

## 1 New cell biological explanations for kinesin-linked axon degeneration

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11 ♦ Please, note that Y.-T.L. is the key contributor to this work as part of her PhD thesis (Liew, 2018).  
12 Unfortunately, we have not been able to establish contact for over two years, so that direct  
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### 23 Abstract

24 Axons are the slender, up to meter-long projections of neurons that form the biological cables wiring  
25 our bodies. Most of these delicate structures must survive for an organism's lifetime, meaning up to  
26 a century in humans. Axon maintenance requires life-sustaining motor protein-driven transport  
27 distributing materials and organelles from the distant cell body. It seems logic that impairing this  
28 transport causes systemic deprivation linking to axon degeneration. But the key steps underlying  
29 these pathological processes are little understood. To investigate mechanisms triggered by motor  
30 protein aberrations, we studied more than 40 loss- and gain-of-function conditions of motor proteins,  
31 cargo linkers or further genes involved in related processes of cellular physiology. We used one  
32 standardised *Drosophila* primary neuron system and focussed on the organisation of axonal  
33 microtubule bundles as an easy to assess readout reflecting axon integrity. We found that bundle  
34 disintegration into curled microtubules is caused by the losses of Dynein heavy chain and the Kif1  
35 and Kif5 homologues Unc-104 and Kinesin heavy chain (Khc). Using point mutations of Khc and  
36 functional loss of its linker proteins, we studied which of Khc's sub-functions might link to microtubule  
37 curling. One cause was emergence of harmful reactive oxygen species through loss of Milton/Miro-  
38 mediated mitochondrial transport. In contrast, loss of the Kinesin light chain linker caused  
39 microtubule curling through an entirely different mechanism appearing to involve increased  
40 mechanical challenge to microtubule bundles through de-inhibition of Khc. The wider implications of  
41 our findings for the understanding of axon maintenance and pathology are discussed.

## 42 Introduction

43 Axons are the long and slender processes of neurons which form the biological cables that wire the  
44 nervous system and are indispensable for its function. In humans, axons can be up to 2 metres long  
45 whilst displaying diameters of only 0.1-15µm (Prokop, 2020). Most of these delicate cellular  
46 processes must survive for an organism's lifetime, meaning up to a century in humans.  
47 Unsurprisingly, mammals lose about 40% of their axon mass towards high age (Calkins, 2013;  
48 Coleman, 2005; Marner et al., 2003). This rate is drastically increased in hereditary forms of  
49 axonopathies (Prokop, 2021).

50 Of particular interest to this article are mutations of microtubule-binding motor proteins that cause  
51 axonopathies, of which the OMIM® database (Online Mendelian Inheritance in Man®; Amberger et  
52 al., 2015) currently lists DYNACTIN 1 (ALS1, OMIM® reference #105400), DYNEIN HEAVY CHAIN  
53 1 (CMT2A1, #614228), KIF1B (CMT20, #118210), KIF5A (SPG10, #604187; ALS, #617921), KIF1A  
54 (SPG30, #610357; HSN2C, #614213), KIF1B (CMT2A1, #118210) and KIF1C (SPAX2, #611302);  
55 in the case of KIF1A, links to HSP and ataxias are likely to be added soon (Nicita et al., 2020). The  
56 listed motor proteins are actively involved in live-sustaining axonal transport of RNAs, proteins, lipids  
57 and organelles (Guedes-Dias and Holzbaur, 2019; Hirokawa et al., 2010). Genetic aberration of such  
58 transport is thought to lead to systemic collapse of axonal structure and physiology, hence  
59 axonopathy. However, we have little understanding of the concrete mechanisms leading to these  
60 pathologies.

61 To bridge this important knowledge gap, we took a novel approach based on two strategic decisions:  
62 Firstly, we used axonal microtubules (MTs) as our main readout. These MTs are arranged into loose  
63 bundles that run all along axons to form the essential highways for axonal transport and to provide  
64 a source of MTs for axon growth and branching at any life stage (Prokop, 2020). Accordingly,  
65 aberrations of MT bundles (presenting as gaps or areas of bundle disorganisation in the form of MT  
66 curling) are sensitive indicators of axonal pathology (Prokop, 2021). Mechanisms that help to  
67 maintain these MT bundles are starting to emerge (Hahn et al., 2019).

68 Our second strategic decision was the use of *Drosophila* primary neurons as a cost-effective and  
69 fast model system, where the complexity of mechanisms can be addressed with powerful genetics,  
70 in ways hard to achieve in vertebrate models (Prokop et al., 2013). For example, in this study alone,  
71 we used over 40 different mutations or transgenic constructs - some of them in genetic combinations  
72 to address functional redundancies or hierarchies (e.g. Beaven et al., 2015; Gonçalves-Pimentel et  
73 al., 2011; Koper et al., 2012). Loss-of-function analyses in *Drosophila* are facilitated by the fact that  
74 key factors, such as Kinesin heavy chain (Khc, kinesin-1), Kinesin light chain (Klc) or Milton, are  
75 each encoded by a single gene in *Drosophila*, as opposed to three, four or two in mammals,  
76 respectively. Furthermore, genetic and pharmacological tools are readily available to manipulate  
77 virtually any genes in question - and all these functional approaches can be combined with efficient  
78 and well-established readouts for axonal physiology and MTs (Hahn et al., 2016; Prokop et al., 2013;  
79 Prokop et al., 2012; Sánchez-Soriano et al., 2010).

80 Here we demonstrate that the losses of three motor proteins cause MT curling: the Kif5A/B  
81 homologue Kinesin heavy chain (Khc), the Kif1A homologue Unc-104, and Dynein heavy chain  
82 (Dhc/DYNC1H1). We find that all three are required for axonal transport of mitochondria and synaptic  
83 proteins. Focussing on Khc and employing available means to dissect its various sub-functions, we  
84 identified two mechanisms linking to MT curling: Firstly, loss of Khc/Milton/Miro-mediated transport  
85 causes harmful reactive oxygen species (ROS) likely linking to mitochondrial transport. Secondly,  
86 loss of the Kinesin light chain linker appears to cause de-inhibition of Khc as a condition that we find

87 to cause MT curling. Both mechanisms align with the recently proposed 'dependency cycle of local  
88 axon homeostasis' as a conceptual model of axonopathy (Prokop, 2021).

89

## 90 **Results**

91

### 92 Loss of Khc, Unc-104 or Dhc cause axonal MT curling

93 To assess whether loss of motor protein function impacts on axonal MT organisation, we tested  
94 mutant alleles for axonal transport-related motor proteins: (a) Dynein heavy chain (Dhc) is an  
95 obligatory component of the dynein/Dynactin complex and essential for most, if not all, MT-based  
96 retrograde transport (Reck-Peterson et al., 2018); (b) Klp64D is an obligatory subunit of  
97 heterodimeric kinesin-2 (KIF3 homologue) reported to mediate anterograde axonal transport of  
98 acetylcholine-related synaptic enzymes or olfactory receptors (Baqri et al., 2006; Jana et al., 2021;  
99 Kulkarni et al., 2017; Ray et al., 1999); (c) the PX-domain-containing type 3 kinesin Klp98A (KIF16B  
100 homologue) was shown to mediate autophagosome-lysosome dynamics and endosomal Wingless  
101 transport in non-neuronal cells but is also strongly expressed in the nervous system (Mauvezin et  
102 al., 2016; Witte et al., 2020; flybase.org: [FBgn0004387](#)); (d) the PH-domain-containing type 3 kinesin  
103 Unc-104 (Kif1A homologue) is essential for synaptic transport in *Drosophila* axons (Pack-Chung et  
104 al., 2007; Voelzmann et al., 2016); (e) Kinesin heavy chain/Khc is the sole kinesin-1 in *Drosophila*  
105 (Kif5A-C homologue) involved in multiple transport functions in *Drosophila* neurons (see details  
106 below; e.g. Bowman et al., 2000; Gindhart et al., 2003; Glater et al., 2006; Loiseau et al., 2010;  
107 Rosa-Ferreira et al., 2018; Saxton et al., 1991).

108 To assess potential roles of these motor proteins in MT regulation, we cultured primary neurons  
109 obtained from embryos lacking these gene functions (see Methods; Fig.1) and analysed them at  
110 5DIV (days *in vitro*). MT curling phenotypes (where bundles deteriorate into curled, intertwined,  
111 crisscrossing MTs; curved arrows and enlarged insets in Fig.1B,F,G) occurred as a moderate  
112 phenotype in Dhc-deficient neurons and were prominent in neurons lacking Khc or Unc-104 (Fig.1H).

113 To assess whether MT curling phenotypes were accompanied by transport defects, we analysed  
114 additional sub-cellular markers in *Khc*<sup>8</sup>, *unc-104*<sup>170</sup> and *Dhc64C*<sup>4-19</sup> homozygous mutant neurons.  
115 First, using the pre-synaptic protein Synaptotagmin (Syt) as an indicator of vesicular transport  
116 (Voelzmann et al., 2016), we found reduced presynaptic spots within axons of neurons mutant for  
117 any one of these three motor proteins, suggesting they all contribute to axonal vesicular transport  
118 (Fig.2A-D,O). Second, the axonal number and distribution of mitochondria (visualised with  
119 mitoTracker; Klionsky et al., 2012), is significantly reduced in neurons lacking either Khc, Unc-104  
120 or Dhc64C function (Fig.2H-K,P). Third, in *Khc*<sup>8</sup> mutant neurons, we also assessed the distribution  
121 of endoplasmic reticulum (ER) using the genomically tagged *Rtnl1-GFP* allele. In wild-type neurons,  
122 ER is distributed evenly along the entirety of the axon (Fig.S1A); loss of Khc does not affect this  
123 distribution, but about three quarters of neurons show an abnormal accumulation of Rtnl1::GFP-  
124 labelled ER material at their tips (Fig.S1B,C; details in legend).

125 In conclusion, the three motor proteins that display MT curling are also required for normal axonal  
126 transport of synaptic vesicles and mitochondria in *Drosophila* primary neurons. In addition, at least  
127 Khc is also required for the normal axonal distribution of ER.

128

129

130 Khc displays strong maternal effects

131 Of these three motor proteins, we performed more detailed analyses on Khc because many genetic  
132 tools are available for the systematic dissection of its various functions (Fig.3B). First, to validate its  
133 MT-related phenotypes, we tested additional mutant alleles (*Khc*<sup>27</sup> and *Khc*<sup>8</sup> in homozygosis or over  
134 deficiency) as well as RNAi mediated knockdown of Khc (via pan-neuronal *elav-Gal4*). In all cases  
135 we found that the MT curling phenotypes were equally present at 5DIV (Fig.S2A,B,D).

136 However, the MT phenotypes were not evident at earlier stages in *Khc*<sup>8/Df</sup> mutant neurons, either at  
137 6 hours *in vitro* (HIV) or at 3DIV (Fig.S2B,D), suggesting that phenotypes either accumulate gradually  
138 (as observed upon loss of *Efa6*; Qu et al., 2019) or are masked by perdurance of maternal product  
139 (meaning wild-type *Khc* gene product deposited in the egg by the heterozygous mothers; Prokop,  
140 2013).

141 To distinguish between these two possibilities, we used a pre-culture technique where neurons are  
142 kept in centrifuge tubes for 5 days to deplete maternal gene product before plated in culture (Prokop  
143 et al., 2012; Sánchez-Soriano et al., 2010). Such pre-cultured neurons displayed prominent MT  
144 curling already at 1DIV (Fig.S2C,E), arguing that Khc has a prominent maternal contribution that  
145 persists for more than 3 days. Similar observations were made with the *Khc*<sup>1ts</sup> mutant allele (details  
146 in Fig.S2C).

147

148 MT sliding functions of Khc do not link to MT curling

149 A C-terminal MT-binding site enables Khc to cross-link MTs and move them against each other  
150 (Fig.3Bi; Andrews et al., 1993; Jolly et al., 2010; Lu et al., 2013; Lu et al., 2015; Winding et al., 2016).  
151 We hypothesised that Khc might contribute to MT bundle maintenance by using its MT sliding  
152 function, for example by shifting MTs to achieve even distribution along axons. The Khc sliding  
153 function is selectively inhibited by the genomically engineered, lethal *Khc*<sup>mutA</sup> allele that abolishes C-  
154 terminal MT binding without interfering with other linkers or autoinhibition of Khc (Fig.3A; Winding et  
155 al., 2016).

156 To test whether Khc-mediated sliding contributes to MT bundle regulation, we cultured *Khc*<sup>mutA</sup>  
157 mutant neurons in different ways: embryo-derived neurons were cultured for 1DIV after 5d pre-  
158 culture or for 5DIV without pre-culture, and neurons from larval brains were cultured for 2DIV. In all  
159 cases, these neurons failed to show enhanced MT curling (Fig.3Ci), suggesting that the *Khc* mutant  
160 phenotype is not caused by the loss of its MT sliding function.

161

162 Loss of Milton and Miro causes MT curling phenotypes

163 Next, we focussed on the transport functions of Khc. For example, Pat1 (Protein interacting with APP  
164 tail-1) had been shown to link Khc to non-vesicular transport in *Drosophila* oocytes (Fig.3Bii; Loiseau  
165 et al., 2010). It is also strongly expressed in the *Drosophila* nervous system (flybase.org:  
166 [FBgn0029878](#)) but potential neuronal cargoes are unknown. Pat1 function can be eliminated by the  
167 gene-specific small deficiencies *Pat1*<sup>robin</sup> and *Pat1*<sup>grive</sup> which represent viable null alleles (Loiseau et  
168 al., 2010). When analysing cultures of larval neurons homozygous for either allele, we did not find  
169 any obvious enhancement of MT curling (Fig.3Cii).

170 The linker protein Milton and its binding partner Miro (a small GTPase) link the C-terminus of Khc to  
171 organelles including mitochondria (Fig.3A,Biii; Harbauer, 2017; Misgeld and Schwarz, 2017; Sheng,

172 2017; Smith and Gallo, 2018) and potentially peroxisomes (Castro et al., 2018; Covill-Cooke et al.,  
173 2017; Okumoto et al., 2018; Tang, 2018).

174 Using the loss-of-function mutant alleles *milt*<sup>92</sup>, *Miro*<sup>Sd32</sup> or *Miro*<sup>B682</sup>, we first confirmed the functional  
175 contributions of Milt and Miro in primary fly neurons. For this, we stained homozygous mutant  
176 neurons with mitoTracker and anti-Syt. We found the axonal localisation of Syt to be unaffected  
177 whereas mitochondria were strongly reduced in number, thus confirming the expected cargo  
178 specificity (Fig.2E-G,L-P). The mitochondrial phenotype was milder for loss of Miro than Milt, as is  
179 consistent also with previous findings in fly neurons *in vivo* as well as mouse neurons (Glater et al.,  
180 2006; Guo et al., 2005; López-Doménech et al., 2018; Russo et al., 2009; Vagnoni et al., 2016). In  
181 *milt*<sup>92</sup> mutant neurons, mitochondria were virtually absent from axons, restricted mostly to cell bodies  
182 and proximal axon segments (Fig.2L). This absence of mitochondria as the major ATP source does  
183 not affect synaptic transport because it is self-sufficient through local glycolysis on transported  
184 vesicles (Fig.S3A,B; Hinckelmann et al., 2016; Zala et al., 2013).

185 Having confirmed that the *milt*<sup>92</sup>, *Miro*<sup>Sd32</sup> or *Miro*<sup>B682</sup> alleles selectively inhibit mitochondrial transport,  
186 we then assessed potential impacts on MT organisation. We found that all three mutant alleles  
187 caused significant increases in MT curling (Fig.3Ciii). This finding suggests that loss of Khc-mediated  
188 organelle transport triggers MT curling (Fig.3Biii).

189

#### 190 Excessive ROS triggers MT disorganisation

191 Reduced numbers of axonal mitochondria can be expected to impair local homeostasis of calcium,  
192 ATP, reactive oxygen species (ROS), and AAA+ protease-mediated protein quality control systems  
193 (Glynn, 2017; Misgeld and Schwarz, 2017; Paupe and Prudent, 2018). We started by manipulating  
194 the ROS homeostasis through the application of DEM (diethyl maleate). DEM is an effective inhibitor  
195 of the anti-oxidant compound glutathione, hence causing the elevation of ROS levels (Fig.4A; Albano  
196 et al., 2015; Dasgupta et al., 2012; Pompella et al., 2003). We found that 12 hr-long application of  
197 100 µM DEM before fixation (from 4.5 to 5DIV) induced robust MT curling (Fig.4F). To validate this  
198 finding, we then used genetic tools to generate loss- or gain-of-function conditions for ROS-  
199 regulating enzymes (yellow highlighted in Fig.4A):

200 Firstly, we used a null allele of Catalase (*Cat*<sup>1</sup>), an enzyme removing hydrogen-peroxide (H<sub>2</sub>O<sub>2</sub>;  
201 Walker et al., 2018), and null alleles of two members of the Superoxide dismutase family, Sod1  
202 (*Sod1*<sup>n1</sup> or *Sod1*<sup>n64</sup>) and Sod2 (*Sod1*<sup>n283</sup>), which convert superoxide anions (O<sub>2</sub><sup>-</sup>) into H<sub>2</sub>O<sub>2</sub> (Palma et  
203 al., 2020). Of these, Catalase is enriched in peroxisomes, copper-zink-dependent Sod1 (linked to  
204 amyotrophic lateral sclerosis/ALS1; #105400; Saccon et al., 2013) is primarily cytoplasmic, and the  
205 manganese-dependent Sod2 enzyme is predominantly mitochondrial (Fig.4A). When assessed in  
206 primary *Drosophila* neurons, the functional deficiencies of either Sod1, Sod2 or Cat caused MT  
207 curling (Fig.4F).

208 Secondly, we used targeted expression (a) of *Nox* (NADPH oxidase) to enhance O<sub>2</sub><sup>-</sup> levels, (b) of  
209 *Sod1* to reduce O<sub>2</sub><sup>-</sup> levels and enhance H<sub>2</sub>O<sub>2</sub>, and (c) of *Duox* (Dual oxidase) to increase H<sub>2</sub>O<sub>2</sub> levels  
210 (Anh et al., 2011; Bedard and Krause, 2007; Zelko et al., 2002). All these manipulations caused  
211 increased MT curling (Fig.4F).

212 Taken together these results clearly indicate that insults to ROS homeostasis have a strong tendency  
213 to trigger MT curling. Upregulation of either O<sub>2</sub><sup>-</sup> or H<sub>2</sub>O<sub>2</sub> seems to cause this effect, although O<sub>2</sub><sup>-</sup> may  
214 elicit its effects through conversion into H<sub>2</sub>O<sub>2</sub> (Bedard and Krause, 2007).

215

216 Harmful ROS appears to relate to mitochondria and links loss of Khc or Milt to MT curling

217 To assess whether harmful ROS might be responsible for linking loss of Khc/Milton/Miro to MT  
218 curling, we treated mutant neurons with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic  
219 acid), an  $\alpha$ -tocopherol/vitamin E analogue that displays beneficial antioxidant effects in many cell  
220 systems by inhibiting fatty acid peroxidation and quenching singlet oxygen and superoxide (Giordano  
221 et al., 2020). Neurons mutant for *Khc*<sup>8</sup> or *milt*<sup>92</sup> were either pre-cultured for a day in the presence of  
222 100 $\mu$ M Trolox, or they were cultured directly for 5 days with Trolox. Under both conditions,  
223 application of Trolox strongly suppressed or even abolished the MT curling phenotype of *Khc*<sup>8</sup> and  
224 *milt*<sup>92</sup> mutant neurons (Fig.5); this indicated harmful ROS to be the main reason for MT bundle  
225 disintegration. It might explain why rat neurons depleted of the Khc homologue KIF5C were reported  
226 to be more vulnerable to H<sub>2</sub>O<sub>2</sub> application (Iworima et al., 2016).

