cortex, they get
409 eliminated by Efa6 ('4' in Fig.3), a cortical collapse factor that associates with the axonal
410 membrane via its C-terminal plekstrin homology domain; when Efa6 is absent, off-track MTs
411 outside axonal MT bundles persist for longer and are higher in number (Qu et al., 2018).
412 Consistent with the known roles of off-track MTs in axon growth, branching and MT
413 disorganisation (see Box 1 and above), neurons lacking Efa6 display longer axons, more
414 branches and prominent MT disorganisation (Fig.5D; Qu et al., 2018).

415 Our model would predict that mutant phenotypes caused by loss of Shot and Efa6 should
416 enhance each other because they are caused through complementary mechanisms of MT
417 bundle regulation. Accordingly, we found a clear increase in MT disorganisation when removing
418 both Shot and Efa6 from the same neurons, in culture and *in vivo* (Qu et al., 2018). We propose
419 therefore that Shot and Eb1 keep MTs away from the membrane, whereas Efa6 acts as a
420 quality control factor eliminating occasional accidental off-track MTs; this elimination seems to
421 occur in moderate, well-balanced amounts so that 'intended' off-track MTs required for axon
422 growth and branching can persist and perform their function.

423 Interestingly, the cortical collapse function of Efa6 is not conserved in vertebrates (Qu et al.,
424 2018). Nevertheless, the concepts derived from Efa6 studies appear relevant, because loss of
425 the unrelated neuronal cortical collapse factor KIF21A (a type 4 kinesin) causes analogous
426 phenotypes in mammalian neurons. Thus, KIF21A mutations linked to the neurodevelopmental
427 disorder CFEOM1 (type 1 congenital fibrosis of the extraocular muscles) affect axon growth and
428 axonal branching just like Efa6 (Qu et al., 2018; van der Vaart et al., 2013) - and might as well
429 cause MT disorganisation, but no data are currently available.

430 However, guidance along cortical actin seems not the only mechanism through which Eb1 and
431 Shot keep MTs on track. This is illustrated by the simple fact that MT disorganisation observed
432 upon loss of Shot or Eb1 (Fig.5B, E) does not occur when removing actin from axon shafts
433 (Alves-Silva et al., 2012; Qu et al., 2017; Sánchez-Soriano et al., 2010). This suggests that both
434 factors perform additional, actin-independent functions or interactions to promote MT bundles.

435 For example, the unusual Shot-PH isoform, which is highly enriched in the nervous system and
436 harbours a plakin repeat region (PRR; conserved in mammalian spectraplakins), is a likely
437 candidate for such roles that still await investigation ('11' in Fig.3; Hahn et al., 2016; Voelzmann
438 et al., 2017). Eb1 has a long list of protein interactors besides Shot (Gupta et al., 2014), and
439 some of them might associate with MTs and guide extending plus ends along pre-existing
440 bundles ('9' in Fig.3); for example, APC or GAS2-LIKE family members (Pickled eggs/Pigs in
441 *Drosophila*) are good candidates, known to bind both MTs and Eb1 in mammals and *Drosophila*
442 (Beaven et al., 2015; Pines et al., 2010; Stroud et al., 2014).

443

444 <u>Potential roles of severing proteins and MT-destabilising kinesins in MT bundle maintenance</u>

445 Apart from cortical MT elimination, also MT severing and/or depolymerisation in the cytoplasm

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may play important roles in maintaining axonal MT bundles. This is supported by axonal MT disorganisation observed upon the losses of *Drosophila* katanin (our unpublished results) or mammalian spastin (Denton et al., 2014; Fassier et al., 2013; Havlicek et al., 2014; Tarrade et al., 2006).

As explained in the previous section, MTs leaving the bundled conformation can drive axonal growth, branching and MT disorganisation, and cortical collapse factors negatively regulate all three processes. In line with this argumentation, also the MT-depolymerising kinesin-13 family member Kif2A (Homma et al., 2003) and MT severing proteins (spastin, katanin and fidgetin) were reported to inhibit neurite growth and/or branching (Leo et al., 2015; Mao et al., 2014; Tao et al., 2016). However, other studies of spastin, katanin and fidgetin led to contradictory findings, describing them as promoters rather than inhibitors of neurite growth and branching (Ahmad et al., 1999; Butler et al., 2010; Havlicek et al., 2014; Karabay et al., 2004; Riano et al., 2009; Stewart et al., 2012; Stone et al., 2012; Wood et al., 2006; Yu et al., 2008). Such stark, potentially context-dependent deviations seem to reflect the complex regulation of severing proteins.

Spastin, katanin and fidgetin are all members of the superfamily of AAA proteins (ATPases associated with diverse cellular activities; McNally and Roll-Mecak, 2018; Sharp and Ross, 2012; Zhang et al., 2007), but their severing activity is differentially regulated through their individual responses to (a) posttranslational MT modifications (in particular acetylation and poly-glutamylation; Bailey et al., 2015; Lacroix et al., 2010; Leo et al., 2015; Shin et al., 2019; Sudo and Baas, 2010; Valenstein and Roll-Mecak, 2016), (b) antagonistic MT shaft-binding proteins such as tau (Qiang et al., 2018; Qiang et al., 2006; Yu et al., 2008), or (c) spatial recruitment through specifically localised proteins such as CAMSAP (Jiang et al., 2018). Furthermore, katanin has the ability to depolymerise MTs in an ATP-independent manner (Belonogov et al., 2019).

Through this precise context-dependent spatiotemporal regulation of their activities, severing proteins can have two diametrically opposed outcomes: they either eliminate MTs and reduce their numbers, or they break them up into stable fragments that serve as seeds for MT amplification (Baas et al., 2016; McNally and Roll-Mecak, 2018; Vemu et al., 2018). In the following, we will briefly discuss how either of these outcomes could be used to prevent MT disorganisation:

First, MT severing proteins could complement roles of cortical collapse factors ('4' in Fig.3) by serving as quality control factors that eliminate disorganised MTs in the cytoplasm ('6' in Fig.3). For example, katanin in plant cells was reported to localise and sever preferentially at MT cross-points, which can be used to take out non-aligned MTs (McNally and Roll-Mecak, 2018).

Second, MT shortening functions of katanin are required at MT minus ends. Thus, in both mammals and *Drosophila*, the minus-end capper CAMSAP/Patronin protects against MT disassembly, and recruits katanin to counterbalance against uncontrolled minus-end polymerisation ('13' in Fig.3; Goodwin and Vale, 2010; Jiang et al., 2018; Nashchekin et al., 2016); uncontrolled minus end extension upon katanin deficiency may cause MTs to go off-track or to buckle through extra forces produced.