227 To understand how loss of Khc and Milt might trigger harmful levels of ROS, we first set out to identify  
228 the potential source. For example, loss of Catalase causes MT curling (Fig.4A,F), potentially  
229 suggesting that peroxisomes are required to keep H<sub>2</sub>O<sub>2</sub> levels down. To test this possibility, we  
230 blocked peroxisome biogenesis using the *Pex3*<sup>2</sup> mutation (Faust et al., 2014). Abolishing  
231 peroxisomes in this way did not induce any obvious MT curling phenotypes, but the *Pex3*<sup>2</sup> mutant  
232 neurons had shorter axons (Fig.S4) potentially due to lack of peroxisomal lipidogenesis (Wanders et  
233 al., 2020). The absence of obvious MT phenotypes seems to contradict MT curling observed upon  
234 Catalase deficiency (Fig.4F), but it might be explained by observations that Catalase can localise  
235 outside peroxisomes in the cytoplasm (Zhou and Kang, 2000).

236 We concluded that MT curling observed upon loss of Khc, Milt or Miro is more likely to link to their  
237 roles in axonal transport of mitochondria; disturbing mitochondrial dynamics could either generate  
238 harmful ROS (via the ETC; Fig.4A) or affect their ability to quench local ROS (via Sod2; Fig.4A).

239

240 ROS-absorbing properties rather than disrupted fission/fusion of mitochondria might provide links to  
241 MT curling

242 Loss of Khc/Milt/Miro might cause harmful ROS by affecting fission/fusion processes required to  
243 maintain a healthy mitochondrial population (Cagalinec et al., 2013; Liu et al., 2009; Wang et al.,  
244 2015). In support of this notion fission/fusion factors are linked to axonopathies; this is the case for  
245 the fission factor DNM1L/DYNAMIN-LIKE PROTEIN 1 (Optic atrophy 5; OMIM® #610708), as well  
246 as the fusion factors OPA1/OPA1 MITOCHONDRIAL DYNAMIN-LIKE GTPase (Optic atrophy 1;  
247 #165500) and MFN/MITOFUSIN (CMT2A2A, CMT2A2B, HMSN6A; #609260, 617087, 601152).

248 To test whether loss of fission/fusion is a condition that affects MT bundling, we used mutant alleles  
249 abolishing the functions of the fly homologues of mammalian DNM1L (*Drp1*<sup>T26</sup>; Dynamin related  
250 protein 1), of mammalian OPA1 (*Opa1*<sup>s3475</sup>; Optic atrophy 1) and of mammalian MFN (*Marf*<sup>B</sup>;  
251 Mitochondrial assembly regulatory factor). In axons of wild-type neurons, mitochondria mostly  
252 displayed dash-like shapes (Fig.S5A) and occasionally appeared dot-like or formed longer lines (not  
253 shown). In contrast, within axons of neurons with impaired fusion (*Opa1*<sup>s3475</sup> or *Marf*<sup>B</sup>) mitochondrial  
254 shapes were primarily short and dot-like (Fig.S5C,D), whereas loss of fission (*Drp1*<sup>T26</sup>) caused string-  
255 of-pearl arrangements where a continuous thread of mitochondria ran all along the main axon but  
256 was excluded from side branches (Fig.S5B). These findings are consistent with reports for  
257 mammalian neurons (Smirnova et al., 2001; Uo et al., 2009; Yu et al., 2011).

258 When analysed for MT organisation, none of the three fission/fusion-deficient conditions caused  
259 curling in axons, neither at 5 DIV nor upon pre-culture (Fig.S5E,F). This might suggest that MT  
260 curling upon loss of Khc/Milt/Miro is unlikely to be caused by mitochondrial fission/fusion defects; the

261 absence of fission/fusion events seems not to affect mitochondria in ways that cause harmful ROS  
262 leakage, as is also consistent with views of other authors (Misgeld and Schwarz, 2017). Accordingly,  
263 also MFN2-deficient mouse neurons seem not to experience oxidative stress (Baloh et al., 2007). A  
264 further argument against the involvement of fission/fusion is based on the observation that  
265 mitochondria in Milt-deficient neurons tend to stay in the cell body: it is unlikely that harmful ROS  
266 generated in the soma were to reach the distal axon via long-range diffusion, especially when  
267 considering the abundance of ROS-buffering systems (Fundu et al., 2019; Kükürt et al., 2021;  
268 Oswald et al., 2018).

269 We prefer therefore the explanation that MT curling in Khc/Milt/Miro-deficient neurons (Fig.2P) might  
270 be caused by the absence of mitochondria from critical positions in the axon, thus depleting these  
271 areas from Sod2 activity (for details see Discussion).

272

### 273 Klc also causes MT curling but through a mechanism distinct from Milt

274 Given the comparable strength of MT phenotypes upon loss of Khc, Milt or Miro (Fig.3Ciii) and their  
275 shared links to harmful ROS production (Fig.5), mitochondrial transport defects seemed to offer the  
276 perfect explanation for why loss of Khc induces MT curling. We expected therefore that loss of the  
277 vesicular transport linker Klc would not cause these MT phenotypes.

278 Surprisingly, we found that also loss of Klc (*Klc*<sup>8ex94</sup> or *Klc*<sup>8ex94/Df</sup> mutant neurons at 5 DIV) caused  
279 MT curling, and that this phenotype was at least as strong as observed with Khc or Milt deficiency  
280 (Fig.3Ciii,iv). We obtained the same results when performing 5d pre-cultures with *Khc*<sup>8</sup>, *Klc*<sup>8ex94</sup> and  
281 *milt*<sup>92</sup> mutant neurons (to deplete their maternal products; Figs.6A-E), confirming that the three  
282 factors generate comparably strong MT curling phenotypes.

283 To establish whether Klc might work synergistically with Khc and Milt, we applied Trolox to *Klc*<sup>8ex94</sup>  
284 mutant neurons. However, in contrast to *Khc*<sup>8</sup> and *milt*<sup>92</sup> mutant neurons, the MT curling in Klc-  
285 deficient neurons was not suppressed by Trolox, neither in pre-cultured neurons nor in 5DIV cultures  
286 (Fig.5). This clearly demonstrated that Klc works through an independent mechanism.

287

### 288 Klc's links to MT curling do not depend on vesicular cargo transport

289 We first tested whether Klc's impact on MT regulation might link to vesicular cargo transport,  
290 capitalising on reports that Khc-mediated vesicular cargo transport requires a protein complex of a  
291 number of factors including Klc and Sunday driver (Syd, the JIP3 homologue; Fig.3Biv; Gindhart et  
292 al., 2003; Horiuchi et al., 2005; Koushika, 2008). Accordingly, functional loss of either Klc or Syd was  
293 shown to abolish Khc-mediated vesicular transport, with motoraxons in peripheral larval nerves  
294 displaying synaptic protein accumulation that were similarly strong upon Klc or Syd deficiency as  
295 observed upon loss of Khc (Bowman et al., 2000; Föger et al., 2012; Gauger and Goldstein, 1993;  
296 Gindhart et al., 1998; Hurd and Saxton, 1996; Pilling et al., 2006). Equally in primary culture, we  
297 found that the number of Synaptotagmin-positive dots in axons was reduced in *Klc*<sup>8ex94</sup> and *syd*<sup>z4</sup> null  
298 mutant neurons, and the phenotypes were similarly strong as observed in Khc-deficient neurons  
299 (Fig.2B,G,O). These effects seem specific to vesicular cargo transport since mitochondrial numbers  
300 in axons of *Klc*<sup>8ex94</sup> mutant neurons appeared normal (Fig.2N,P).

301 Our data confirm therefore that Klc, Syd and Khc closely co-operate during vesicular cargo transport  
302 in primary *Drosophila* neurons. However, in contrast to severe MT curling in Klc- and Khc-deficient  
303 neurons, *syd*<sup>z4</sup> or *syd*<sup>z4/Df</sup> mutant neurons at 5DIV failed to show similar phenotypes (Fig.3Civ). This

304 observation was further supported by experiments where we knocked down GAPDH  
305 (glyceraldehyde-3-phosphate dehydrogenase), an enzyme required for glycolysis that is known to  
306 fuel the axonal transport of vesicles but not of mitochondria (details in Fig.S3A,B; Zala et al., 2013).  
307 When GAPDH was knocked down in primary *Drosophila* neurons, axons displayed a reduction in  
308 synaptic dots but not in mitochondrial numbers (Fig.S3C), as is consistent with analyses in larval  
309 nerves (Zala et al., 2013). However, GAPDH knock-down did not cause obvious MT curling  
310 phenotypes (Fig.S3C), thus mirroring results obtained with Syd deficiency.

311 We concluded that blocking vesicular axonal transport appears not to be a cause for MT curling, and  
312 that loss of Klc is likely to trigger its MT phenotypes through a different mechanism. This view was  
313 also supported by genetic interaction studies using trans-heterozygous pairings of *Khc*<sup>8</sup>, *milt*<sup>92</sup> and  
314 *Klc*<sup>8ex94</sup> (i.e. combining heterozygosity for two genes at a time in the same neurons). Of the three  
315 constellations, only *Khc*<sup>8/+</sup> *milt*<sup>92/+</sup> trans-heterozygote mutant neurons generated a MT curling  
316 phenotype that was significantly enhanced over single heterozygous conditions (Fig.6G), supporting  
317 functional links between Khc and Milt but not with Klc.

318 Taken together, MT curling upon loss of Khc appears to relate to Milt/Miro-mediated mitochondrial  
319 transport as explained before, but not to Klc-mediated vesicular transport. Milt, Miro and Khc seem  
320 to have comparably strong mutant phenotypes because their loss leads to the same transport defect,  
321 whereas phenotypes observed upon loss of Klc (which has binding sites on Khc that overlap with  
322 those of Milt; details in Fig.3A) seem not to relate to its function as a transport linker but work through  
323 an entirely different mechanism.

324

#### 325 Excessive pools of active Khc might explain the Klc-deficient MT phenotype

326 We hypothesised that MT curling upon loss of Klc may relate to its roles in regulating the activation  
327 state of Khc. Thus, Khc pools that are not linked to cargo tend to be auto-inhibited and detached  
328 from MTs. This inactivation requires intramolecular loop formation via binding of the N- to the C-  
329 terminus, and this also involves the association with Klc (co-regulated through its own auto-  
330 inhibition/activation mechanism; Figs.3A,Biv; Bowman et al., 2000; Koushika, 2008; Verhey and  
331 Hammond, 2009; Verhey et al., 1998; Wong and Rice, 2010; Yip et al., 2016). In non-neuronal cells,  
332 overriding auto-inhibition of the Khc-Klc complex causes MT curling (Paul et al., 2020; Randall et al.,  
333 2017).

334 To test whether Klc-deficient MT curling in neurons might involve excessive pools of active Khc, we  
335 first targeted the expression of GFP-tagged constructs of Khc to neurons. We found that full-length  
336 Khc::GFP was homogeneously distributed along axons and failed to increase MT curling when  
337 analysed at 5DIV (Fig.7), consistent with the idea that extra pools of Khc tend to be inactive and  
338 detached from MTs.

339 We then expressed two non-inactivating Khc derivatives (*Khc*<sup>1-811</sup>::GFP and *Khc*<sup>82-711</sup>::GFP; top of  
340 Fig.3A) which both lack the C-terminal domain needed for auto-inhibition but also for their roles in  
341 cargo transport and MT sliding (dark grey and green in Fig.3A). When analysed in neurons at 5DIV,  
342 both constructs accumulated at axon tips, as is typical of non-inactivating kinesins (Niwa et al., 2013).  
343 Of these, *Khc*<sup>1-850</sup>::GFP caused a very mild MT phenotype, suggesting that extra pools of free-  
344 running Khc *per se* cause little harm (Fig.7B,D). In contrast, *Khc*<sup>82-711</sup>::GFP caused severe MT curling  
345 (Fig.7C,D), potentially because this truncated form also has a small N-terminal deletion – and short  
346 deletions of the N-terminus have been shown to display damaging effects on MTs (Budaitis et al.,  
347 2021; details in Fig.3A). In principle, our findings with this dys-regulated construct supported our



348 previously published hypothesis that the activity of kinesins is harmful to axonal MT bundles and can  
349 explain MT curling (Hahn et al., 2019; Prokop, 2021).

350 However, since all essential C-terminal binding sites were removed in  $Khc^{1-850}::GFP$  and  $Khc^{82-711}::GFP$  (Fig.3A), our experiments so far only assessed free-running Khc that could not engage in  
351 movement of any cargo (Fig.3A,B). We hypothesised that active transport would be expected to  
352 generate higher forces than free-running Khc and, hence, be more challenging to MT bundles. We  
353 therefore tested the genomically engineered  $Khc^{E177K}$  and  $Khc^{E177R, R947E}$  mutant alleles (Fig.3A),  
354 which have point mutations in the E177 and R947 residues that are known to form a required salt  
355 bridge with each other during auto-inactivation (Kaan et al., 2011; Kelliher et al., 2018); these mutant  
356 alleles cause lethality and distal accumulations of Khc (Brendza et al., 1999; Kelliher et al., 2018).  
357 When analysing  $Khc^{E177K}$  and  $Khc^{E177R, R947E}$  homozygous mutant neurons at 3DIV, we found that  
358 they display robust MT curling (Fig.7D). This clearly indicated that extra pools of actively engaging  
359 Khc harm MT bundles, which might therefore explain the *Klc* mutant phenotype (see Discussion).  
360

361

## 362 Discussion

363

### 364 Using MT bundles of *Drosophila* neurons as a powerful approach to dissect motor-related patho- 365 mechanisms

366 Motor proteins involved in axonal transport clearly are key drivers of neuronal survival, yet their links  
367 to axonopathies remain poorly understood and speculative (Coleman, 2005; Guo et al., 2020;  
368 Kawaguchi, 2013; Sleight et al., 2019). Here, we aimed to unravel concrete mechanisms through  
369 which motor protein loss can affect axons.

370 Our approach was unprecedented in that we performed a systematic genetic study in one  
371 standardised neuron system and used MT bundles as key readout. We studied the organisation of  
372 axonal MT bundles because they are good indicators of axon integrity (Prokop, 2020) that are easy  
373 to quantify and have an intricate interdependent relationship with motor proteins (Prokop et al.,  
374 2013). The key phenotype we observed upon motor manipulation is MT curling which appears  
375 conserved across species, since depleting Dynein or the kinesin-1 linker JIP3 causes the same kind  
376 of curling in axons of mammalian neurons (Ahmad et al., 2006; Rafiq et al., 2020).

377 The easily accessible and quantifiable MT curling readout allowed us to determine roles of motor  
378 proteins and their interactors, or of proteins regulating potential downstream processes - and many  
379 of these factors have known links to neurodegeneration or axonopathies. Our approach was  
380 facilitated by using a standardised neuronal culture system in which findings could be integrated and  
381 were highly accessible to powerful *Drosophila* genetics. The additional advantage of this system is  
382 low genetic redundancy of factors involved in axonal transport, with one *Drosophila* gene having on  
383 average almost 3 mammalian orthologues (*Khc/Kif5*: 1 paralogue in fly vs. 3 paralogues in mammals;  
384 *Miro/RHOT*: 1 vs. 2; *Milt/TRAK*: 1 vs. 2; *Klc*: 1 vs. 4; *Marf/MFN*: 1 vs. 2; *Unc-104/Kif1*: 1 vs. 3). This  
385 low redundancy in fly enormously facilitates loss-of-function analyses and combinatorial genetics.

386 Capitalising on these advantages, our unprecedented strategy enabled us to generate new  
387 understanding and conceptual explanations. So far, we found that deficiencies of three motor  
388 proteins (*Khc*, *Unc-104*, *Dhc*) cause MT curling. Notably, the homologues of all three factors have  
389 OMIM®-listed links to human axonopathies (see Introduction) potentially reflecting evolutionarily  
390 conserved mechanisms of axon pathology that might even be shared between these motor protein  
391 classes.

392 For example, we found that phenotypes upon loss of Khc and Unc-104 are very similar with respect  
393 to enhanced MT curling and the reduction in axonal numbers of mitochondria and synaptic dots  
394 (Figs.1, 2), and both seem involved in mRNA transport (L.M.P.C., unpublished results; Lyons et al.,  
395 2009). This functional overlap is in agreement with reports that kinesin-1 and -3 collaborate during  
396 transport (Arpağ et al., 2019; Zahavi et al., 2021). Consequently, these two motors might therefore  
397 link to axonopathy through comparable mechanisms.

398

### 399 An intricate relationship: kinesins simultaneously harm and care for MT bundles

400 The challenges of studying axonal transport are ample due to (1) the parallel involvement of different  
401 motor protein classes (which might act redundantly; Hirokawa et al., 2010; see previous section), (2)  
402 the enormous wealth of their cargoes, (3) the involvement of many different linkers (that might  
403 interact promiscuously with different motors; Brady and Morfini, 2017; Drerup et al., 2016; Gindhart,  
404 2006; Hirokawa et al., 2010; Maday et al., 2014), (4) additional roles in slow transport (transient  
405 'hitchhiking' of proteins on transported vesicles; Roy, 2020; Tang et al., 2013), and (5) complications  
406 caused by the interdependence of kinesins and dynein/Dynactin (Hancock, 2014; Moughamian et  
407 al., 2013; Twelvetrees et al., 2016; potentially explaining the rather counter-intuitive observation that  
408 loss of anterograde Khc transport causes distal ER accumulations; details in legend of Fig.S1).

409 For kinesin-1 alone (Fig.3B), we tested roles of Khc in MT sliding (*Khc<sup>mutA</sup>*), roles of Khc/Milt/Miro in  
410 mitochondrial/peroxisomal transport, of Khc/Klc/Syd/GAPDH in vesicular transport, of Khc/Pat1 in  
411 potential non-vesicular transport, and potential roles of Klc and certain Khc domains/residues in Khc  
412 auto-inhibition. These extensive studies still left out further known linkers, such as SKIP/SNW1/SKIIP  
413 and Arl8 (lysosome transport; Keren-Kaplan and Bonifacino, 2021; Rosa-Ferreira and Munro, 2011;  
414 Rosa-Ferreira et al., 2018) or Tropomyosin (mRNA transport; Fig.3A; Dimitrova-Paternoga et al.,  
415 2021; Veeranan-Karmegam et al., 2016). Nevertheless, the analyses we performed suggested two  
416 distinct mechanisms:

417 Firstly, Khc/Milt/Miro-mediated transport is required to uphold ROS homeostasis, with harmful ROS  
418 being a strong inducer of MT curling (demonstrated by our studies with DEM, Trolox and ROS-  
419 regulating enzymes; Figs.4, 5). Secondly, we found that the movement and active transport of Khc  
420 along MTs damages axonal bundles, as demonstrated by the expression of Khc deletion constructs  
421 and analyses of non-inactivating *Khc* mutant alleles (Fig.7). These latter findings align with published  
422 *in vitro* experiments demonstrating kinesin-1-induced MT damage (Andreu-Carbó et al., 2021;  
423 Budaitis et al., 2021; Dumont et al., 2015; Triclin et al., 2021; VanDelinder et al., 2016), MT curling  
424 observed in kinesin-1-based gliding assays *in vitro* (Hahn et al., 2019; Lam et al., 2016), and the  
425 curling observed upon kinesin-1 activation in non-neuronal cells (Paul et al., 2020; Randall et al.,  
426 2017). Notably, MT curling is not specifically linked to motor proteins, but is similarly observed upon  
427 loss of various MT-binding and -regulating proteins (Hahn et al., 2019) and in a model of  
428 chemotherapy-induced peripheral neuropathy (Rozario et al., 2021).

429 All these causes of MT curling, including the two mechanisms described in this work, can be  
430 explained with the previously proposed "local axon homeostasis" model (Hahn et al., 2019) and the  
431 subsequently derived "dependency cycle of axon homeostasis" (Prokop, 2021; details in Fig.8).  
432 These models propose that kinesins that trail along MT bundles during axonal transport ('2' in Fig.8)  
433 pose a mechanical challenge that leads to MT curling ('3'); active machinery of MT-regulating  
434 proteins and support through the cortical actin-spectrin sleeve is therefore required to prevent  
435 disintegration and maintain these bundles long-term ('4'). However, the machinery that maintains MT  
436 bundles is itself dependent on materials and physiology provided by axonal transport ('5'), thus

437 establishing a cycle of mutual dependency where interruption at any point will have a knock-on effect  
438 on all other aspects of axon physiology and function (Prokop, 2021). The mechanisms we described  
439 here act in either direction of this cycle (thick read arrows in Fig.8).

440

#### 441 Mitochondria regulate ROS homeostasis required for MT bundle maintenance

442 Our finding that harmful ROS is a key trigger of MT curling aligns with reports that actin as well as  
443 MTs are modified or even damaged by ROS (Goldblum et al., 2021; Wilson et al., 2016; Wioland et  
444 al., 2021) and that oxidative stress induces axon swellings in models of Parkinson's disease, multiple  
445 sclerosis or ALS (Czaniecki et al., 2019; Nikić et al., 2011; Song et al., 2013). Unfortunately, a more  
446 generalised statement cannot be made because axonal MTs have rarely been analysed in oxidative  
447 stress experiments (De Vos et al., 2007; Debattisti et al., 2017; Fischer et al., 2012; Saccon et al.,  
448 2013; Song et al., 2013).

449 Pinpointing the precise source of harmful ROS upon Khc/Milt/Miro loss is a tedious task when  
450 considering (1) the intricate network of ROS regulation (Fig.4A) where manipulations of very different  
451 regulators caused comparable phenotypes (Fig.4F), and (2) the spectrum of organelles involved in  
452 ROS homeostasis regulation: these involve the finely tuned mitochondria-peroxisome system  
453 (Fransen et al., 2017; Pascual-Ahuir et al., 2017), but also the ER which contains oxidases required  
454 for protein folding (Hudson et al., 2015). Unfortunately, removing or affecting the ER to assess its  
455 involvement is not trivial (O'Sullivan et al., 2012; Yalcin et al., 2017); but our studies of *Pex3* mutant  
456 conditions suggested that peroxisomes are unlikely to link to MT curling (Fig.S4). This said,  
457 peroxisomes certainly play important further roles in maintaining healthy axons (Wali et al., 2016).

458 In our view, the most likely organelles involved in MT curling are the mitochondria. We were surprised  
459 to find that not the presence of damaged mitochondria leaking harmful ROS seems to trigger MT  
460 curling (Fig.S5), but rather the absence of mitochondria. This is best illustrated by *milt* mutant  
461 neurons where mitochondria are mostly restricted to cell bodies (Fig.2L,P), yet strong ROS-induced  
462 MT curling occurs in axons (Figs.3Ciii, 5, 6E).

463 As already mentioned in the Results part, we believe that the best model combining all observations  
464 is the absence of mitochondria and Sod2 as their ROS scavenger from critical locations in axons.  
465 For example, we know from live imaging experiments that MT curling starts at growth cones or  
466 branch points (A.V., unpublished data), and both are typical sites where mitochondria localise  
467 (Bunge, 1973; Mandal and Drerup, 2019). Failure to quench harmful ROS in these critical locations  
468 could therefore promote the initiation of MT curling; this would also explain why drastic mitochondrial  
469 depletion upon Milt deficiency triggers similarly strong MT curling as moderate depletion upon loss  
470 of Khc or Miro (Figs.2P, 3C): not the number of mitochondria is essential but their adequate  
471 localisation, and this aspect is regulated through a Khc/Milt/Miro-dependent mechanism (Misgeld  
472 and Schwarz, 2017).

473 The drastic depletion of axonal mitochondria upon Milt deficiency as compared to the moderate  
474 number reductions upon loss of Khc, Unc-104 or Dhc (Fig.2P) might suggest Milt as a 'master linker'  
475 for mitochondrial transport in fly neurons. Indeed, Milt is known to link to Khc and Dynein in both flies  
476 and mammals (Russo et al., 2009; van Spronsen et al., 2013), whereas there are currently no such  
477 reports for Unc-104; its mammalian homologue Kif1 was reported so far to perform mitochondrial  
478 transport through KBP (Kif1 binding protein; Campbell et al., 2014; Nangaku et al., 1994; Tanaka et  
479 al., 2011; Wozniak et al., 2005).

480

481 Khc activation as a further factor leading to MT curling

482 As discussed above, our data with non-inactivating constructs and mutant alleles of Khc strongly  
483 suggested that excess engagement of this motor can trigger MT curling (Fig.7). Since Klc is involved  
484 in Khc auto-inhibition, the MT curling phenotype we observe in axons of Klc-deficient neurons might  
485 therefore link to this mechanism as a potential cause for axonopathy.

486 Also Kif1A/Unc-104 undergoes inactivation involving intramolecular loop formation and the KBP  
487 linker (Cong et al., 2021; Kevenaar et al., 2016). KBP mutations impair axon growth, are disruptive  
488 to axonal MT bundles (Lyons et al., 2008) and cause the devastating neurological disorder Goldberg-  
489 Shprintzen syndrome in humans (Chang et al., 2019; Hirst et al., 2017). Similarly, non-inactivating  
490 mutations of Kif1A cause spastic paraplegia (Chiba et al., 2019; Gabrych et al., 2019). Also KLC2  
491 has been linked to neuropathy (SPOAN; #609541) and KLC4 mutations have recently been reported  
492 to cause excessive axon branching (Haynes et al., 2021). Axonopathy-linked human mutations of  
493 Kif5A were mapped exclusively to the motor domain or the very C-terminal end so far, but none were  
494 reported in the auto-inactivation domains or the KLC binding site (Nicolas et al., 2018). However,  
495 this does not mean that such mutations are not detrimental: mutations affecting auto-inhibition might  
496 rather confer lethality (as is the case in *Drosophila*; Brendza et al., 1999; Kelliher et al., 2018) and  
497 therefore escape the spectrum of diagnosed diseases.

498 The modest curling observed upon overexpression of Khc<sup>1-850::GFP</sup>, versus the strong phenotype  
499 with genomically engineered *Khc*<sup>E177K</sup> and *Khc*<sup>E177R, R947E</sup> mutant alleles (Fig.3A), suggests that free-  
500 running Khc is insufficient to cause a strong phenotype; instead it seems that the C-terminus of Khc  
501 has to interact with cargo to generate forces strong enough to affect MT bundles. But what cargoes  
502 might be involved?

503 Some insights might come from *milt*<sup>92/92</sup> *Klc*<sup>8ex94/8ex94</sup> double-mutant neurons which show an intriguing  
504 pattern: loss of Klc and Milt trigger MT curling through completely different mechanisms (Fig.8), and  
505 their phenotypes should therefore be additive. However, double-mutant neurons show the same  
506 amount of curling as the single mutants (Figs.5B, 6F). Even more, MT curling in double-mutant  
507 neurons is partly cured by Trolox, although Klc-deficiency alone does not respond to Trolox (Fig.5B).  
508 The easiest explanation for these findings is that surplus pools of activated Khc triggered by loss of  
509 Klc engage in force-generation that depends on mitochondria-derived ATP (unlike vesicular  
510 transport; Fig.S3A). Since mitochondria and their ATP are absent from axons of *milt* *Klc* double-  
511 mutant neurons, the extra pool of Khc lacks the necessary fuel to contribute to the joint MT curling  
512 phenotype. So far, our attempts to pinpoint such force-generating activities of surplus Khc pools  
513 have not been successful: they seem not to involve microtubule sliding and Pat1-mediated transport  
514 (Fig.3Bi,ii) as suggested by failed suppression of MT curling in *Khc*<sup>mutA/mutA</sup> *Klc*<sup>8ex94/8ex94</sup> or *Pat1*<sup>robin/robin</sup>  
515 *Klc*<sup>8ex94/8ex94</sup> double-mutant neurons (details in Fig.S6).

516

517 Conclusion

518 Using our unconventional strategy (MT curling as key readout for systematic genetic analyses in a  
519 standardised *Drosophila* primary neuron system) we were able to develop new concepts for how  
520 molecular motor mutations might trigger axonopathies. Given the breadth of genetics versus lack of  
521 mechanistic detail of our studies, our results are certainly more suggestive than definite. But they  
522 are astonishingly consistent with many reports in the field (as mentioned throughout this work) and  
523 align well with the 'dependency cycle of local axon homeostasis' as a model describing the  
524 fundamental principle of axon maintenance and pathology (Prokop, 2021). We hope therefore that  
525 our findings and ideas will stimulate further studies in which the model and proposed mechanisms

526 are put to the test, incorporating also other aspects of axon physiology, such as ATP and calcium  
527 regulation. Whatever the outcome, such studies will be highly informative and contribute to the battle  
528 against a class of diseases that are of enormous socioeconomic burden and personal hardship.

529

### 530 **Conflict of Interest**

531 None of the authors has a conflict of interests

532

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540

### 541 **Methods**

#### 542 Fly stocks

543 All human homology statements are based on information listed on flybase.org (Marygold et al.,  
544 2016; Millburn et al., 2016), all statements about genetic links to human diseases on information  
545 provided by [www.omim.org](http://www.omim.org) (Online Mendelian Inheritance in Man®; Amberger et al., 2015). The  
546 following stocks were used in this study (reference and source provided in brackets; BL indicates  
547 Bloomington *Drosophila* Stock Collection): null mutant alleles (unless indicated differently) we used  
548 were

- 549 • *unc-104*<sup>170</sup> (Pack-Chung et al., 2007; Tom Schwarz)
- 550 • *Klp64D*<sup>K1</sup> (Ray et al., 1999; hypomorphic allele; BL #5578)
- 551 • *Klp64D*<sup>n123</sup> (Perez and Steller, 1996; BL #5674)
- 552 • *Klp98A*<sup>A47</sup> (Derivery et al., 2015; Marcos Gonzalez-Gaitan)
- 553 • *Dhc64C*<sup>4-19</sup> (Gepner et al., 1996; BL #5274)
- 554 • *Khc*<sup>8</sup> (Saxton et al., 1991; BL #1607)
- 555 • *Khc*<sup>27</sup> (Saxton et al., 1991; Isabel Palacios)
- 556 • *Klc*<sup>1ts</sup> (Saxton et al., 1991; BL #31994; a temperature-sensitive allele which is homozygous  
557 viable at 18°C but usually kept over balancer)
- 558 • *Khc*<sup>mutA</sup> (Winding et al., 2016; Vladimir Gelfand; confirmed by lethality of hetero-allelic  
559 *Khc*<sup>mutA/B</sup> animals)
- 560 • *Khc*<sup>E177K</sup> and *Khc*<sup>E177K,R947E</sup> (Kelliher et al., 2018; Jill Wildonger)
- 561 • *Df(Khc)* (*Df(2R)BSC309*; Cook et al., 2012; BL #23692)
- 562 • *milt*<sup>92</sup> (Cox and Spradling, 2006; Stowers et al., 2002; Tom Schwarz)
- 563 • *Df(milt)* (*Df(2L)ED440, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3}ED440*; Ryder et al., 2004;  
564 Kyoto #150498)
- 565 • *Miro*<sup>Sd32</sup> (Guo et al., 2005)
- 566 • *Miro*<sup>B682</sup> (Guo et al., 2005; BL #52003)
- 567 • *Df(Miro)* (*Df(3R)Exel6197*; Parks et al., 2004; BL #7676)
- 568 • *Klc*<sup>8ex94</sup> (Gindhart et al., 1998; BL #31997)

- 569 • *syd<sup>4</sup>* (Bowman et al., 2000; BL #32016)  
570 • *Df(3L)syd<sup>A2</sup>* (Bowman et al., 2000; deleting C-terminus; BL #32017)  
571 • *Pat<sup>grive</sup>* and *Pat<sup>robin</sup>* (Loiseau et al., 2010; Isabel Palacios)  
572 • *Sod1<sup>n1</sup>* (Phillips et al., 1989; BL #24492)  
573 • *Sod1<sup>n64</sup>* (Phillips et al., 1995; BL #7451)  
574 • *Rtnl1-YFP (PBac{681.P.FSVS-1}Rtnl1CPTI001291*; Cahir O’Kane; O’Sullivan et al., 2012)  
575 • *Drp1<sup>T26</sup>* (Verstreken et al., 2005; BL #3662)  
576 • *Mar<sup>B</sup>* (Sandoval et al., 2014; Hugo Bellen)  
577 • *P{lacW}Opa1<sup>s3475</sup>* (Spradling et al., 1999; BL #12188)  
578 • *Cat<sup>n1</sup>* (Mackay and Bewley, 1989; Matthias Landgraf)  
579 • *Sod2<sup>n283</sup>* (Duttaroy et al., 2003; BL#34060)  
580 • *Pex3<sup>2</sup>* (Faust et al., 2014; BL#64251)

581 Gal4 driver lines used were the

- 582 • *elav-Gal4* (Luo et al., 1994)  
583 • *tubP-Gal4* (Lee and Luo, 1999; Liqun Luo)

584 UAS lines

- 585 • *UAS-Khc<sup>FL</sup>::GFP* (3<sup>rd</sup>, unpublished; Isabel Palacios)  
586 • *UAS-Khc<sup>82-711</sup>-GFP* (2<sup>nd</sup>, BL #9648; constitutively active Khc consisting of base pairs 248-  
587 2134 / aa 82-711; fused with EGFP sequence; flybase.org: [FBrf0198610](#))  
588 • *UAS-Khc1-850-GFP* (Loiseau et al., 2010; Isabel Palacios)  
589 • *UAS-Khc-RNAi* (Lu et al., 2013; Vagnoni et al., 2016; BL #35770)  
590 • *UAS-Sod1* (J. Hu and J.P. Phillips, unpublished)  
591 • *UAS-Duox* (Ha et al., 2005; Matthias Landgraf)  
592 • *UAS-Gapdh-IR (Gapdh1<sup>GD7467</sup>*; Vienna *Drosophila* Resource Centre)

#### 593 Cloning of UAS-Nox-YPet

594  
595 *10xUAS-IVS-Nox::YPet* was generated by using the *pJFRC12-10xUAS-IVS-myr-GFP*  
596 vector (Addgene 26222; Pfeiffer et al., 2010) as a backbone which was modified by substituting GFP  
597 with YPet (Nguyen and Daugherty, 2005) plus an N-terminal flexible linker, amplified from  
598 dFlex\_YPet\_phase0 (Gärtig et al., 2019) using primer ML1 and ML2, and inserted by the Klenow  
599 Assembly Method ([tinyurl.com/4r99uv8m](https://www.tinyurl.com/4r99uv8m)) into the XbaI/BamHI sites producing Vector 1: *pJFRC12-*  
600 *10xUAS-IVS-myr-linker-YPet*. Nox cDNA was amplified from a DGRC (*Drosophila* Genomics  
601 Resource Center) cDNA library clone using primers ML5 and ML6, located in a *pOTB7* vector  
602 backbone, and inserted into BamHI/XhoI sites of Vector 1. Constructs were sent to FlyORF  
603 for transgenesis, and targeted via PhiC31-mediated site-specific insertion to the *PBac{y<sup>+</sup>-attP-*  
604 *3B}VK00040* landing site (Bloomington line #9755) on the third chromosome (3R, 87B10).

605

ML1	gacatcatcagaccacgcgatccggctccgcccggctccgcccggctccggcgagttcgtgtcca agggcgag
ML2	gttcctcacaagatcctctagattactgtacagctcgttcattgccc
ML5	ggagccggcgagccggatccgaagcactccttacgaaaggcaaatccgt
ML6	cttcaggcgccgcccggctcgagaatcaaatgaacgcgaccaggagtc

606

607 Drosophila primary cell culture

608 *Drosophila* primary neuron cultures were performed as published previously (Prokop et al., 2012;  
609 Qu et al., 2017). In brief, stage 11 embryos were treated for 1 min with bleach to remove the chorion,  
610 sterilized for ~30 s in 70% ethanol, washed in sterile Schneider's/FCS, and eventually homogenized  
611 with micro-pestles in 1.5 centrifuge tubes containing 21 embryos per 100µl dispersion medium and  
612 left to incubate for 5 min at 37°C. Cells were washed with Schneider's medium (Gibco), spun down  
613 for 4 mins at 650g, supernatant was removed and cells re-suspended in 90µl of Schneider's medium  
614 containing 20% fetal calf serum (Gibco). 30µl drops were placed on cover slips. Cells were allowed  
615 to adhere for ~2hrs either directly on glass or on cover slips coated with a 5 µg/ml solution of  
616 concanavalin A, and then grown as a hanging drop culture for hours or days at 26°C as indicated in  
617 each experiment.

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

622 Cells were treated with 100 µM Trolox (Sigma; stepwise diluted from a 100mM stock solution in  
623 ethanol) or 100 µM DEM prepared in 100% ethanol. For controls (vehicle treatment), equivalent  
624 concentrations of vehicle (sterile H<sub>2</sub>O or 100% ethanol) were diluted in cell culture medium. All  
625 reagents were purchased from Sigma-Aldrich, unless otherwise stated.

626 For visualisation of mitochondria, cell cultures were incubated with 400nM MitoTracker Red CMXRos  
627 (Invitrogen; Klionsky et al., 2012) for 30min at room temperature (RT); stock solutions were prepared  
628 in DMSO and diluted in cell culture medium to the final concentration. Following incubation, cultures  
629 were then fixed and stained following the procedures below.

630

631 Immunohistochemistry

632 Primary fly neurons were fixed in 4% paraformaldehyde (PFA) in 0.05M phosphate buffer (PB; pH  
633 7–7.2) for 30min at room temperature (RT). Antibody staining and washes were performed with PBT.  
634 Staining reagents: anti-tubulin (clone DM1A, mouse, 1:1000, Sigma; alternatively, clone YL1/2, rat,  
635 1:500, Millipore Bioscience Research Reagents); anti-Syt (1:1000; rabbit; Sean Sweeney); anti-GFP  
636 (1:500, rabbit, ab290, Abcam); Cy3-conjugated anti-HRP (goat, 1:100, Jackson ImmunoResearch);  
637 FITC-, Cy3- or Cy5-conjugated secondary antibodies (1:200; donkey, purified, Jackson Immuno  
638 Research); F-actin was stained with Phalloidin conjugated with TRITC/Alexa647, FITC or Atto647N  
639 (1:200; Invitrogen and Sigma). Specimens were embedded in ProLong Gold Antifade mounting  
640 medium.

641

642 Microscopy and data analysis

643 Standard documentation was performed with AxioCam monochrome digital cameras (Carl Zeiss  
644 Ltd.) mounted on BX50WI or BX51 Olympus compound fluorescent microscopes. To determine the  
645 degree of MT disorganisation in axons we used the "MT disorganisation index" (MDI) (Qu et al.,  
646 2017): the area of disorganisation was measured using the freehand selection tool in Fiji/ImageJ;  
647 this value was then divided by axon length (see above) multiplied by 0.5 µm (typical axon diameter,  
648 thus approximating the expected area of the axon if it were not disorganised). To quantify the number  
649 of synaptic densities in mature neurons in culture, we used ImageJ, first thresholding to select  
650 synaptic densities from axons of single isolated cells, followed by particle analysis. For statistical

651 analyses, Kruskal–Wallis one-way ANOVA with *post hoc* Dunn’s test or Mann–Whitney Rank Sum  
652 Tests were used to compare groups. The data used for our analyses will be made available on  
653 request from the authors.

654

#### 655 Ethical statement

656 An ethical statement is not required.

657

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1315 **Figures**

1316

1317 **Fig.1** Deficiencies of three motor proteins cause MT curling. **A-G)** Examples of neurons of different  
1318 genotype (indicated top right) and stained for tubulin at 5DIV; asterisks indicate cell bodies, arrow  
1319 heads axon tips, curved arrows areas of MT curling, white rectangles shown as twofold magnified,  
1320 yellow emboxed insets; scale bar in A represents 20µm in all images. **H)** Quantification of MT curling  
1321 phenotypes measured as MT disorganisation index (MDI) and normalised to wild-type controls (red  
1322 stippled line); mean ± SEM is indicated in blue, numbers of analysed neurons in orange, results of  
1323 Mann Whitney rank sum tests are shown in grey/black.

1324

1325 **Fig.2** Impacts of motor protein and linker mutations on numbers of axonal mitochondria and synaptic  
1326 spots. **A-N)** Examples of neurons of different genotype (indicated top right) and stained at 5DIV for  
1327 tubulin (tub, magenta) and either Synaptotagmin (Syt, green in A-G) or with mitoTracker (green in  
1328 H-N); scale bar in A represents 20µm in all images. **O,P)** Quantification of axonal numbers of Syt-  
1329 positive spots (O) or mitochondria (P), all normalised to wild-type controls (red stippled line); medians  
1330 are indicated in blue, numbers of analysed neurons in orange, results of Mann Whitney rank sum  
1331 tests are shown in grey/black.