Third, MT elimination functions could prevent MT bundle senescence. For example, MTs suffer from damage through tear-and-wear (Dumont et al., 2015; Peet et al., 2018; Schaedel et al., 2015; Triclin et al., 2018; VanDelinder et al., 2016a), which might cause bundle aberration by abrogating interactions with MT-binding proteins (red cross at '16' in Fig.3). MT fractures or holes can be repaired through mechanisms involving katanin or spastin (Davis et al., 2002;

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492 Diaz-Valencia et al., 2011; Gasic and Mitchison, 2018; Triclin et al., 2018; Vemu et al., 2018).
493 More subtle features of senescence (e.g. irreversible modifications, loss of tubulin C-tails) might
494 require selective elimination of ageing MTs through severing factors (as similarly suggested for
495 kinesin-8 or -13; Gardner et al., 2011), followed by compensatory polymerisation ('14' in Fig.3).
496 For example, spastin deficiency in the *Sp$^{\Delta}$* mouse model causes a drop in MT polymerisation
497 (which might reflect reduced turn-over) accompanied by a rise in MT disorganisation (which
498 might be due to precocious MT senescence; Fassier et al., 2013).

499 However, the MT phenotypes observed in the *Sp$^{\Delta}$* mouse model could likewise be explained
500 through the opposite role of spastin in MT multiplication. Thus, in the absence of spastin-
501 mediated amplification, MT numbers might gradually decline and cause transport interruptions;
502 this, in turn, would affect MT bundle organisation and eventually cause axonal pathology (see
503 section on motor proteins; Wali et al., 2018; Wali et al., 2016). Curiously, axon swellings in this
504 model were reduced with low doses of MT-stabilising or -destabilising drugs (Fassier et al.,
505 2013), therefore failing to provide any clues as to whether spastin works through MT turn-over
506 or amplification in this context.

507 Understanding spastin is important because it is by far the most prominent factor linking to
508 spastic paraplegias worldwide (Koh et al., 2018; Schüle et al., 2016), and axonal swellings are a
509 hallmark of the disease (Blackstone, 2018; Zempel and Mandelkow, 2015). Most SPG4-linked
510 mutations lie within the AAA-ATPase domain (Shoukier et al., 2009), suggesting that MT
511 severing is key to the disease pathology. However, point mutations might generate versions of
512 spastin, which either act as dominant negative alleles (forming dysfunctional complexes that
513 titrate out other spastin-interacting factors), or acquire gain-of-function qualities by diffusing
514 away to perform very different roles. One such MT-independent role of spastin is the isoform-
515 specific regulation of the endoplasmic reticulum ('15' in Fig.3), including its shape, its interaction
516 with the endosome and its production of lipid droplets (Allison et al., 2017; Papadopoulos et al.,
517 2015; Park et al., 2010; Solowska and Baas, 2015). It is therefore difficult to exclude that at
518 least part of those SPG4-linked mutations triggers axon decay through other routes than the
519 direct induction of MT aberrations ('2' or '3' versus '1' in Fig.4).

520

521 <u>Potential roles of MT-MT cross-linkage in MT bundle maintenance</u>

522 MT-MT cross-linkage ('12' in Fig.3) is likely the oldest mechanistic concept put forward by
523 neurobiologists to explain MT bundles (Lee and Brandt, 1992) and appears an obvious means
524 of suppressing MT disorganisation. Physical cross-linking strands between axonal MTs were
525 observed decades ago (Hirokawa, 1982; Hirokawa, 1986), and mathematical models support
526 MT-MT cross-linkage as an important structural feature of axons (e.g. de Rooij and Kuhl, 2018;
527 Lazarus et al., 2015; Li et al., 2018; Peter and Mofrad, 2012). To illustrate this point, axons have
528 been described as a "*stiff spring in series with a viscoelastic (Voight) element composed of a*
529 *less stiff spring in parallel with a fluid dashpot*" (Heidemann et al., 1990), meaning that axons
530 are under rest tension and combine elastic and viscous properties. A central structural
531 component underpinning such properties is likely provided by networks of MT-MT cross-linkers
532 (Fig.2), where each linker is able to detach upon super-threshold pull or compression, and re-
533 attach thereafter (slip-bonds). However, the molecular players mediating MT-MT cross-linkage
534 remain surprisingly controversial to this day, as explained in the following.

535 First, showing that a neuronal linker expressed in non-neuronal cells induces MT bundling, is
536 insufficient proof: MT bundling can even be achieved through expression of isolated MT-binding
537 domains, or the application of the MT-stabilising drug taxol which causes bundles with

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ultrastructural cross-bridges that are indistinguishable from those induced by tau or MAP2 (Chapin et al., 1991; DeBonis et al., 2015; Goriounov et al., 2003; Kader et al., 2017; Lee and Brandt, 1992).

Second, dynamin is linked to Charcot-Marie-Tooth disease and has been shown to bundle MTs *in vitro*; however, the physiological relevance of this is questionable, because dynamin *in vivo* seems to bind primarily membranes (Scaife and Margolis, 1990; Shpetner and Vallee, 1989; Züchner et al., 2005).

Third, MTLC1 and MAP1B (Futsch in *Drosophila*) appear ideal cross-linkers, because they both possess an N- and a C-terminal MT-binding domain; they were shown to induce MT bundles upon expression in non-neuronal cells (with MAP1B being a weak bundler; Kader et al., 2017; Penazzi et al., 2016; Satake et al., 2017), and the fly homologue Futsch promotes looped MT bundles at synaptic terminals (Roos et al., 2000). Upon loss-of-function, MTLC1 causes MT disorganisation at the axon initial segment, strongly supporting its role as MT-MT cross-linker in this specific compartment (Satake et al., 2017). In contrast, the long history of MAP1B/Futsch research is mostly dedicated to aspects of axon development (Hummel et al., 2000; Migh et al., 2018; Villarroel-Campos and Gonzalez-Billault, 2014), but we are aware of only one isolated report showing axonal bundle defects (upon loss of Futsch; Bettencourt da Cruz et al., 2005).

Fourth, the other conserved linker candidate tau, has only one central MT-binding region, but it achieves physical MT-MT linkage *in vitro* through N-terminal dimerisation (Chung et al., 2016; Méphon-Gaspard et al., 2016; Rosenberg et al., 2008). However, its dwell time on MTs seems very short (Janning et al., 2014; Samsonov et al., 2004); similar to MAP1B/Futsch, reported tau-deficient phenotypes in neurons mainly concern developmental defects (Penazzi et al., 2016), but we are aware of only one report of bundle aberration (in *C. elegans*; Krieg et al., 2017; Penazzi et al., 2016).

Pinpointing roles of tau or MAP1B/Futsch in MT-MT cross-linkage is enormously complicated by the fact that both proteins seem to perform a whole array of further molecular functions relevant for MT dynamics. For example, tau can protect MTs from severing by katanin (Qiang et al., 2006), bind tubulin hetero-dimers (Shin et al., 2018), switch between bundled and single MT states (Prezel et al., 2018), cross-link MTs with actin or the cortex (Biswas and Kalil, 2018; Cabrales Fontela et al., 2017; Maas et al., 2000), stabilise MTs during axon initiation (Brandt, 1998), maintain labile domains along MT shafts (Qiang et al., 2018), regulate End-binding proteins (Sayas et al., 2015), compete with kinesins (Trinczek et al., 1999), and promote MT nucleation and polymerisation (Penazzi et al., 2016). A similarly broad pleiotropy has been reported for MAP1B (Villarroel-Campos and Gonzalez-Billault, 2014).

Pinpointing relevant MT-MT cross-linking activities of specific factors is also complicated by functional redundancies. For example, *MAP1B* and *tau* mutations have enhanced growth phenotypes when combined in double-mutant mouse neurons (Takei et al., 2000), and co-expression of Futsch and Tau causes enhanced phenotypes in the *Drosophila* CNS (Hummel et al., 2000). Functional redundancies likely extend to further potential cross-linkers. For example, Kinesin-5 (KIF11), kinesin-6 (KIF23, Pavarotti in *Drosophila*) and kinesin-12 (KIF15) slide anti-parallel MTs in the mitotic spindle (Baas, 1999); in axons MTs are arranged in parallel, and these kinesins seem to inhibit sliding (Dong et al., 2019; Lin et al., 2012; Liu et al., 2010; Lu et al., 2013; Myers and Baas, 2007; Nadar et al., 2012), indicating that they cross-link MTs. In support of this idea, we observe that loss of *Drosophila* Pavarotti causes axonal MT disorganisation which might reflect potential linker function (unpublished data).

In conclusion, MT-MT cross-linkage is a widely accepted concept, but experimental support for

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584 its existence in axons and our knowledge of the molecular players involved is insufficient. We
585 even cannot fully exclude a model where MT bundles are held together by the corset of
586 contractile cortical actin rings (Fig.1), and cross-linkers merely separate MTs to generate space
587 for transport (Fan et al., 2017). If we are to decipher the true molecular nature of MT-MT cross-
588 linkage in axons, future studies will have to address the challenges of functional redundancies
589 between different classes of linker candidates.

590

591 Does MT bundle maintenance involve their anchorage to the axonal surface?

592 Apart from cross-linking MTs within axonal bundles, they might also be anchored to the axon
593 wall, thus achieving an even more stable structure that can prevent MT buckling and bundle
594 deformation caused by the enormous forces imposed by axonal cargo transport. Relevant in
595 this context are observations in developing vertebrate and fly neurons of a gradual flow of MT
596 bundles towards the distal axon tip (Miller and Sheetz, 2006; O'Toole et al., 2008; Reinsch et
597 al., 1991; Roossien et al., 2013). Forces contributing to this process could be derived from an
598 increase in MT volume through polymerisation along the axon shaft (Sánchez-Soriano et al.,
599 2010), pulling forces in the rear of growth cones (O'Toole et al., 2015), thermal motion of MT-
600 MT cross-linkers (Lansky et al., 2015), kinesins actively sliding MTs along other MTs ('B' in
601 Fig.3; Lu and Gelfand, 2017), or dyneins sliding MTs along cortical F-actin ('10' in Fig.3; Ahmad
602 et al., 2006; He et al., 2005; Myers et al., 2006; Roossien et al., 2014).

603 Potential MT sliding along cortical actin would represent one form of tethering MT bundles to the
604 axonal surface. Such anchorage is also suggested by observed co-drift of the axolemma with
605 the axon core (Lamoureux et al., 2010; Popov et al., 1993; Zheng et al., 1991). But anchorage
606 would not have to be static; for example, it might involve an interface of slip-bonds, as similarly
607 suggested for actin networks that flow across, whilst dynamically anchoring to, relatively stable
608 focal adhesion sites (Case and Waterman, 2015). MTs could anchor to cortical actin (Fig.1; '2' in
609 Fig.3; Xu, 2013 #6895} or to membrane-associated or transmembrane proteins including ion
610 channels, ion transporters or adhesion factors (Fig.1; '3' in Fig.3). Links to transmembrane
611 proteins could be used as mechano-sensing modules (Yap et al., 2018) to measure local shear
612 forces generated between MT bundles and the axonal environment (Fig.1). Such mechano-
613 sensing could explain local regulation phenomena: for example, net rates of mitochondrial
614 movement along the axon are fairly constant, but the slow transport component (driven by MT
615 bundle flow) is low in proximal and high in distal axon segments; this gradual increase in the
616 amount of slow transport is compensated for by fast transport (high proximal, low distal; Miller
617 and Sheetz, 2006). The regional amount of fast mitochondrial transport could potentially be
618 regulated by mechano-sensing, measuring the local MT drift rate relative to the outer axonal
619 environment.

620 Apart from dynein (see above), other potential anchoring mechanisms can be deduced from the
621 literature. For example, spectraplakins are good candidates, as suggested by distal shift of
622 axonal MTs in fly neurons lacking the *Drosophila* spectraplakin Shot and treated with the MT-
623 stabilising drug taxol (Voelzmann et al., 2017). Three distinct mechanisms could account for
624 spectraplakin-mediated MT anchorage: Firstly, spectraplakins could directly cross-link actin and
625 MTs ('2' and '5' in Fig.3). Secondly, they could link to membrane-associated proteins; thus, the
626 mammalian spectraplakin dystonin can link to ß4-integrin and transmembrane collagen XVII ('3'
627 in Fig.3; Voelzmann et al., 2017), and *Drosophila* Shot is able to regulate the axonal localisation
628 of the cell adhesion molecule Fasciclin 2, potentially cross-linking Fasciclin 2 to MT bundles
629 (Bottenberg et al., 2009; Prokop et al., 1998). Thirdly, spectraplakins were shown in non-
630 neuronal cells of fly and mammals to anchor MT minus ends to the cortex ('1' in Fig.3;

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631 Nashchekin et al., 2016; Ning et al., 2016; Noordstra et al., 2016); this mechanism requires
632 interaction with the MT minus end-stabilising factor CAMSAP/Patronin, a factor that is known to
633 be relevant for neuronal morphology (Yau et al., 2014).

634 Also other MT-binding proteins, such as tau, MAP1B, APC and dynamin, might be involved in
635 anchorage since they were also reported to bind to actin or to the cortex ('2' in Fig.3; Biswas
636 and Kalil, 2018; Blanchoin and Michelot, 2012; Brandt et al., 1995; Elie et al., 2015; Gu et al.,
637 2010; Maas et al., 2000; Mohan and John, 2015; Villarroel-Campos and Gonzalez-Billault,
638 2014). But potential MT-actin cross-linkage in the axon may not only occur at the cortex, but as
639 well at central longitudinal actin trails (Fig.1; Leterrier et al., 2017), thus further contributing to
640 the intricate cross-linking networks expected to stabilise MT bundles. Deciphering MT bundle
641 cross-linkage, internally or with the axonal surface, stays a major challenge for future
642 experimentation that will teach us important lessons about axon biology and pathology.

643

644 <u>Conclusions and future perspectives</u>

645 Here we have presented a conceptual view by describing a functional interactome that
646 integrates the enormous complexity of cross-regulatory networks acting at the local level in
647 axons. We propose that there has to be a fine balance between damaging effects inflicted by
648 life-sustaining motor movements ('associated', 'A-E' in Fig.3) and the activities that maintain the
649 highways required for this movement (MT-'taming' mechanisms; '1-15' in Fig.3); both are fine-
650 tuned through a number of cross-regulatory mechanisms ('a-e' in Fig.3).