1332

1333 **Fig.S1** Endoplasmic reticulum accumulates at axon tips upon loss of Khc. **A,B)** Primary neurons  
1334 at 5 DIV carrying the genomically tagged *Rtn1-YFP* allele labelling endoplasmic reticulum (ER; del  
1335 Castillo et al., 2019; O'Sullivan et al., 2012), either in wild-type (wt; A) or *Khc<sup>8</sup>* mutant background  
1336 (B); inset with blue outline in A displays the green channel of the neuron (reduced to 50% in size) to  
1337 illustrate the continuous nature of *Rtn1::GFP*-labelled ER throughout its neurites; the yellow  
1338 emboxed area in B is shown as twofold increased inset of the green channel to illustrate the netlike  
1339 organisation of ER visible in axonal swellings. Asterisks indicate cell bodies and arrow heads axon  
1340 tips (note that there are two neurons in B), white/orange chevrons point at strong/weak axonal tip  
1341 accumulations of ER. Accumulations might indicate an imbalance of antero- and retrograde  
1342 organelle movement potentially caused by loss of Khc-dependent Dynein transport to axon tips  
1343 (Moughamian et al., 2013; Twelvetrees et al., 2016) expected to reduce the retrograde drift of ER.  
1344 The scale bar in A represents 20 µm in A and B. **C)** Quantification of axonal tip accumulation of ER:  
1345 numbers of neurons analysed are shown in orange, numbers in bars the rounded percentages of  
1346 neurons with no/weak/strong accumulations, the number above bars show the P value of the X<sup>2</sup> test.

1347

1348 **Fig.3** Assessing contributions of Khc subfunctions to MT regulation. **A)** Schematic representation of  
1349 *Drosophila* Khc drawn to scale. Domains are colour-coded and start/end residues are indicated by  
1350 numbers: motor domain (red; according to Sablin et al., 1996), coiled-coil domains required for  
1351 homo- and/or heterodimerisation (green; as predicted by Ncoils in ensembl.org), the C-terminal ATP-  
1352 independent MT-binding motif (blue; according to Winding et al., 2016), and the C-terminal auto-  
1353 inactivation domain (dark grey; according to Kaan et al., 2011); grey lines above the protein scheme  
1354 indicate the three expression constructs used in this study; below the protein scheme further details  
1355 are shown: the sequence of the C-terminal MT-binding domain (*mutA* mutations indicated in orange;  
1356 Winding et al., 2016), the sequence of the auto-inactivation domain (indicating the IAK motif and  
1357 R947E mutation; Kelliher et al., 2018), the binding areas (darker green coiled-coils) of Klc (according  
1358 to Veeranan-Karmegam et al., 2016), Mlt (known to overlap with Klc; Glater et al., 2006; Verhey et  
1359 al., 1998) and Tropomyosin 1 (Dimitrova-Paternoga et al., 2021), and the two-fold enlarged motor

1360 domain. The secondary structure of the motor domain is indicated below ( $\alpha$  helices in black,  $\beta$  sheets  
1361 in blue, loops/L in red); this map was generated by matching the resolved structure of Khc (UniProt  
1362 code: P17210, PDB id 2y65) with descriptions of the kinesin consensus (Sablin et al., 1996);  
1363 regions/motifs that bind ADP/ATP (nt, orange; according to Cao et al., 2017; Gigant et al., 2013;  
1364 Sablin et al., 1996) and/or MTs (dark red; according to Hunter and Allingham, 2020) are also  
1365 indicated below; N1-4 in the motor domain indicate highly conserved motifs (according to Sablin et  
1366 al., 1996); abbreviations above the motor domain indicate the locations of the cover strand (CS;  
1367 according to Budaitis et al., 2021), P-loops (PL) and switch domains I and II (SI, SII; according to  
1368 Cao et al., 2017; Gigant et al., 2013; Sablin et al., 1996). The N-terminal deletion of the above  
1369 *Khc(82-711)* construct is shown in pink: it does not affect MT-binding sites, but it removes the cover  
1370 strand (known to affect kinesin's MT affinity and processivity; Budaitis et al., 2021) and the first P-  
1371 loop (with potential impact on the ATP/ADP cycle); it might also affect the behaviour of the second  
1372 P-loop which was shown to accelerate Khc movement when harbouring the T94S mutation (Cao et  
1373 al., 2017; Higuchi et al., 2004). **B**) Schematic representation of some sub-functions of Khc (details  
1374 and abbreviations in main text; red and stippled black lines indicate processive transport; for further  
1375 sub-functions see Discussion): via a C-terminal MT-binding domain Khc can slide MTs (i),  
1376 associating with Pat1 (and potentially Klc) it is expected to transport non-vesicular cargoes including  
1377 mRNA (ii), with Milt and Miro organelle transport (iii), and with a protein complex containing Klc and  
1378 Syd vesicular transport (iv); in the absence of such associations Khc is auto-inhibited and detaches  
1379 from MTs assisted by Klc (v); to interfere with these subfunctions in this study, different genes were  
1380 genetically removed (orange crosses) or specific *Khc* mutant alleles used (italic orange text). **C**)  
1381 Quantified effects on MT curling caused by specific mutations affecting Khc sub-functions (numbers  
1382 in grey circles indicate which function in A is affected): MT curling is quantified as MT disorganisation  
1383 index (MDI) normalised to wild-type controls (red stippled line); bars at bottom indicate type of culture  
1384 ('5 DIV', embryonic neurons 5 days *in vitro*; 'L3 1/2 DIV', late larval neurons 1/2 days *in vitro*; '5d pre  
1385 1 DIV', embryonic neurons pre-cultured for 5 days and cultured for 1 day); mean  $\pm$  SEM is indicated  
1386 in blue, numbers of analysed neurons in orange, results of Mann Whitney rank sum tests are shown  
1387 in grey/black.

1388

1389 **Fig.S2** Validation of Khc's MT phenotype and demonstration of maternal contribution. **A-C**)  
1390 Quantification of MT curling phenotypes measured as MT disorganisation index (MDI) and  
1391 normalised to wild-type controls (red stippled line); mean  $\pm$  SEM is indicated in blue, numbers of  
1392 analysed neurons in orange, results of Mann Whitney rank sum tests are shown in grey/black; A)  
1393 shows data for *Khc*<sup>27</sup> in homozygosity (27/27) or over deficiency (27/Df), for Khc knock-down  
1394 (*elav>Khc-IR*) and wild-type (wt) and driver line (*elav*) controls; B) shows data for *Khc*<sup>8</sup> over  
1395 deficiency (8/Df) and wild-type controls at different culture times (HIV, hours *in vitro*; DIV, days *in*  
1396 *vitro*); C) shows data for *Klc*<sup>8/Df</sup> and *Klc*<sup>1ts</sup> at 1DIV following 5d pre-culture; note that *Klc*<sup>1ts</sup> is a  
1397 temperature-sensitive allele (see methods) and was pre-cultured at 26°C and cultured at 29°C. **D,E**)  
1398 Examples of neurons at different times in culture (D; relating to data in B) and after pre-culture (E;  
1399 relating to C); asterisks indicate cell bodies, arrow heads axon tips, curved arrows areas of MT  
1400 curling; scale bar in D represents 20 $\mu$ m in D and E.

1401

1402 **Fig.4** ROS enhancing manipulations cause MT curling phenotypes. **A**) Scheme illustrating the  
1403 complexity of ROS-regulating systems in *Drosophila*; ROS-generating factors (bold green): two  
1404 cytoplasmic NADPH oxidases (Nox/NADPH Oxidase, Duox/Dual oxidase with its essential  
1405 maturation factor Mol/Moladietz; Khan et al., 2017); enzymes of the mitochondrial EMT/electron  
1406 transport chain (Wong et al., 2017; Zorov et al., 2014); peroxisomal ACOX1/acyl-CoA oxidase 1

1407 (Walker et al., 2018); Xanthine/aldehyde oxidases (Rosy, AOX1, AOX2, AOX3, AOX4; all jointly  
1408 silenced by loss of Mal/Maroon-like sulfurtransferase; Marelja et al., 2014); ROS removal  
1409 mechanisms (red): superoxide dismutases turn superoxide ( $O_2^{\bullet-}$ ) into  $H_2O_2$  (cytoplasmic CuZn-  
1410 dependent Sod1, mitochondrial Mn-dependent Sod2, extracellular Sod3);  $H_2O_2$  is scavenged by  
1411 peroxisomal Cat/Catalase and neuronal peroxiredoxins (Jafrac1, Prx5; Cao and Lindsay, 2017; Orr  
1412 et al., 2013; Smith et al., 2019; Stapper and Jahn, 2018) and the GSH transferase Gzf (GST-  
1413 containing FLYWCH zinc-finger protein; Smith et al., 2019; Stapper and Jahn, 2018); the latter three  
1414 depend on the redox cycle of the Glu-Cys-Gly tripeptide GSH/Glutathione, synthesised by  
1415 glutathione synthetases (Gss1, Gss2) and Gclc/Glutamate-cysteine ligase (Smith et al., 2019;  
1416 Stapper and Jahn, 2018) and regenerated via Thioredoxins (primarily Trx-2 in neurons; Orr et al.,  
1417 2013; Tsuda et al., 2010) and Thioredoxin reductases (primarily TrxR-1 in neurons; Orr et al., 2013;  
1418 Smith et al., 2019); pharmacological agents (black italics): DEM/diethyl maleate blocks the GSH  
1419 system (Pompella et al., 2003); agents/factors used in our study are highlighted in yellow. **B-E**  
1420 Examples of neurons, either wild-type (wt) expressing Sod1 or Duox (driven by *elav-Gal4*) or  
1421 homozygous for *Ca<sup>1</sup>*, all cultured for 1DIV and stained for actin (act, magenta) and tubulin (tub;  
1422 green); asterisks indicate cell bodies, arrow heads axon tips, curved arrows areas of MT curling;  
1423 yellow emboxed areas are shown as 1.5-fold enlarged insets (green channel only); scale bar in B  
1424 represents 20 $\mu$ m in B-E. **F**) Quantification of MT curling phenotypes measured as MT disorganisation  
1425 index (MDI) and normalised to wild-type controls (red stippled line); bars at bottom indicate type of  
1426 culture ('1/3/5 DIV/HIV', embryonic neurons 1/3/5 days/hours *in vitro*); mean  $\pm$  SEM is indicated in  
1427 blue, numbers of analysed neurons in orange, results of Mann Whitney rank sum tests are shown in  
1428 grey/black.

1429

1430 **Fig.S4** MT bundle and axon length phenotypes of *pex3<sup>2</sup>* mutant neurons. Quantification of  
1431 phenotypes of wild-type (wt) and *Pex<sup>3</sup>* homozygous mutant neurons: **A**) MT curling phenotypes  
1432 measured as MT disorganisation index (MDI); **B**) axon length; both measured are normalised to wild-  
1433 type controls (red stippled line); mean  $\pm$  SEM is indicated in blue, numbers of analysed neurons in  
1434 orange, results of Mann Whitney rank sum tests are shown in grey/black.

1435

1436 **Fig.5** Ameliorating effects of Trolox on mutant MT curling phenotypes. Quantification of MT curling  
1437 phenotypes measured as MT disorganisation index (MDI) and normalised to wild-type controls (red  
1438 stippled line); neurons of different genotype (indicated below) were cultured for 1day after preculture  
1439 (A) of for 5 days (B) in the presence of vehicle (blue) or 100 $\mu$ m Trolox (green; molecule depicted in  
1440 A); mean  $\pm$  SEM is indicated in blue/green, numbers of analysed neurons in orange, results of Mann  
1441 Whitney rank sum tests are shown in grey/black.

1442

1443 **Fig.S5** Impaired fission/fusion of mitochondria does not affect MT bundles. **A-D**) Neurons at 5 days  
1444 *in vitro* (DIV) and stained with anti-tubulin (tub, magenta) and mitoTracker (green); they are wild-type  
1445 (wt) or homozygous mutant for the mitochondrial fission factor Drp or the mitochondrial fusion factors  
1446 Marf or Opa, as indicated; asterisks indicate cell bodies, arrow heads axon tips, yellow emboxed  
1447 areas are shown as 2-fold enlarged insets (green channel only), and the scale bar in A represents  
1448 20 $\mu$ m in A-D; note that mitochondria tend to appear as dashed lines in controls (A), as a continuous  
1449 string of pearls excluded from side branches upon loss of fission (B), and as sparse dots upon loss  
1450 of fusion (C,D). **E**) Quantification of MT curling phenotypes from experiments shown in A-D,  
1451 measured as MT disorganisation index (MDI) and normalised to wild-type controls (red stippled line).  
1452 **F**) Similar experiments with the same mutations using 5 day pre-culture and culture for 1 day. In E



1453 and F, mean  $\pm$  SEM is indicated in blue, numbers of analysed neurons in orange, results of Mann  
1454 Whitney rank sum tests are shown in grey/black.

1455

1456 **Fig.6** Genetic studies of functional links between Khc, Milt and Klc. **A-D)** Examples of neurons, either  
1457 wild-type (wt) or homozygous for *Khc*, *milt* or *Klc* null mutant alleles, cultured for 1DIV following 5d  
1458 pre-culture (to deplete maternal product) and stained against tubulin (tub); asterisks indicate cell  
1459 bodies, arrow heads axon tips, curved arrows areas of MT curling; scale bar in D represents 20 $\mu$ m  
1460 in A-D. **E-G)** Quantification of MT curling phenotypes measured as MT disorganisation index (MDI)  
1461 and normalised to wild-type controls (red stippled line) shown for precultured neurons (E; as in A-D),  
1462 single/double-homozygous mutant neurons (F) and upon genetic interaction (G; single heterozygous  
1463 and trans-heterozygous); mean  $\pm$  SEM is indicated in blue, numbers of analysed neurons in orange,  
1464 results of Mann Whitney rank sum tests are shown in grey/black.

1465

1466 **Fig.S3** Phenotypes upon *Gapdh1* knock-down in primary neurons at 5 DIV. **A)** Illustration of the  
1467 NADH- and ATP-generating steps of glycolysis; names of proteins are shown in bold, other  
1468 molecules in italics: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PGK  
1469 (phosphoglycerate kinase), GAP (glyceraldehyde-3-phosphate), 1,3 BPG (1,3-biphosphoglycerate),  
1470 3-PGA (3-phosphoglycerate). **B)** GAPDH and PGK are present on transported vesicles together with  
1471 other factors relevant for glycolysis (Hinckelmann et al., 2016; Zala et al., 2013) providing ATP to  
1472 drive kinesin-mediated processive transport (red and stippled black lines). **C)** In the absence of  
1473 *Gapdh1*, the transport of synaptic vesicles but not mitochondria is impaired (assessed via anti-Syt  
1474 and mitoTracker staining; see Fig.2), as is consistent with *in vivo* observations in *Drosophila* (larval  
1475 motor nerves; Zala et al., 2013). **D)** Absence of GAPDH does not cause MT curling. Quantification  
1476 of MT curling phenotypes in C and D is measured as MT disorganisation index (MDI) and normalised  
1477 to wild-type controls (red stippled line); median in C and mean  $\pm$  SEM in D are indicated in blue,  
1478 numbers of analysed neurons in orange, results of Mann Whitney rank sum tests are shown in  
1479 grey/black.

1480

1481 **Fig.7** Impacts of activated Khc on MT curling. **A-C)** Examples of neurons at 5DIV expressing different  
1482 Khc constructs (indicated bottom left; compare Fig.3A) and stained for tubulin (tub, magenta) and  
1483 GFP (green), also shown as greyscale single channel images on the right; asterisks indicate cell  
1484 bodies, arrow heads axon tips, curved arrows areas of MT curling; scale bar in A represents 20 $\mu$ m  
1485 in all images. **D)** Quantification of MT curling phenotypes measured as MT disorganisation index  
1486 (MDI) and normalised to wild-type controls (red stippled line); genotypes are shown below, also  
1487 indicating the culture period (5DIV, 3DIV); mean  $\pm$  SEM is indicated in blue, numbers of analysed  
1488 neurons in orange, results of Mann Whitney rank sum tests are shown in grey/black.

1489

1490 **Fig.8** Mapping findings on the dependency cycle of local axon homeostasis. The numbered green  
1491 arrows and red T-bar make up the previously published 'dependency cycle of local axon  
1492 homeostasis' (Prokop, 2021): 1) axonal transport provides materials, components and organelles  
1493 required for axon function; 2) this transport requires MT bundles as the essential highways; 3)  
1494 however, this live-sustaining transport damages MT bundles; 4) the axonal cortex and MT binding  
1495 proteins (MTBPs) support and maintain MT bundles (boxed names in orange and grey at bottom  
1496 left list factors that were shown in the *Drosophila* neuron culture system to be involved in bundle-  
1497 maintaining cortical and MT regulation; Alves-Silva et al., 2012; Hahn et al., 2021; Qu et al., 2019;

1498 Qu et al., 2017); 5) bundle maintenance requires transport-dependent components and physiology,  
1499 thus closing the circle. The original model of 'local axon homeostasis' comprised arrows 1-4 (Hahn  
1500 et al., 2019). Khc contributes to the MT bundle damage, and this is enhanced by non-inactivating  
1501 mutations (vibrating red arrow and *Khc* alleles top left). Loss of function of Khc, Milt, Miro, Unc-104  
1502 and Dhc contribute to mitochondrial transport (large green arrow, top right). Mitochondria harbour  
1503 Sod2 that can quench harmful ROS (green T-bar); 8 independent pharmacological and genetic  
1504 manipulation of ROS regulation (bottom right) demonstrated that dysregulation of ROS causes MT  
1505 curling (dashed red arrow). Examples of mammalian factors that can be mapped onto this cycle are  
1506 explained in the Discussion and previous reviews (Hahn et al., 2019; Prokop, 2021).

1507

1508 **Fig.S6** *Pat1* and *Khc<sup>mutA</sup>* mutations fail to suppress the *Klc*-deficient MT curling phenotype.  
1509 Quantification of MT curling phenotypes measured as MT disorganisation index (MDI) and  
1510 normalised to wild-type controls (red stippled line); genotypes are shown below, also indicating the  
1511 culture period (2DIV, 5DIV); mean  $\pm$  SEM is indicated in blue, numbers of analysed neurons in  
1512 orange, results of Mann Whitney rank sum tests are shown in grey/black. The fact that *Khc<sup>mutA</sup>* and  
1513 *Pat1<sup>robin</sup>* fail to suppress *Klc<sup>8ex94</sup>*-induced MT curling suggests that potential surplus pools of non-  
1514 inactivated Khc do not engage in MT sliding or Pat1-mediated transport to cause MT curling.