651 Our model integrates a broad range of findings from the literature, but its original foundations
652 are derived from our own work in *Drosophila* neurons as one consistent cellular system. Like
653 other genetic invertebrate models, *Drosophila* provides a cost-effective and fast system to
654 unravel the functional overlap and interface of different genetic factors - ideal to dissect complex
655 machinery and deliver data that then often apply to axons of higher animals (Beaven et al.,
656 2015; Prokop, 2018; Prokop et al., 2013).

657 This strategy offers one feasible strategy towards solving the daunting task of disentangling the
658 enormous complexity of axonal MT bundle regulation. For this, the model of local axon
659 homeostasis could provide a useful basis, helping to develop testable working hypotheses; a
660 good starting point might be to break down the local axon homeostasis machinery into
661 classifiable sub-machineries, like those discussed in the different sections of this review.

662 This approach also means that the discovery of new molecular mechanisms should no longer
663 be the only gold standard for axon research, but we need to recognise the value of long-term
664 approaches that gradually assemble known and newly discovered molecular mechanisms into
665 an integrated understanding of how regulation at the cellular level can be orchestrated. In our
666 opinion, this would be a much needed strategy shift, providing understanding of axons at the
667 organisational level at which axonopathies become manifest. As B.A. Cohen put it: "Research
668 that results in models that reliably and quantitatively predict the outcomes of genetic,
669 biochemical, or pharmacological perturbations should be valued highly, and rewarded,
670 regardless of whether such models invoke novel phenomena" (Cohen, 2017).

671 For the studies of MTs in neurons, we need to take into consideration that knowledge derived
672 from non-neuronal cells might not apply (Beaven et al., 2015). Furthermore, the interactome
673 shown in Fig.3 makes clear that we will need quantitative approaches: we know increasingly
674 well how factors bind to MTs and partly understand how they might compete with each other.
675 But how crowded can a single MT be, how many molecules are there in its surrounding at any

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676 time point, and how much dynamic exchange is taking place? Computational modelling will be
677 an unavoidable means to make sense of existing data and make reasonable predictions to
678 inform experimentation (Cohen, 2004; Gunawardena, 2014).

679 Integrated understanding of axon biology will also improve our knowledge of the next higher
680 level of complexity, i.e. the mechanisms that orchestrate axon homeostasis and that maintain
681 balance even during phases of change (e.g. when switching from growth to differentiation, or
682 during stress, injury, regeneration) - or that tip the balance and induce degeneration. Obviously,
683 signalling networks or dynamic changes of systemic factors such as second messengers or the
684 'tubulin code' will be key players to this end (Baas et al., 2016; Park and Roll-Mecak, 2018;
685 Schelski and Bradke, 2017; Wilson and Gonzalez-Billault, 2015) - and glial cells will likely act as
686 important external influencers of such processes (Pan and Chan, 2017).

687 Finally, MTs have been recognised as promising therapeutic targets (Baas and Ahmad, 2013;
688 Eira et al., 2016; Zempel and Mandelkow, 2015), and urgently needed advance on this
689 translational path will be facilitated by a better understanding of the axonal MT homeostasis
690 system. A larger focus of the research community on MTs, and generation of more and relevant
691 data that can be incorporated into our understanding, would be a key prerequisite to make such
692 progress.

693

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704

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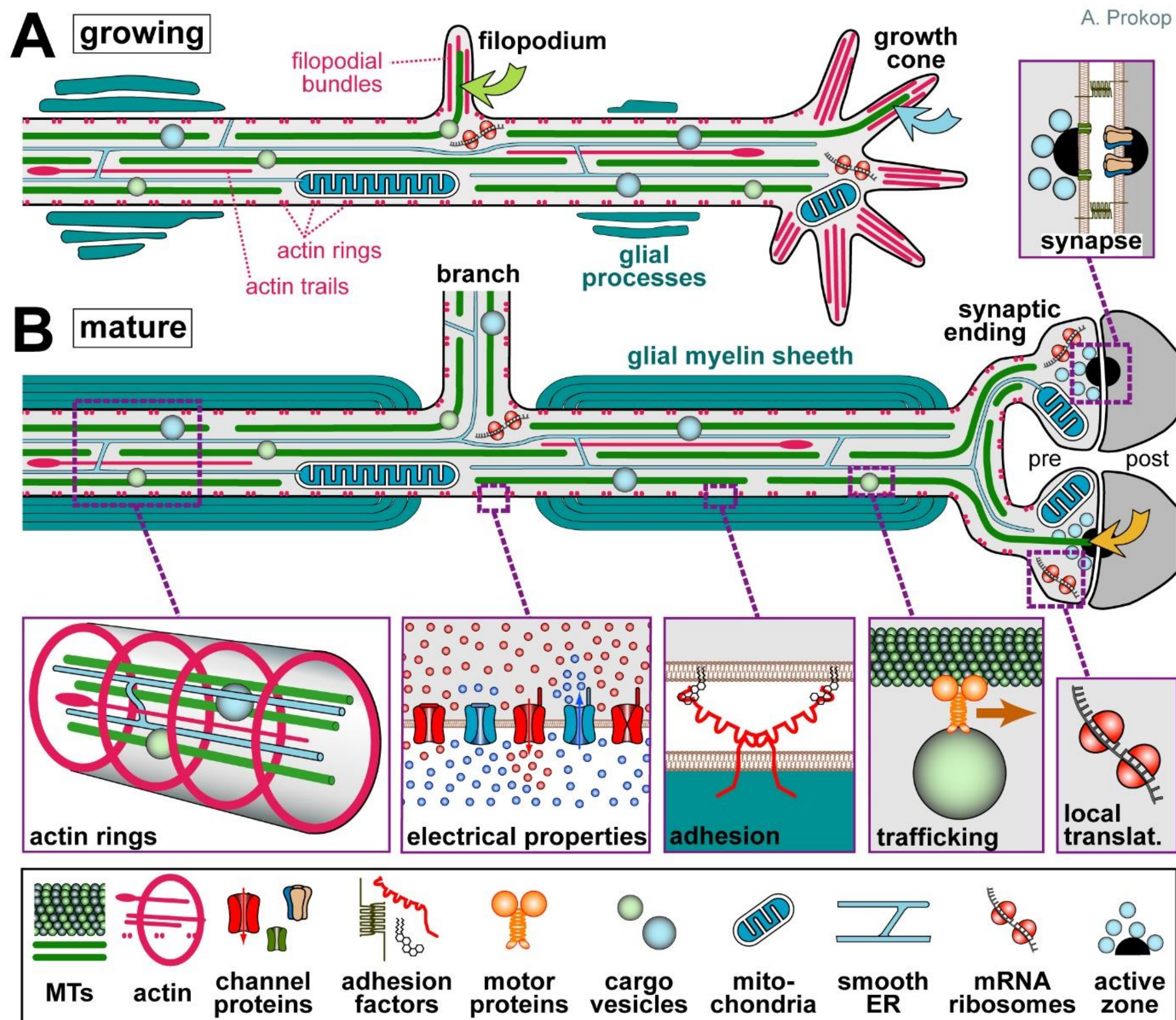
1815 **Figures**

1816



1817

1818 **Fig. 1** Specific properties of axons. Axons during the growth cone stage are shown in (**A**) and after
1819 synaptic maturation in (**B**), differing primarily in certain stage-specific specialisations including growth
1820 cones, synapses, electrical properties and glial interactions (here myelination; Meyer and Kaspar, 2017;
1821 Pan and Chan, 2017). The core machinery in the axon shaft can be expected to be similar at both stages:
1822 parallel continuous bundles of extended but discontinuous MTs run all along axons serving as a structural
1823 backbone (see Fig.2), a transport highway for axonal trafficking (driven by motor proteins), and a source
1824 for 'off-track' MTs contributing to morphogenetic processes including branch formation, directed axon
1825 growth and synapse formation/plasticity (green, orange, blue curved arrows); MT bundles are
1826 interspersed with longitudinal actin trails (Leterrier et al., 2017), continuous networks of (smooth) ER
1827 (Gonzalez and Couve, 2014), and other membranous organelles including mitochondria (Saxton and
1828 Hollenbeck, 2012); axonal membranes display regularly spaced periodic rings of cortical actin (Qu et al.,
1829 2017; Xu et al., 2013; Vassilopoulos et al., 2019), an unusually high number of ion-specific channel
1830 proteins and transporters to conduct nerve impulses (Kandel et al., 2012), as well as adhesions with
1831 external structures including parallel axons (not shown), glial processes (Pronker et al., 2016) and
1832 synaptic partner cells (Koper et al., 2012); a degree of independence from cell-body derived proteins is
1833 provided by local translation machinery (Cioni et al., 2018; Giuditta et al., 2002b; Shigeoka et al., 2018) or
1834 supply from surrounding glia cells (not shown; Court et al., 2011; Frühbeis et al., 2013; Giuditta et al.,
1835 2002a; Rajendran et al., 2014) . Note that the axon diameter in the region between glia cells in B (referred
1836 to as Node of Ranvier) usually has a much smaller diameter than the rest of the axon (Hoffman, 1995).

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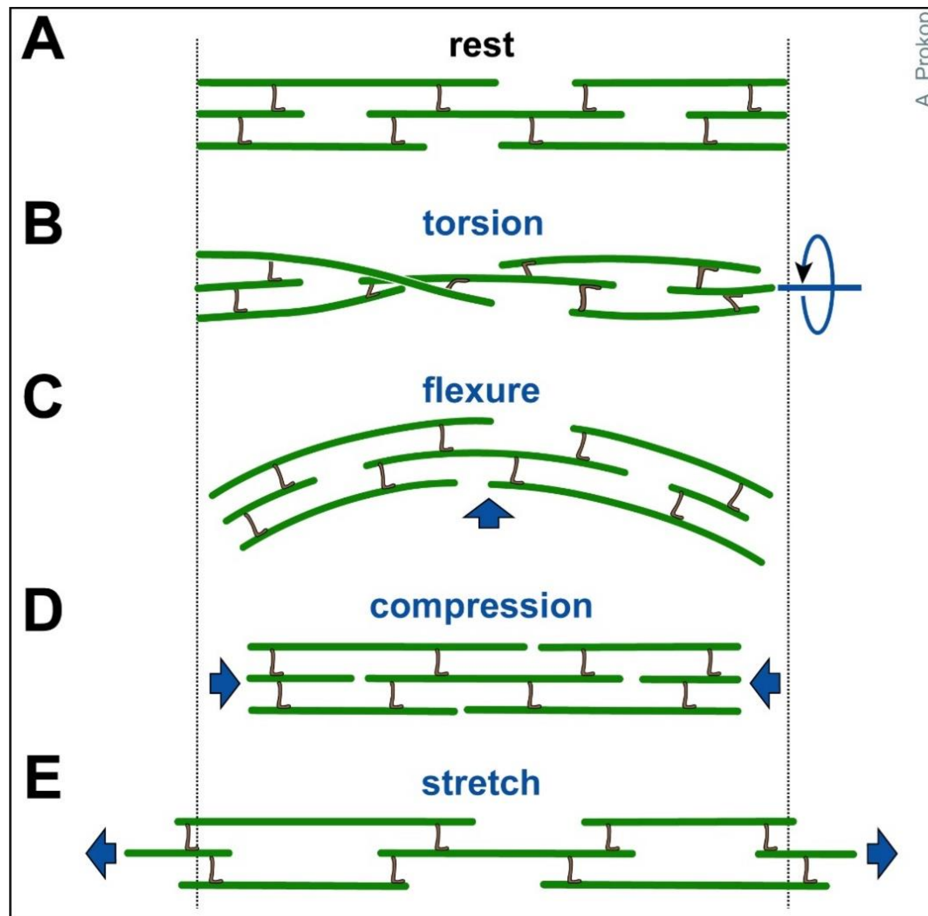

1837

1838 **Fig. 2** Axonal response to mechanical challenges. Continuous bundles of discontinuous MTs which are
1839 flexibly cross-linked (likely involving slip-bonds) are thought to provide a structural element that can
1840 respond to different forms of mechanical impact (as indicated in blue).

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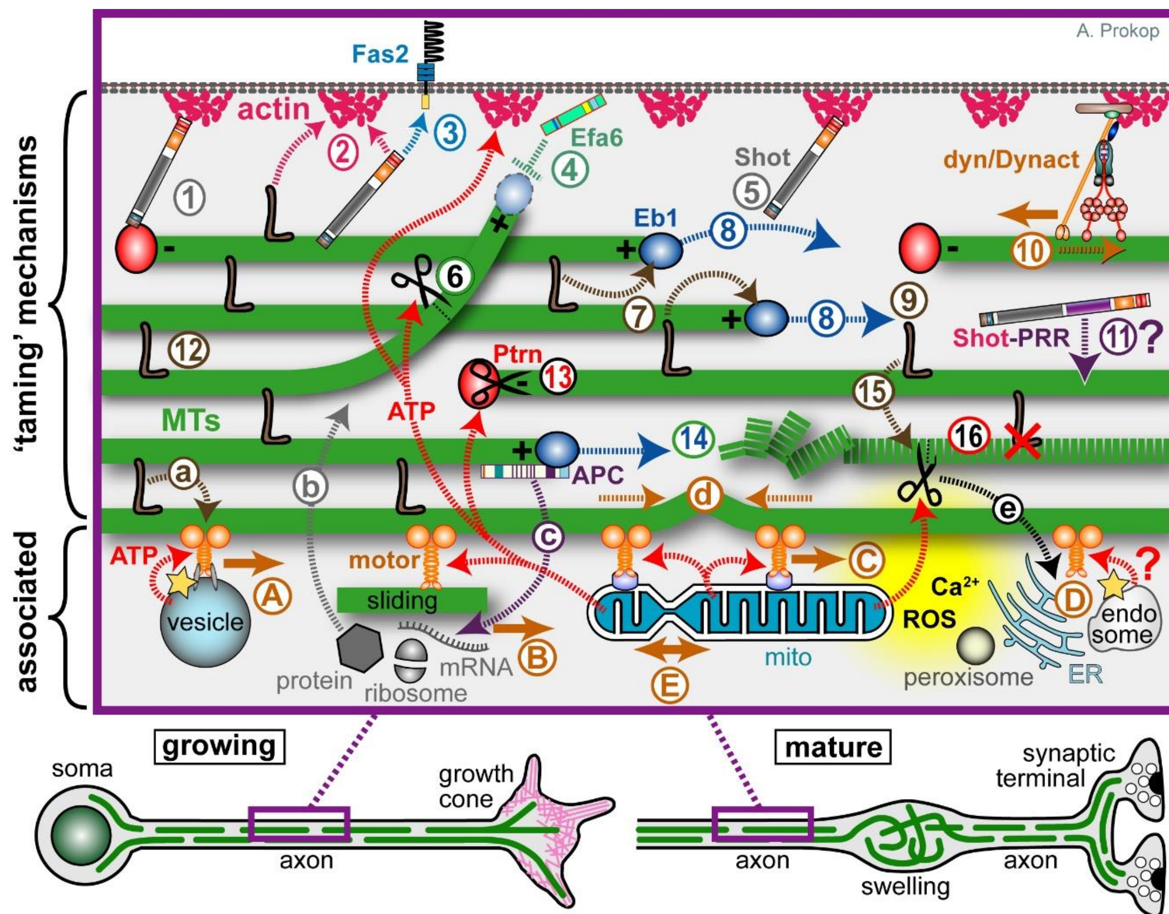