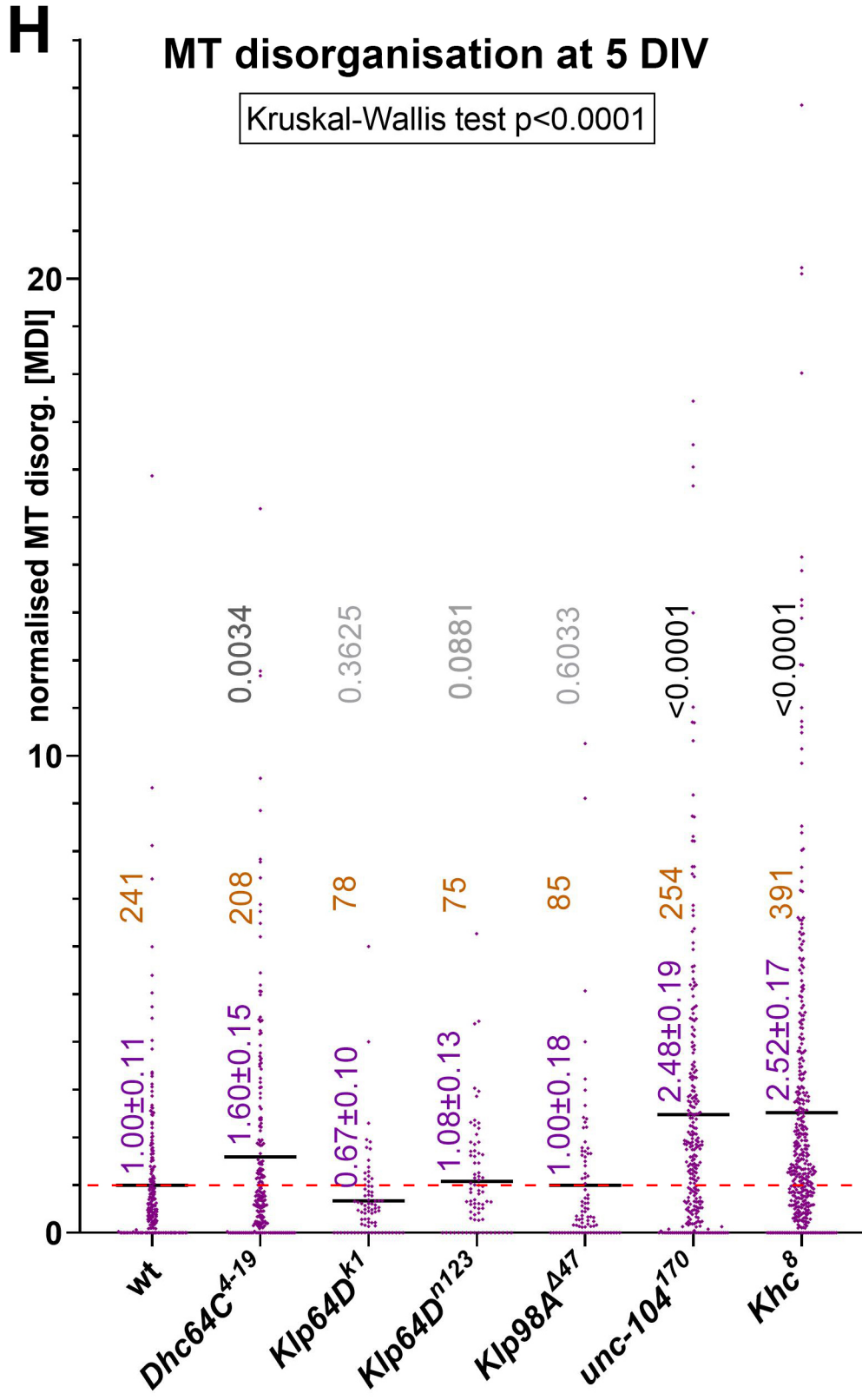
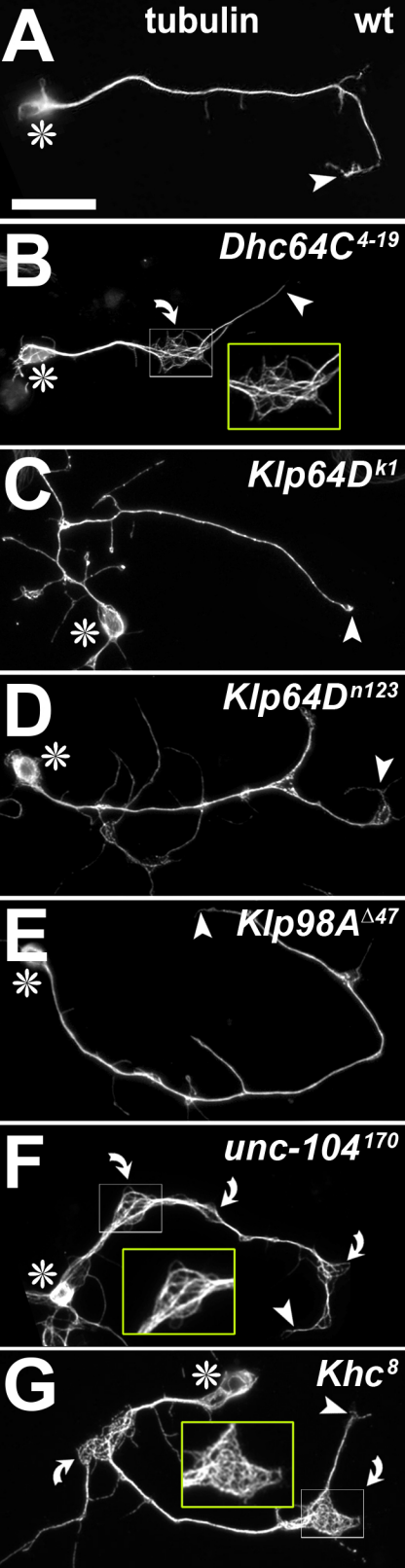
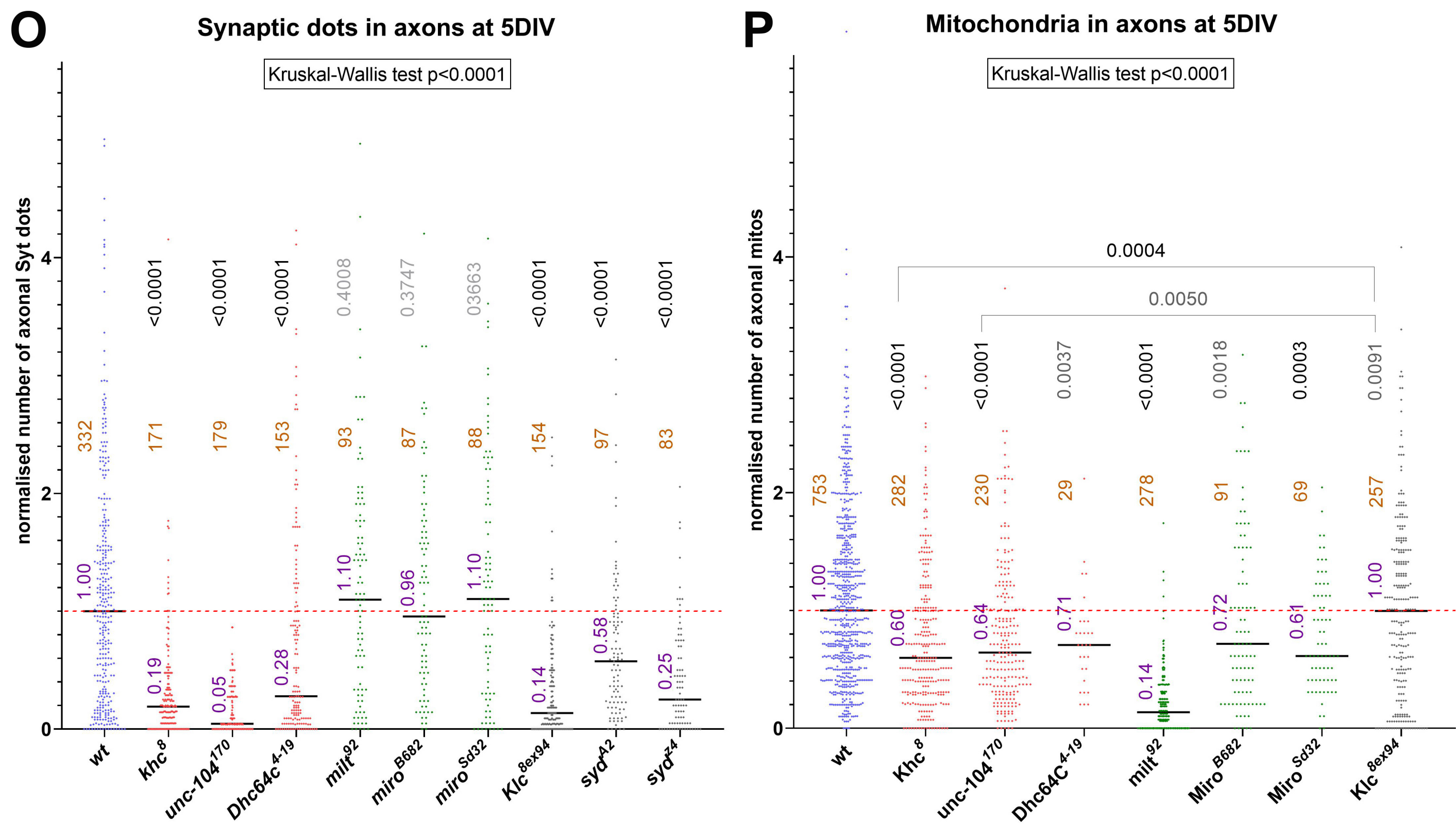
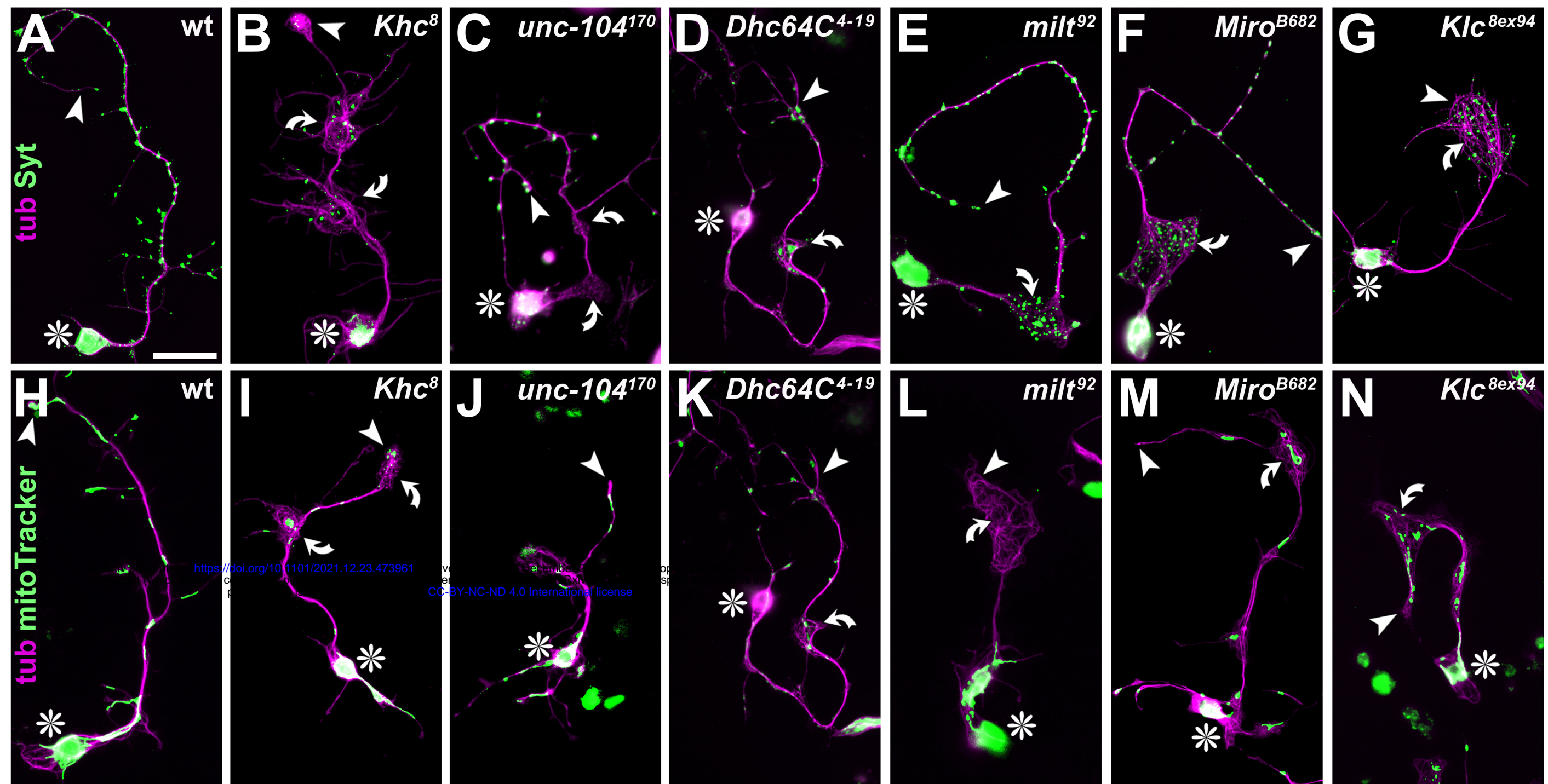


Fig. 1 Liew et al.



**Fig. 2 Liew et al.**

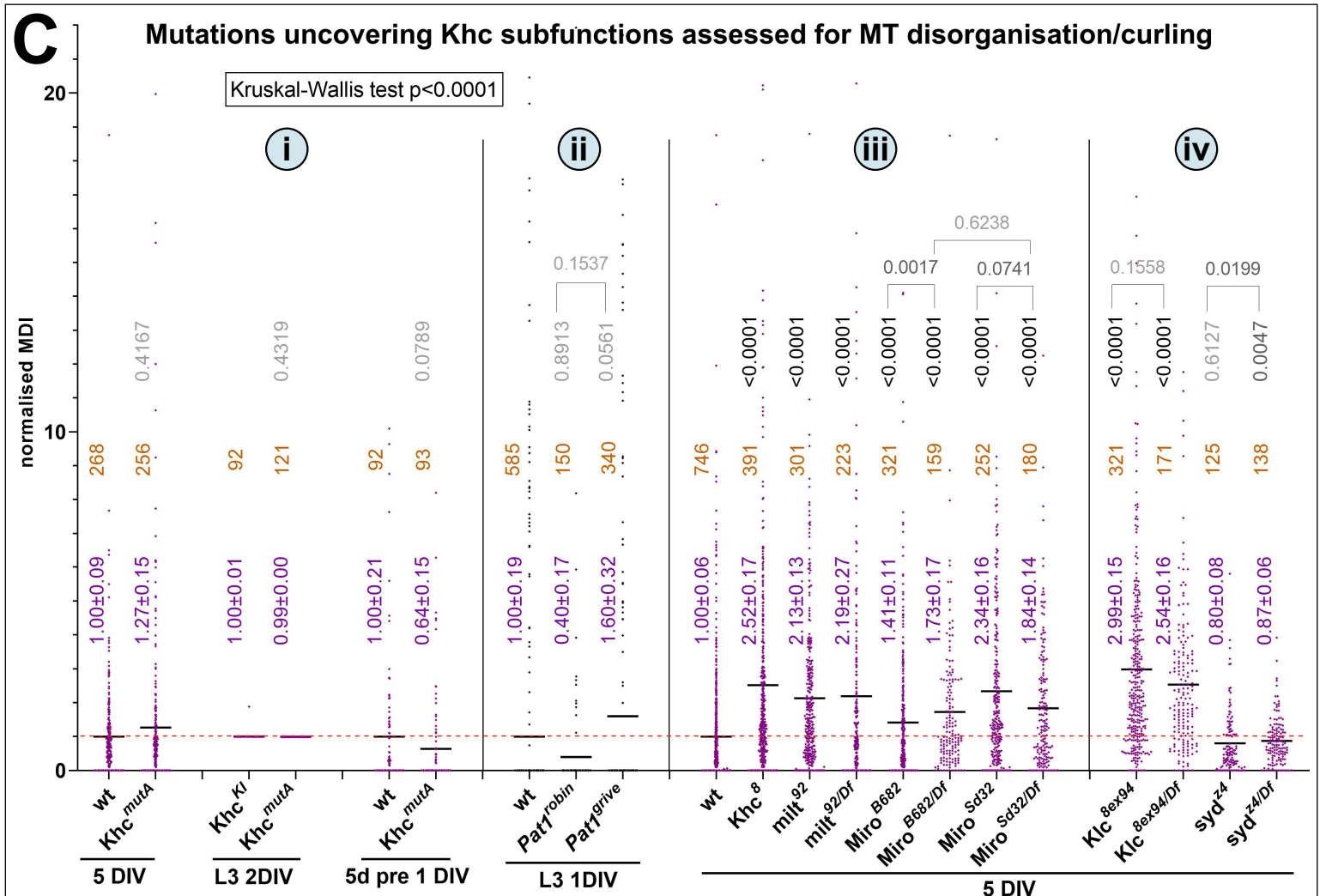
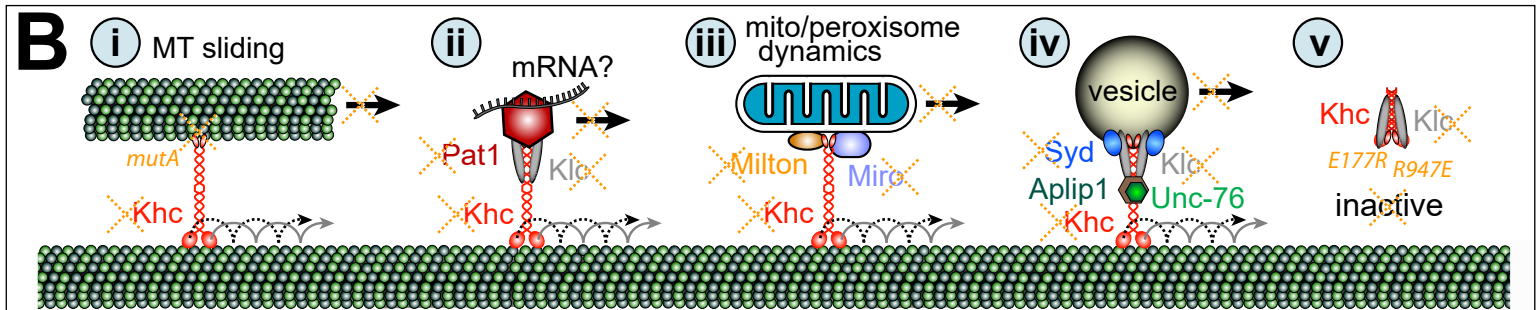
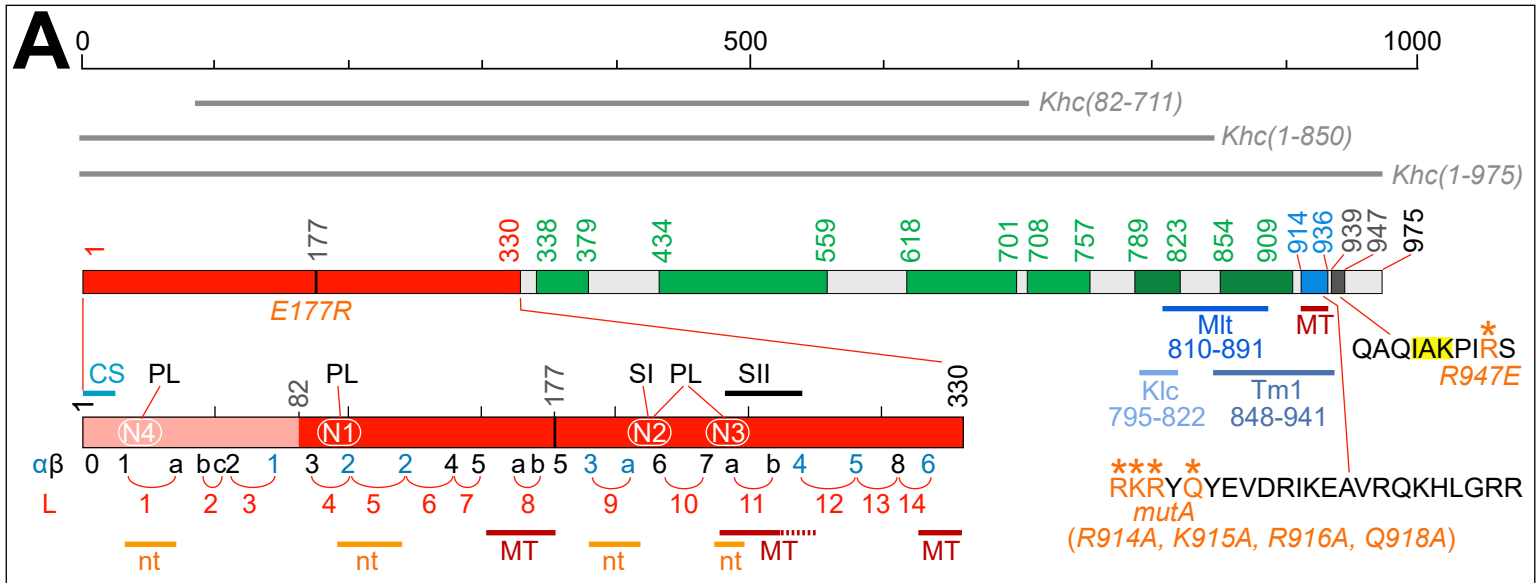
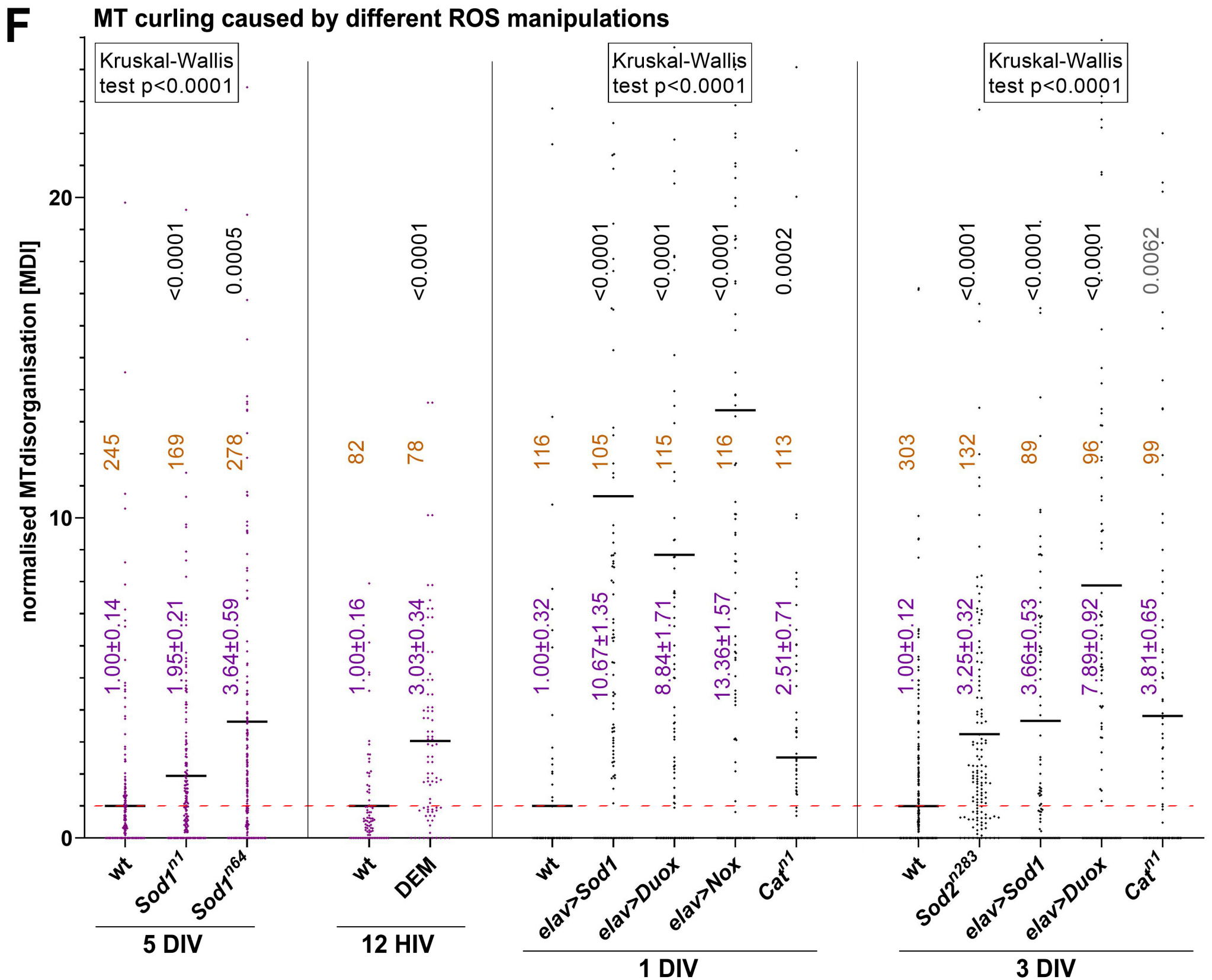
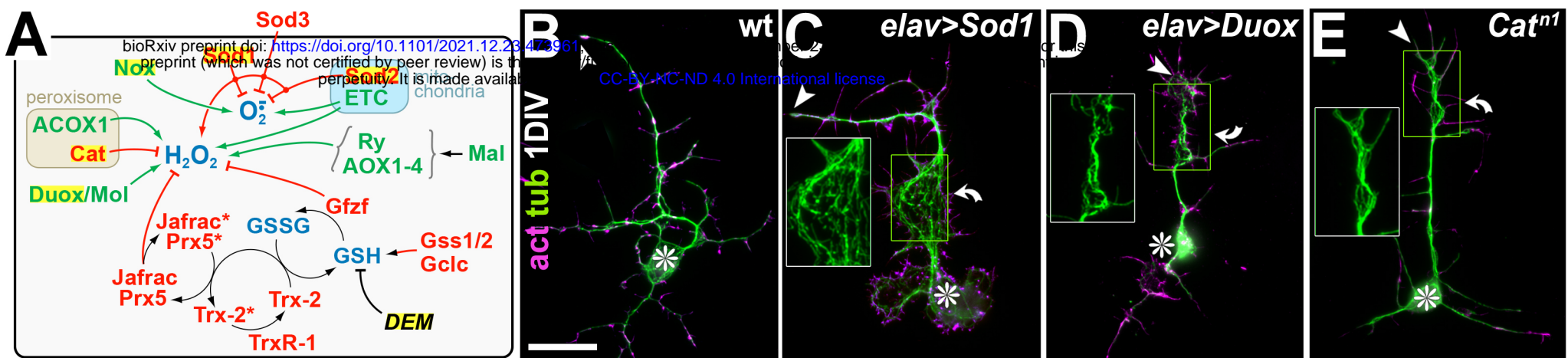