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1842 **Fig. 3** An interactome of MT-regulating and -associated mechanisms expected to contribute within the
1843 model of local axon homeostasis. Developing and mature neurons are shown at the bottom indicating that
1844 the close-up (magenta frame) might apply in both contexts. **1**-**16**) Potential mechanisms that can 'tame'
1845 MTs into bundled conformation: MT polymerisation (blue stippled arrows) is driven by molecular
1846 machinery centred on Eb1 (blue balls), further influenced by the tubulin-supply machinery (not shown)
1847 and shaft-binding proteins (**7**); polymerisation generates new MTs required for bundle formation (**8**) and
1848 turn-over (**14**); to integrate into bundles, extending MTs require guidance via actin-Eb1 cross-linkage
1849 along the axonal surface (**5**; Shot) or along pre-existing MTs through MT-MT cross-linkers (**9**; brown L).
1850 The same or other cross-linkers provide the structural glue that holds MT bundles together (**12**; brown L);
1851 some of them can also bind to actin (**2**), they protect from (or recruit) MT severing activity (**15**), and
1852 influence motor protein dynamics (**a**). MTs which have escaped any cross-linkage are eliminated by
1853 cortical collapse factors when approaching the axonal surface (**4**; Efa6) or by MT severing factors at MT-
1854 MT cross-points (**6**). The bundled MTs are discontinuous; their free minus ends are stabilised by
1855 CAMSAP/Patronin (Ptrn) together with katanin (black scissors; **13**), whereas non-polymerising MT plus
1856 ends are stabilised by other factors (not shown; e.g. CLASP or the Dynactin subunit p150/Glued; Hur et
1857 al., 2011; Lazarus et al., 2013). The dynein/Dynactin complex is believed to link cortical actin to MT
1858 bundles and drive them anterogradely (**10**), whereas Ptrn at minus ends may anchor MTs via
1859 spectraplakins to the axon cortex (**1**); spectraplakins may also link MTs directly to cortical actin (**2**) or to
1860 transmembrane receptors (**3**), and they are expected to perform further, still unexplored actin-
1861 independent bundle-promoting roles through their PRR domains (**11**). Tear-and-wear damages MTs
1862 (dashed green line), potentially affecting interaction with MT-binding proteins (**16**; red X); MT severing
1863 proteins might selectively eliminate such MTs (**16**; scissors) or MTs undergo repair (not shown). **A**-**E**)
1864 Mechanisms closely 'associated' with MT bundles: MT-associated motor proteins ('motor', solid orange
1865 arrows) drive axonal transport of (protein-loaded) vesicles (**A**), cytoplasmic factors including proteins,
1866 translational machinery (ribosomes) or RNAs (**B**), move other MTs (**B**, sliding), and position/rearrange
1867 organelles including mitochondria (**C**, mitos), ER, peroxisomes and endosome (**D**) - and this likely
1868 includes mitochondrial fission and fusion (**E**). **a**-**e**) The motor-associated functions all act downstream of

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1869 MT bundles because they require them to walk on; but they also act upstream: for example, the forces
1870 they generate (stippled orange arrows) are the potential cause for MT disorganisation (buckling shown in
1871 **d**); transport delivers required regulators and building blocks for bundle-maintaining processes (**b**); the
1872 proper regulation of organelles/endocytic compartments provides systemic factors that can orchestrate
1873 taming mechanisms, including intracellular free calcium or reactive oxygen species (Ca^{2+}, ROS; yellow
1874 cloud) as well as ATP required for many processes including actin dynamics, MT severing and MT motor
1875 activity (red stippled arrows; note that vesicular transport uses glycolysis to generate its own ATP; yellow
1876 star); *vice versa*, the MT severer spastin also regulates the ER through ATP-independent mechanisms
1877 (**e**), and MT-associated proteins (APC) regulate local translation events (**c**).

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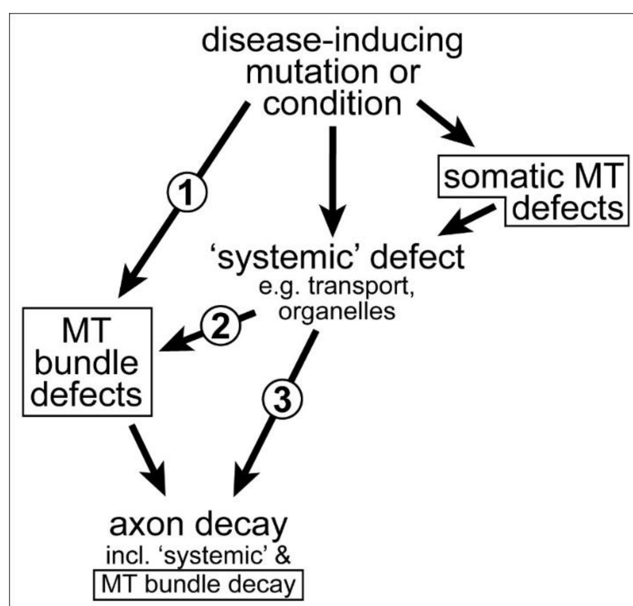

1882

1883 **Fig. 4** MT bundle defects as cause or consequence of axon decay. **1**) Disease-inducing
1884 mutations/conditions can affect a MT-bundle regulator (e.g. dystonin; Voelzmann et al., 2017), thus
1885 causing MT bundle defects first which can, in turn, trigger axon decay. **2**) Disease-inducing
1886 mutations/conditions can affect systemic factors which, in turn cause MT bundle defects as an
1887 intermediate causative step in the cascade leading to axon decay (e.g. axonal transport fails, leading to
1888 MT bundle defects which then contribute to axon decay (e.g. Alzheimer's disease or ALS; Brandt and
1889 Bakota, 2017; Farah et al., 2003; Zempel and Mandelkow, 2015); this may occur even if MT regulators
1890 are affected, but these regulators mainly act in the cell body (e.g. dysregulation of the Golgi; Ferrier et al.,
1891 2013). **3**) MT bundle deterioration may be a mere consequence of axon decay, although this case will be
1892 difficult to disentangle from option 2, since MT bundle disintegration and axonal disassembly may occur in
1893 parallel, as observed in developmental or injury-induced axon degeneration; Bradke et al., 2012; Wang et
1894 al., 2012; Yaron and Schuldiner, 2016). All MT-related phenotypes in this graph are emphasised with a
1895 frame.