Fig. 3 Liew et al.



**Fig. 4 Liew et al.**

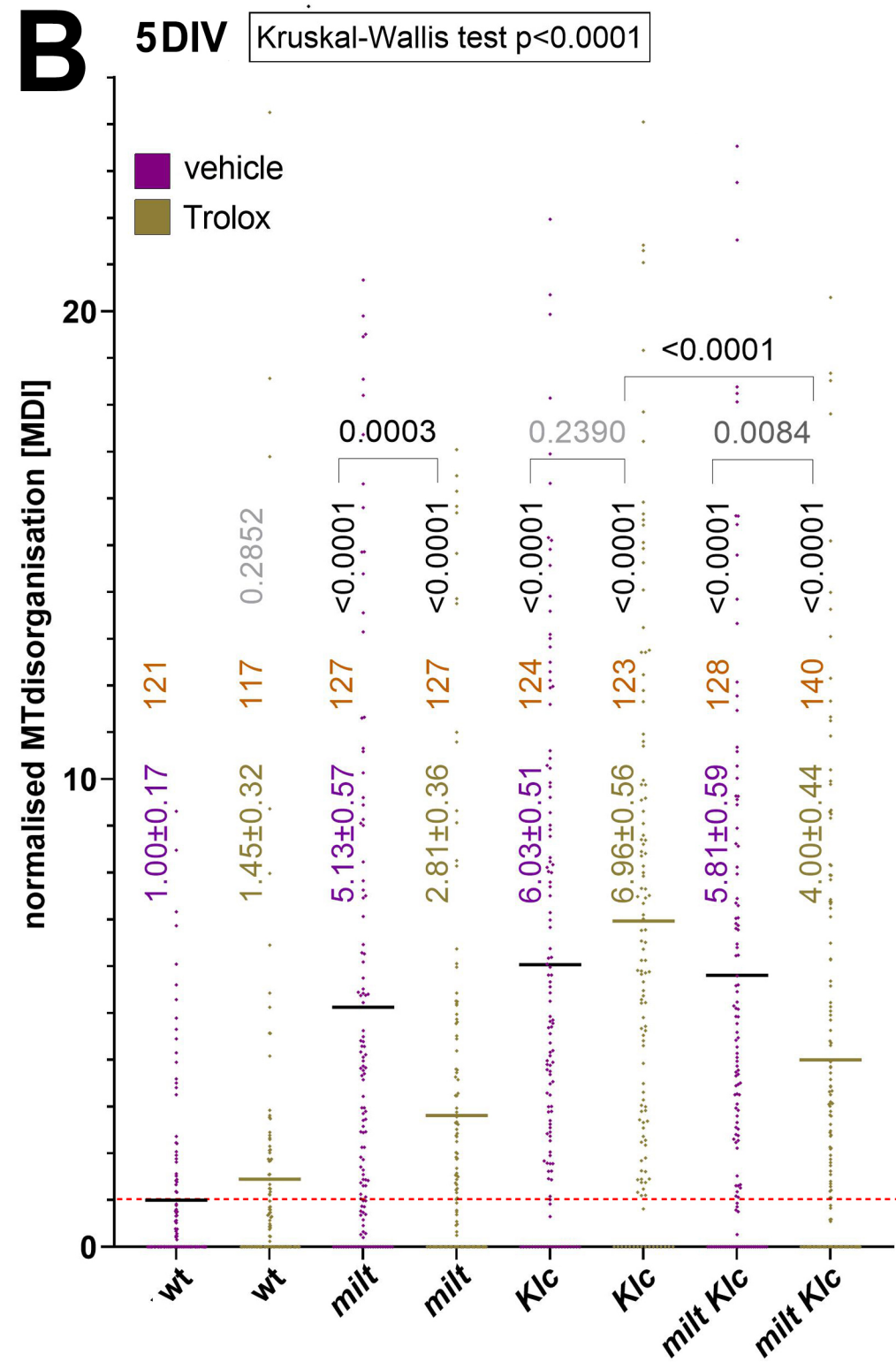
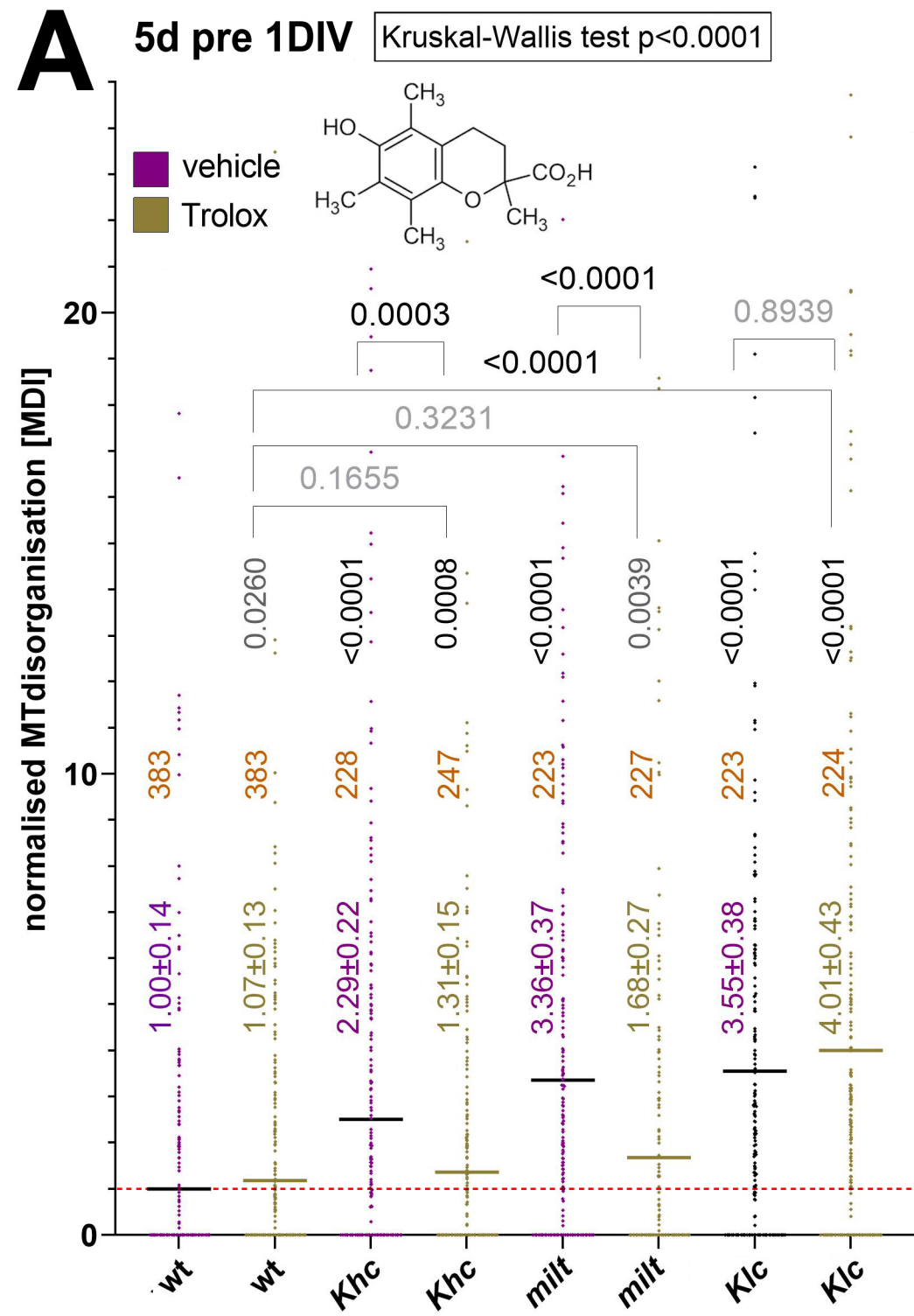


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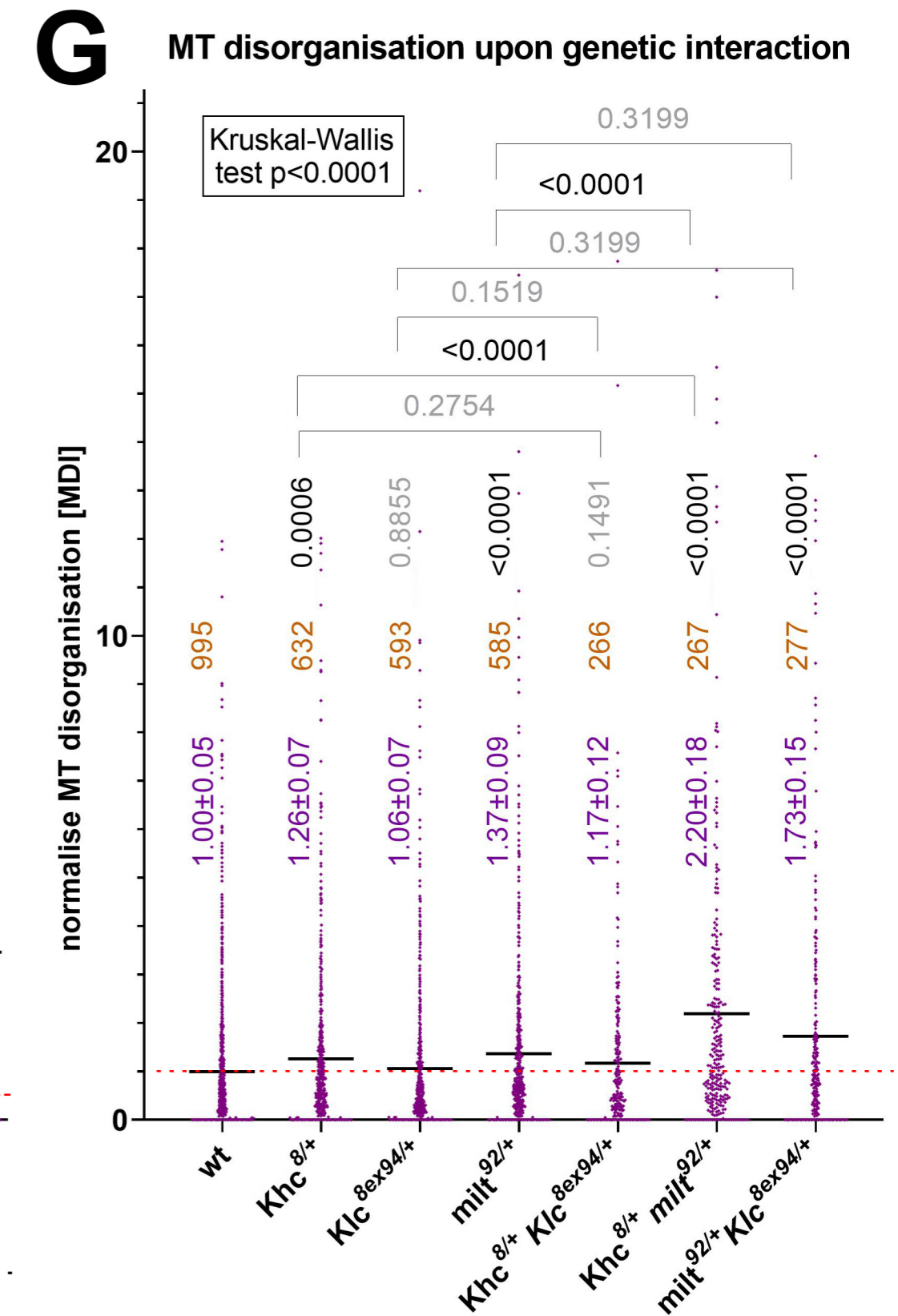
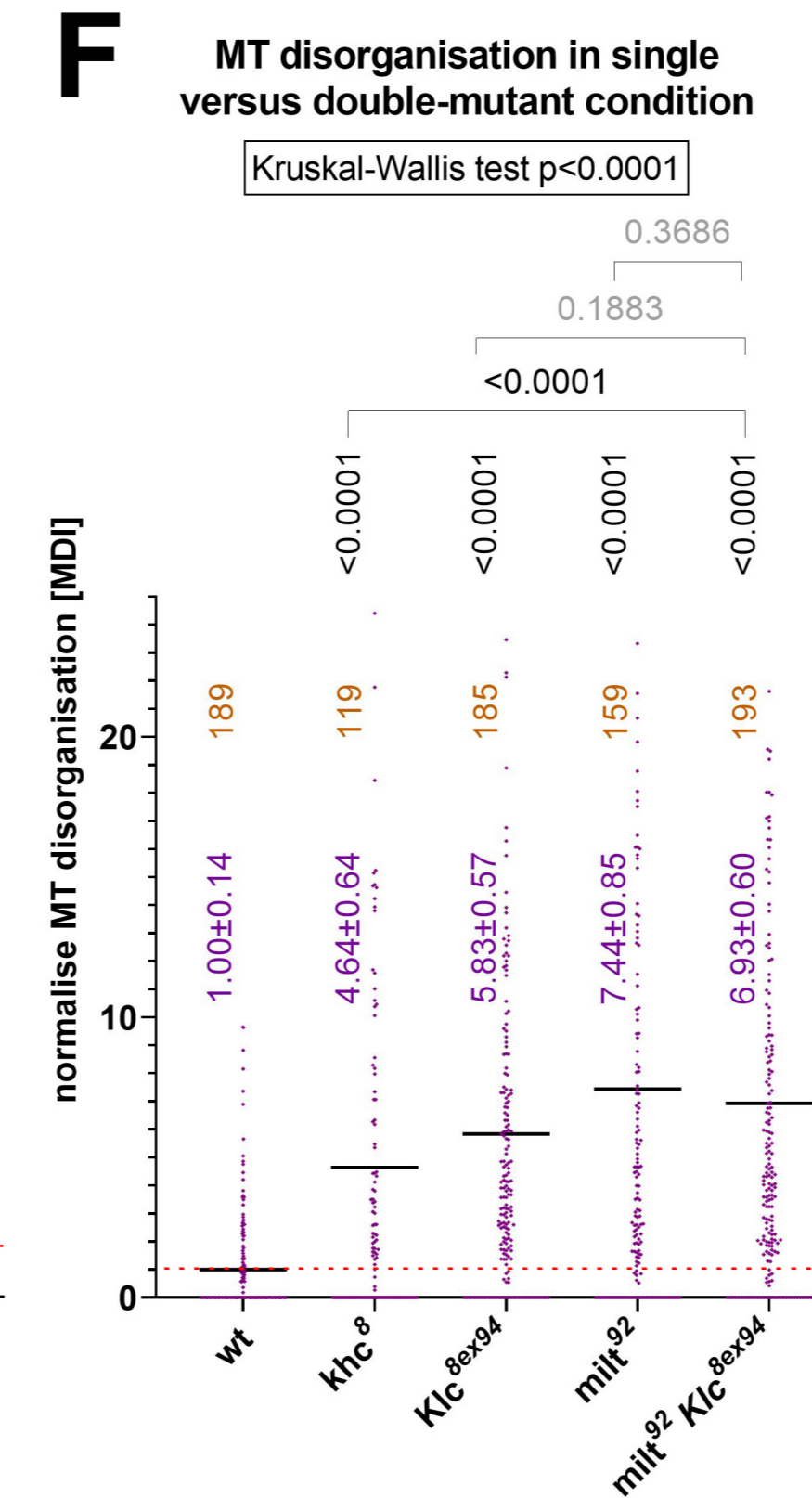
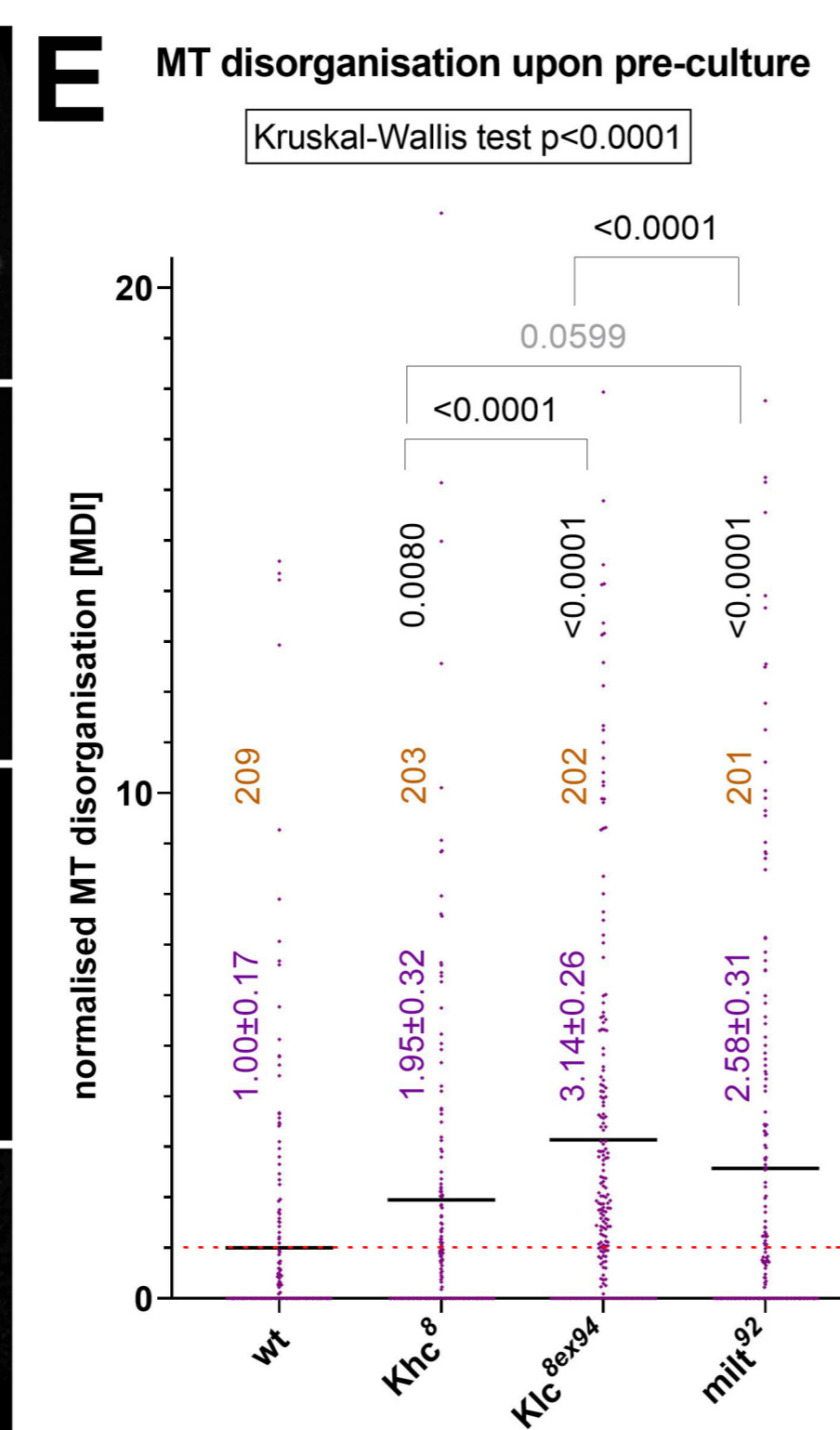
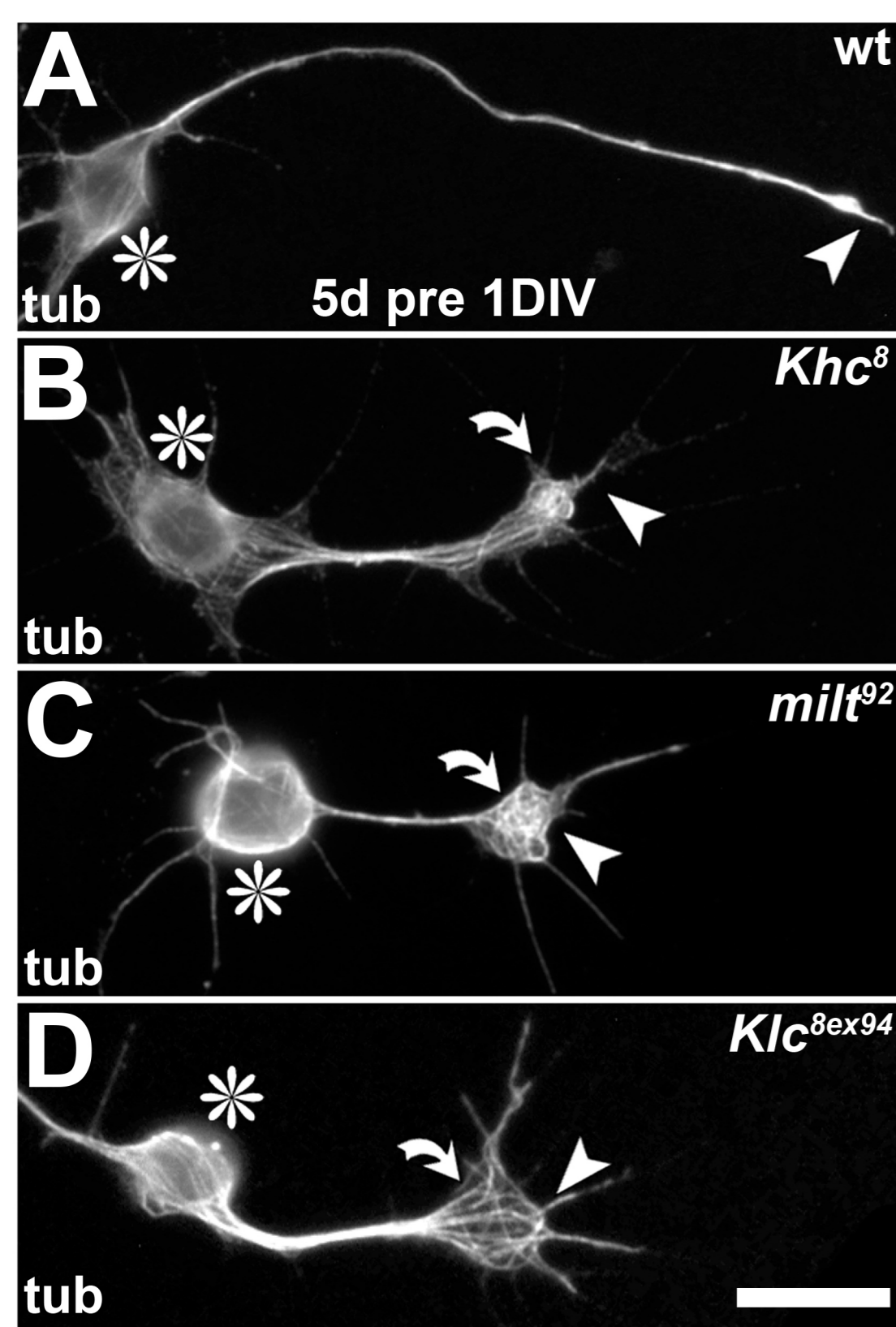
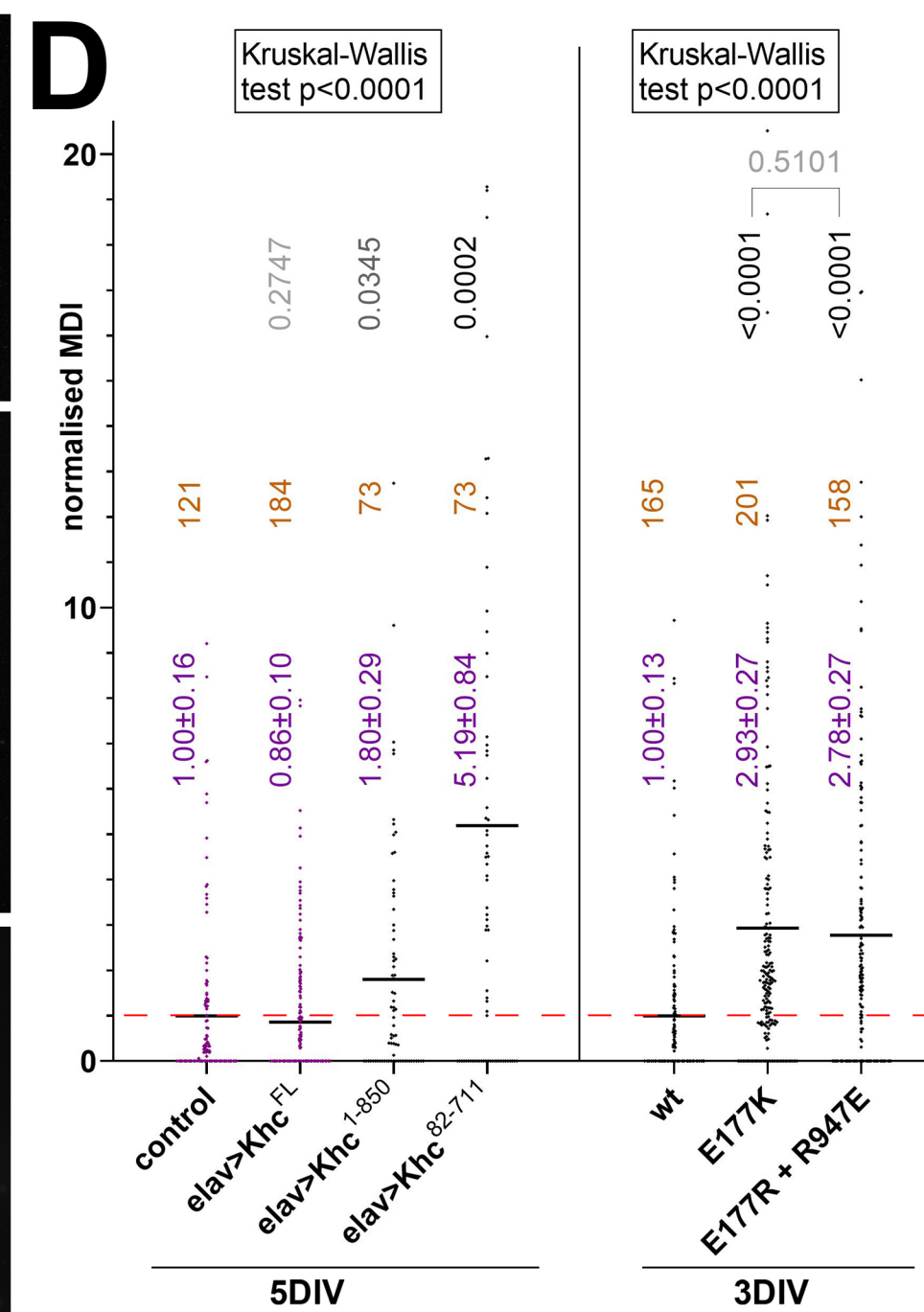
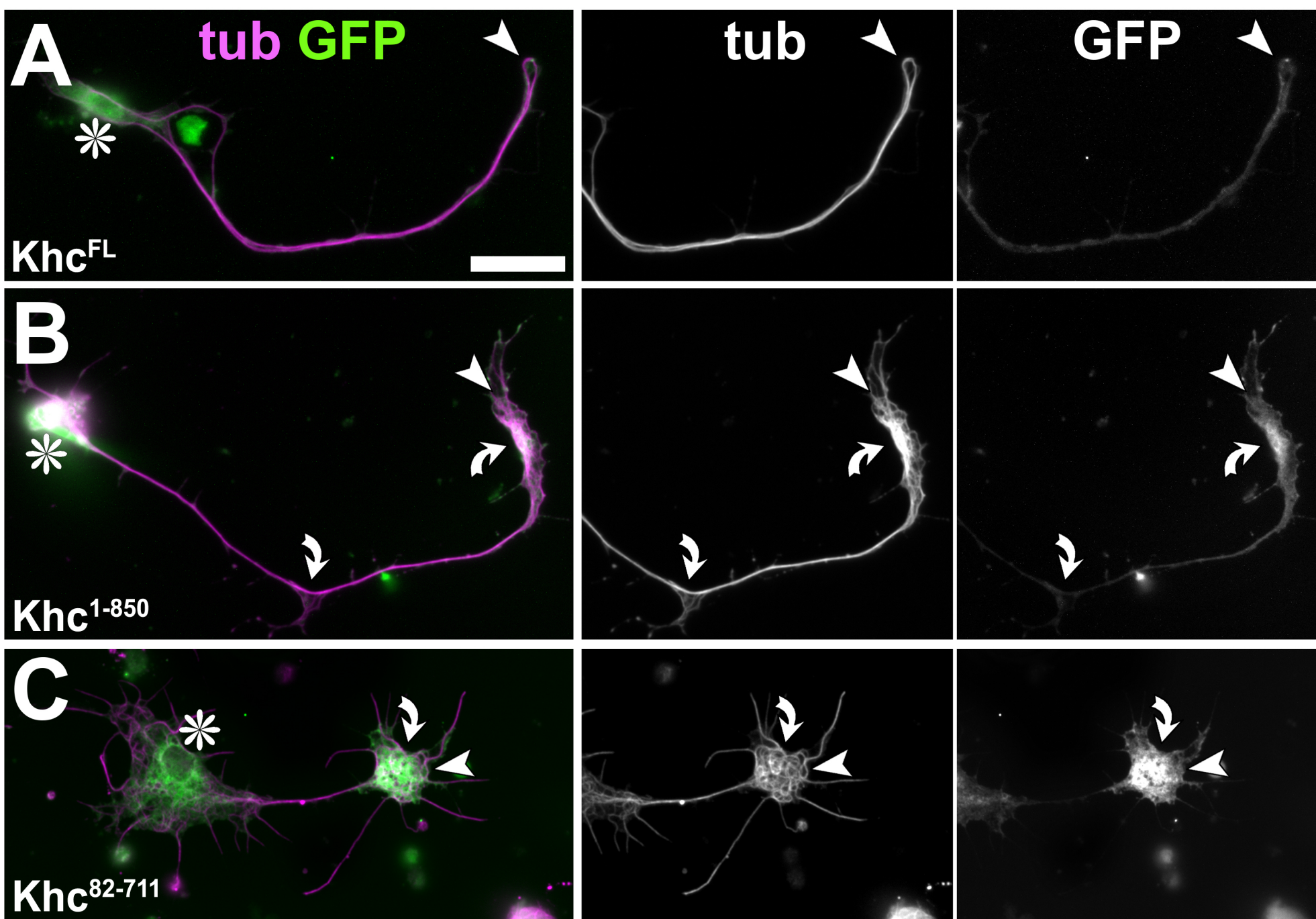
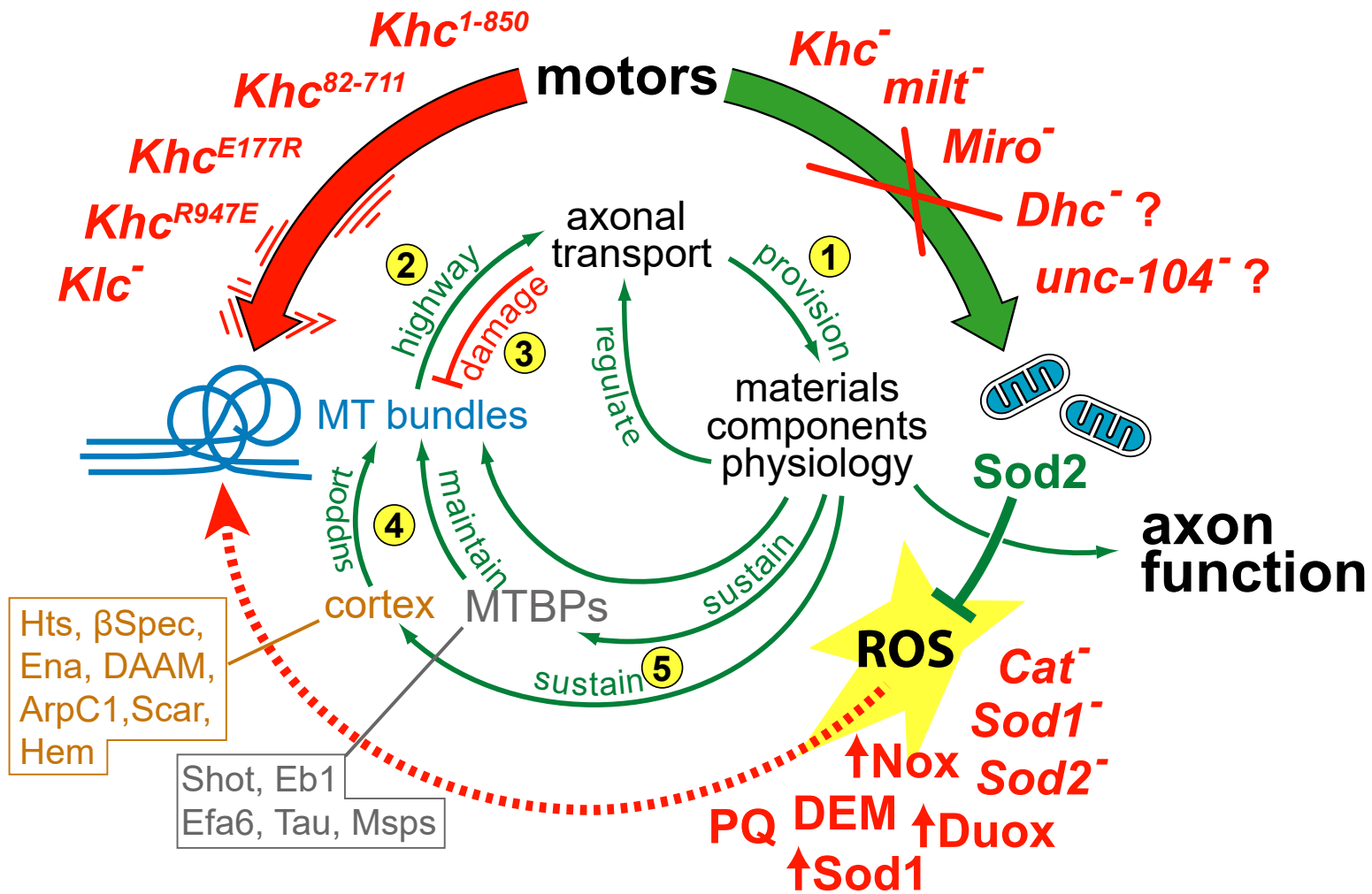


Fig. 6 Liew et al.