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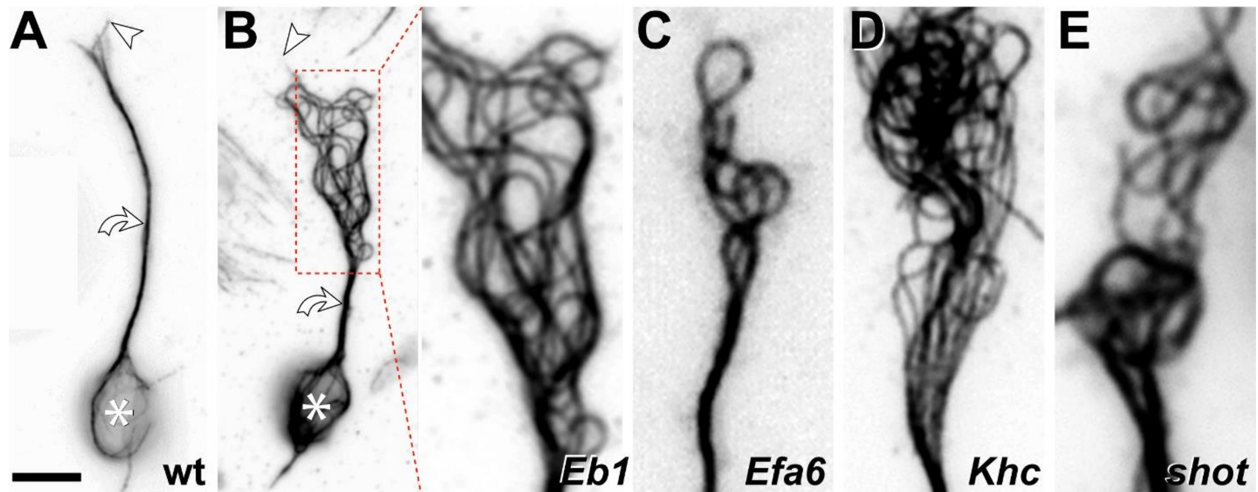
1896 

1897 **Fig. 5** Disorganisation of axonal MTs upon loss of different MT regulators in *Drosophila* primary neurons.
1898 **A)** Normal neuron (wild-type, wt) with soma (asterisk), axon shaft (curved arrow) and growth cone (tip of
1899 most distal MT indicated by arrow head). **B)** $Eb1^{5}$ mutant neuron where the area of MT disorganisation is
1900 framed by a red stippled box and shown as close-up on the right. **C-E)** Similar close-ups shown for
1901 $Efa6^{GX6[w-]}$, Khc^{27} and $shot^{3}$ mutant neurons. Note that the four mutated factors perform fundamentally
1902 different molecular functions, with Eb1 being a MT plus-end binder ('8' in Fig.3), Efa6 a cortical collapse
1903 factor ('4' in Fig.3), Khc a kinesin-1 motor protein ('A-E' in Fig.3) and Shot a multi-functional cross-linker
1904 ('1-3, 5, 11' in Fig.3). All neurons were derived from wild-type or homozygous mutant embryos,
1905 mechanically and chemically dissociated, kept for 7days in pre-culture in a centrifuge tube to deplete any
1906 maternal gene product, mechanically and chemically dissociated again, cultured on concanavalin A-
1907 coated glass coverslips for 1day at 21°C, fixed and stained with anti-α-tubulin (DM1A, Sigma; procedures
1908 detailed elsewhere: Prokop et al., 2012); images were taken using STED (stimulated emission depletion)
1909 microscopy. Scale bar in A represents 10 µm for the two neurons and 4 µm in close-ups.

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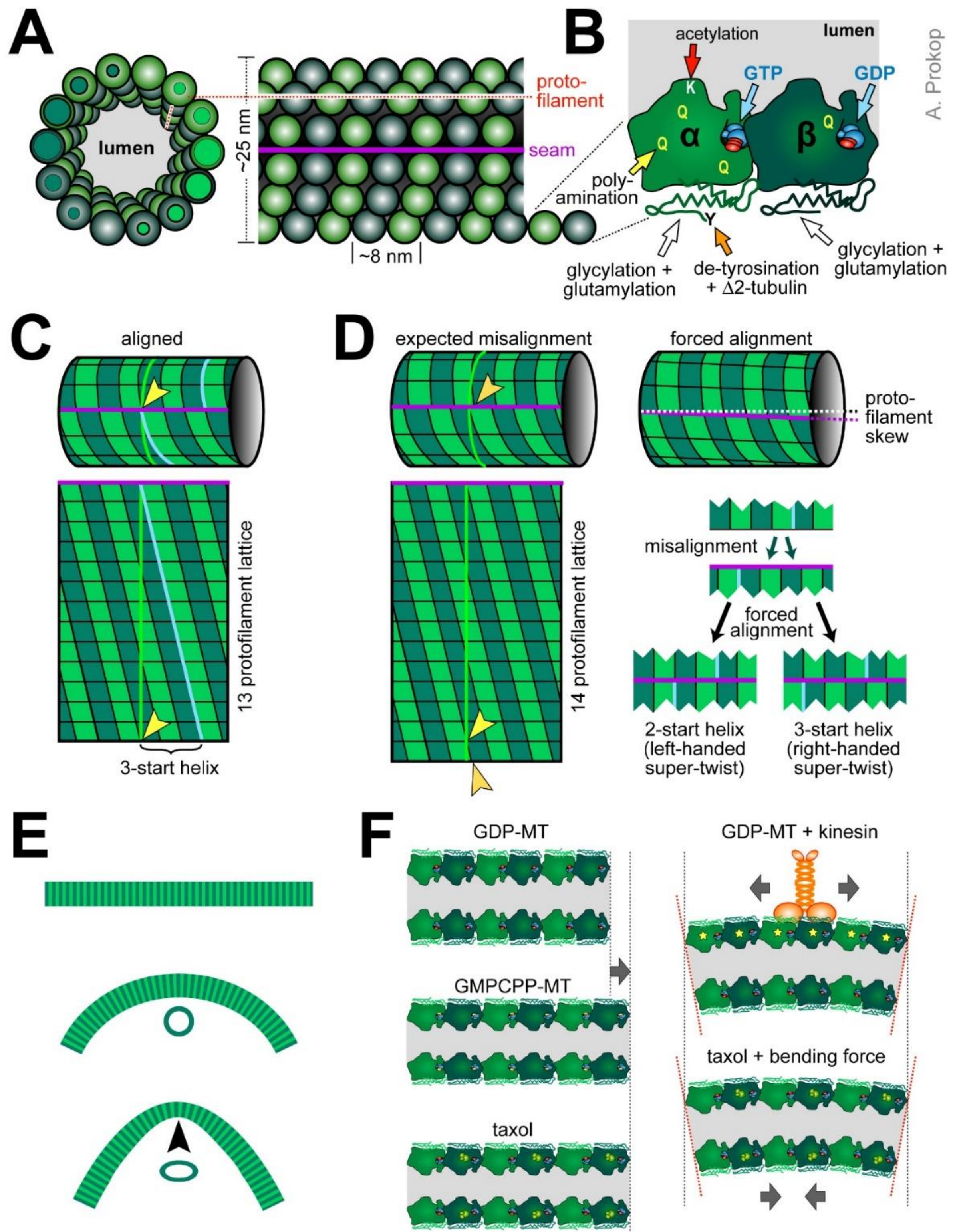

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1911 **Fig. 6** A molecular perspective of microtubule properties. **A)** Cross-section of a MT with 14 protofilaments
1912 (PF) and lateral view of a 13 PF MT, both in B-lattice configuration, where α-tubulins make lateral bonds
1913 with α-tubulins and ß with ß, except at the seam (magenta line: seam; dashed red line: PF). **B)** Close-up
1914 of an α/ß-tubulin heterodimer showing the various post-translational modification sites as indicated; note
1915 that the GTP of ß-tubulin in lattices is usually hydrolysed (GDP). **C)** A 13 PF MT (top), cut open at the
1916 seam and rolled out (bottom); the yellow line shows the diameter, the blue line follows the helical rise of
1917 laterally bonded tubulins; in 13 PF MTs, tubulins are precisely aligned at the seam (yellow arrow head)
1918 but shifted by three positions (3-start helix). **D)** When deviating from the 13 PF prototype, tubulins are
1919 misaligned at the seam (orange arrow head); when forced into alignment, the PFs skew, causing a super-

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twist of the MT as described by the 'lattice accommodation model' (Chrétien and Fuller, 2000; Langford, 1980); for certain PF numbers, MTs can form two alternative alignments, of which usually the version with the lower helix start value (left) has a left-handed super-twist, the higher value is right-handed (Chrétien and Fuller, 2000). **E)** MTs behave like rigid rods with a persistence length of up to 10 mm, but can be bent down to diameters of ~1µm before they break; it has been reported that their cross-sectional profile may flatten above a certain threshold (black arrow head), thus softening the tube. **F)** Lattices of GDP-tubulin are 1-3% shorter than MTs that were polymerised with the non-hydrolysable GTP analogue GMPCPP, or stabilised with taxol (orange structure binding α-tubulin 1:1, according to Nogales et al., 1995); binding of kinesin-1 causes similar lengthening of tubulin (and additional compactions in the tubulin structure: yellow stars) which may cause cooperative binding of further kinesins and induce curvature if occurring only on one side of the MT; in extended taxol-bound MTs, bending forces were suggested to transfer tubulins on the concave side into their short conformation as an energetically favoured condition. For further references see main text.

experimental conditions	**diameters of curvature [µm] [a]**	**comments**	**ref.**
kinesin-1 carpets			
standard tub, 10-20 µm taxol (after?) [b] polym.	1-1.4 [c]	waves and curls upon pinning	[1]
standard tub, 50 µM taxol during & after polym.; high MT density (2.5 MTs/μm^2)	1-5	loops form through collision; loop duration up frequently >5 min; strong increase in loops at high MT conc.; decreasing loop radius with increasing contour	[2]
rhodamine-tub, 10 µm taxol after polym.; exposing to air bubble or n-heptane	1.1 (heptane), 1.8 (air)	MTs become reversibly unstable in non-polar conditions: 50% of MTs form loops as long as close to air bubble; effect absolutely requires kinesins	[3]
rhodamine-tub, 10 µM taxol after polym.	2.5-3.75 [c]	left-handed supertwist favours CCW rotation of loops; CCW rotation is preserved in spools	[4]
biotin-tub, 10 µm taxol after polym.; SA-linked	1-12.6, mean 3.9		
biotin-tub, 10 µm taxol after polym.; SA-linked	1-5, mean 2.3	up to 25 µm long straight bundles; pinning of tip induces spools or fishtailing; rare “unspooling” events	[5]
biotin-tub, 10 µm taxol after polym.; SA-linked; 1600, 870, 270 and 90 kinesins/μm^2	ca. 2.4-4	highest spool density & lowest spool diameter @ highest kinesin density; pinning as main cause for spool formation	[6]
biotin-GTP-tub, 10 µm taxol after polym.; SA-linked	5.7 (@ 10.8 µm length), 3 (@ 3,7 µm)	spool diameters increase with MT length per condition; spool diameters: GMP-MTs (taxol) < GMPCPP-MTs (no taxol) <	[7]

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biotin-GMPCPP-tub, 10 µm taxol after polym.; SA-linked	18.8 (@ 10.3 µm length), 5.8 (@ 3.4 µm)	GMPCPP-MTs (taxol)	
biotin-GMPCPP-tub, no taxol; SA-linked	8.2 (@ 10 µm length), 4.3 (@ 3.4 µm)		
biotin-GTP-tub, 10 µm taxol (after?)[b] polym.; SA-linked	3.2 µm (@ 6µm length)	live imaging: pinning & collisions (simultaneous sticking) cause spool formation; spool formation is not activated by a Brownian ratchet type process	[8]
biotin-tub, 10 µm taxol after polym.; SA-linked; microfluidic device	2.7 (pinning), 6.2 (collisions)	live imaging: pinning & collisions (simultaneous sticking) cause spools of different diameters; pinning more frequent in flow cells than microfluidic device	[9]
biotin-tub, (taxol?)[b] polym.; SA-quantum dot-linked	1.2, mean 3.4	left/right-handed super-twist: CCW/CW rotation; rings form intertwined wreath-like structures; tendency to disassemble involving MT breakage, kinesins pulling (blocked by AMP-PNP), counteracted by SA (enhanced by biotin)	[10]
biotin-tub, 10 µm taxol after polym.; SA-quantum dot-linked; patterned kinesin carpets	1-5.3 and 3.1	smallest spool diameters on constrained carpets: 1-5.3µm on 5 µm stripes, 3.1 µm on 2 µm wide squares	[11]
axonemal dynein carpet			
Cy3-tub, 10µM taxol	straight	forming vortices in mm range	[12]

1938 **Tab. 1** MT loop or spool formation in gliding assays under different conditions. a) Primarily the lower
1939 range of mentioned diameters is listed; b) not clear from experimental section; c) measured from images.
1940 References [1] (Amos and Amos, 1991), [2] (Liu et al., 2011), [3] (Rashedul Kabir et al., 2012), [4]
1941 (Kawamura et al., 2008), [5] (Hess et al., 2005), [6] (Lam et al., 2014), [7] (Wada et al., 2015), [8] (Luria et
1942 al., 2011), [9] (VanDelinder et al., 2016b), [10] (Liu et al., 2008), [11] (Liu and Bachand, 2013), [12]
1943 (Sumino et al., 2012). Note that a number of mathematical models were put forward to describe loop or
1944 spool dynamics in gliding assays (Crenshaw et al., 2011; Gosselin et al., 2016; Luria et al., 2011; Pearce
1945 et al., 2018; Ziebert et al., 2015).