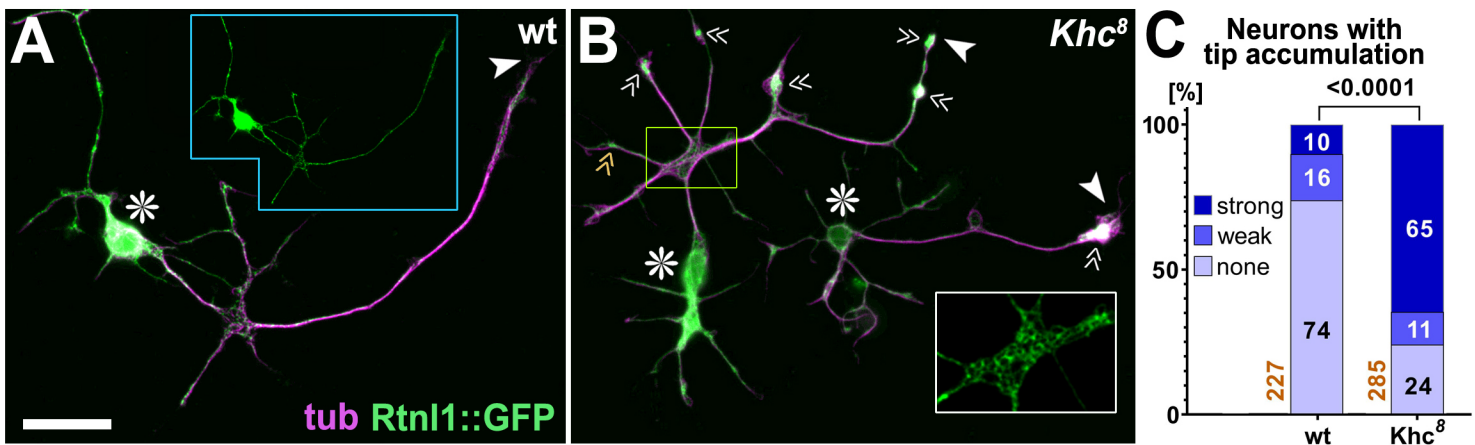




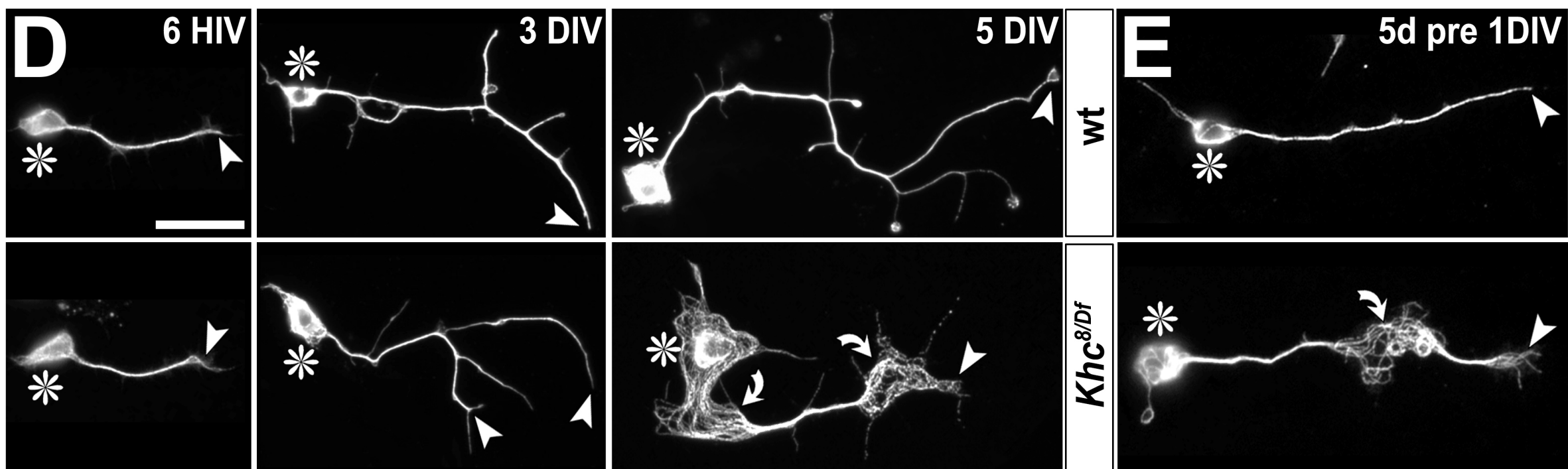
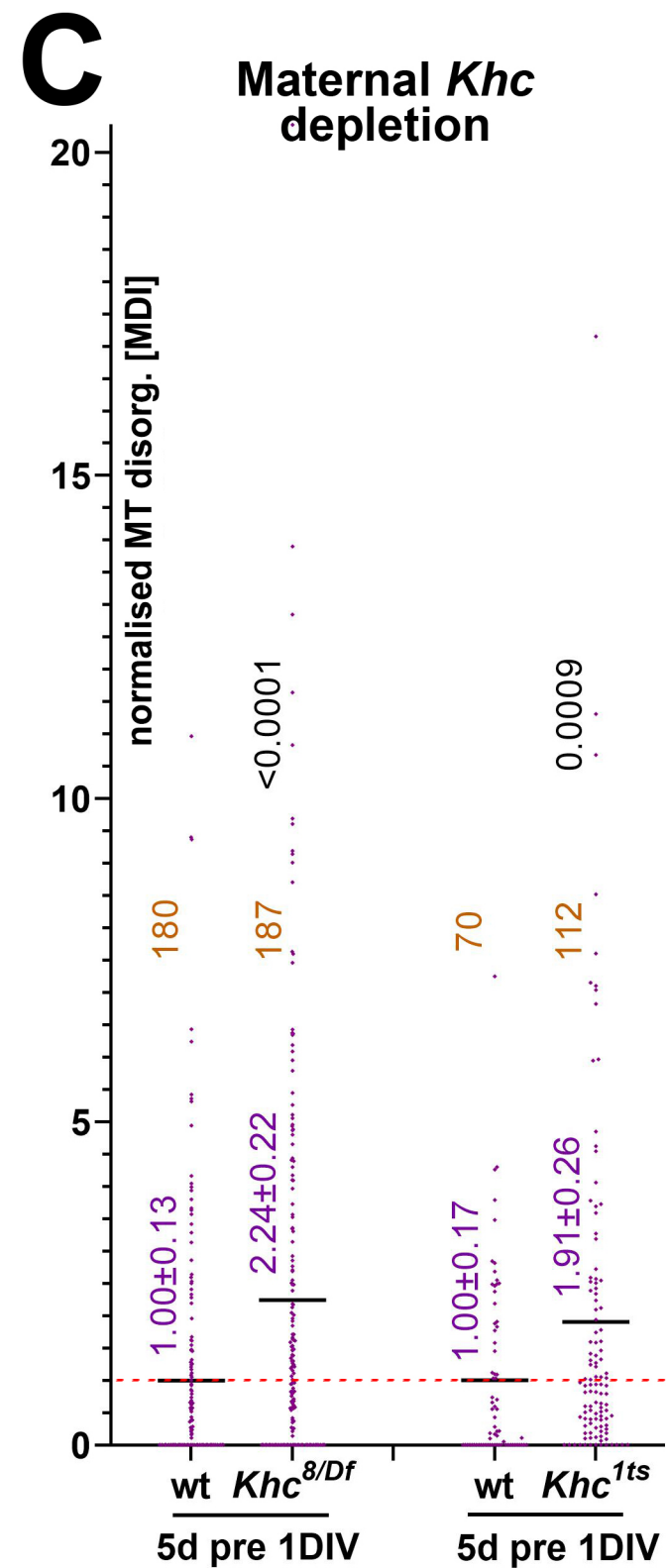
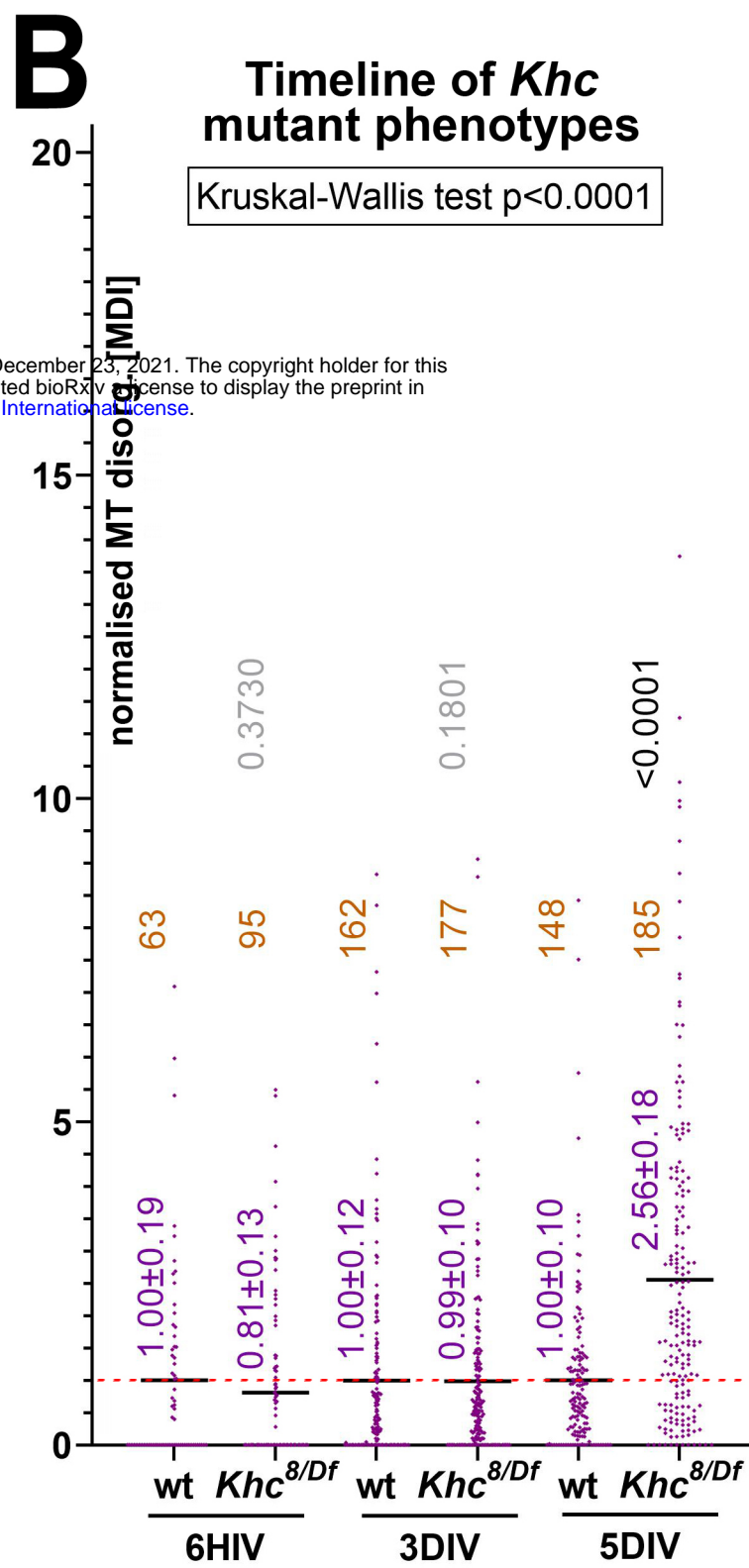
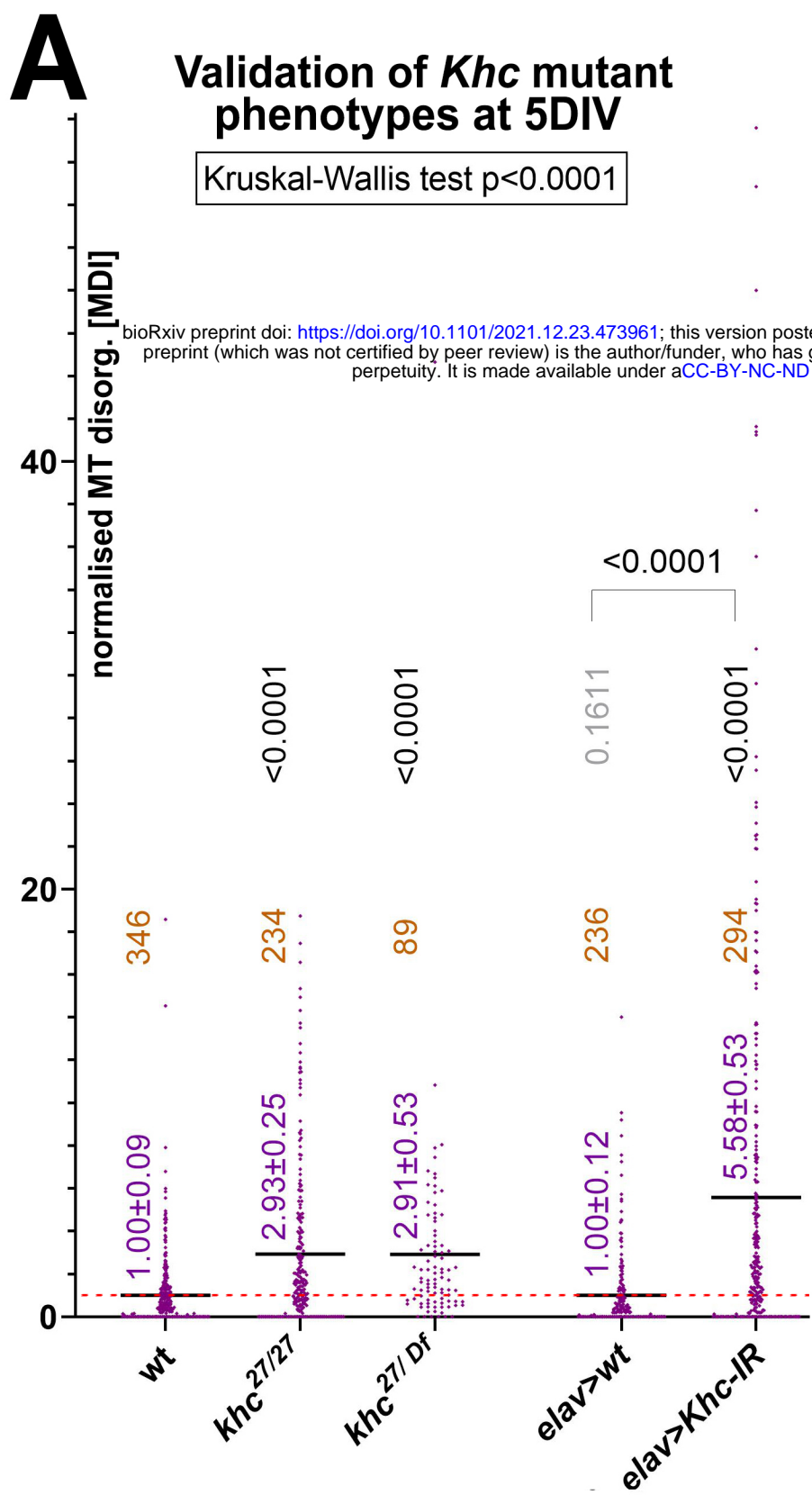
**Fig. 7** Liew et al.



**Fig. 8 Liew et al.**



**Fig. S1** Liew et al.



**Fig. S2** Liew et al.

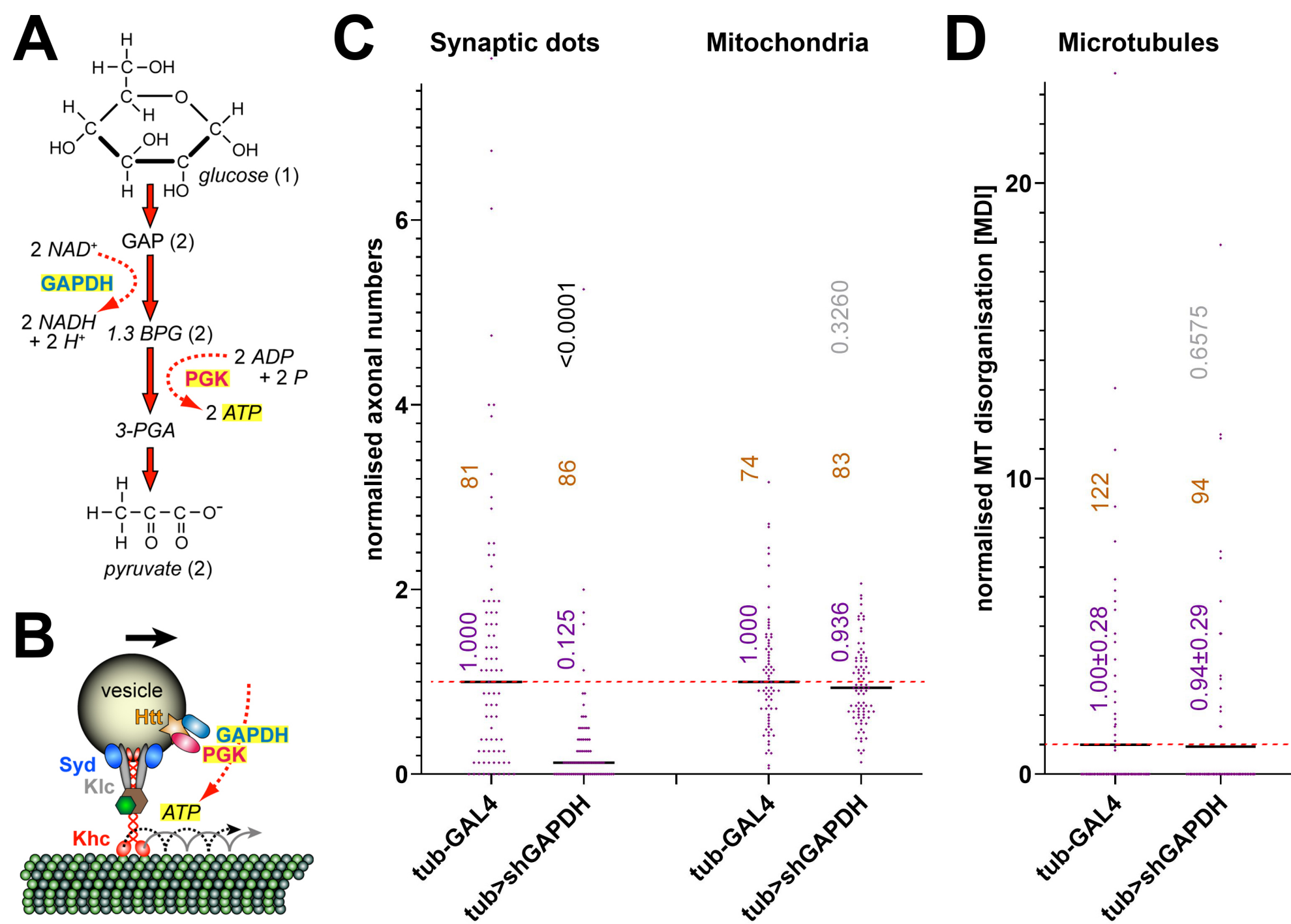


Fig. S3 Liew et al.

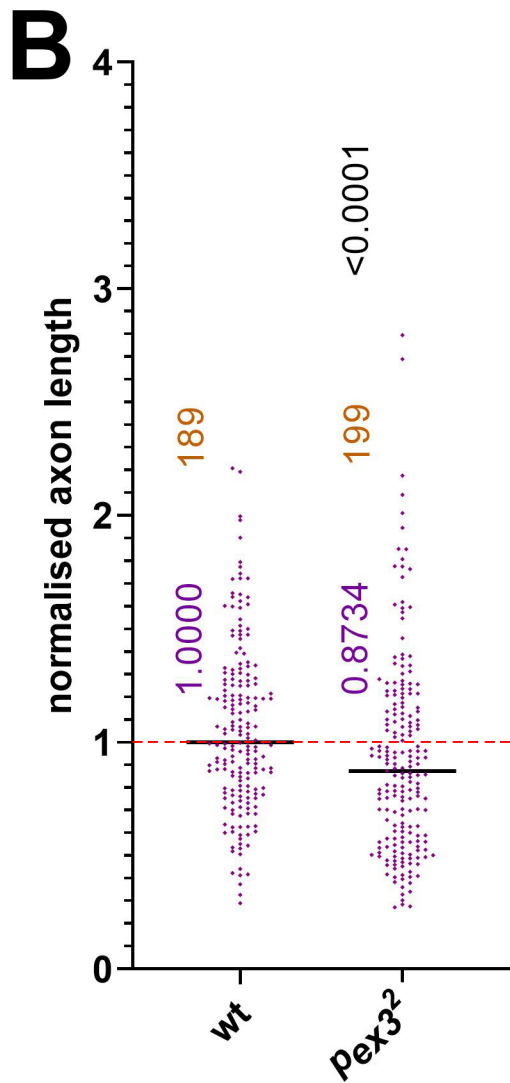
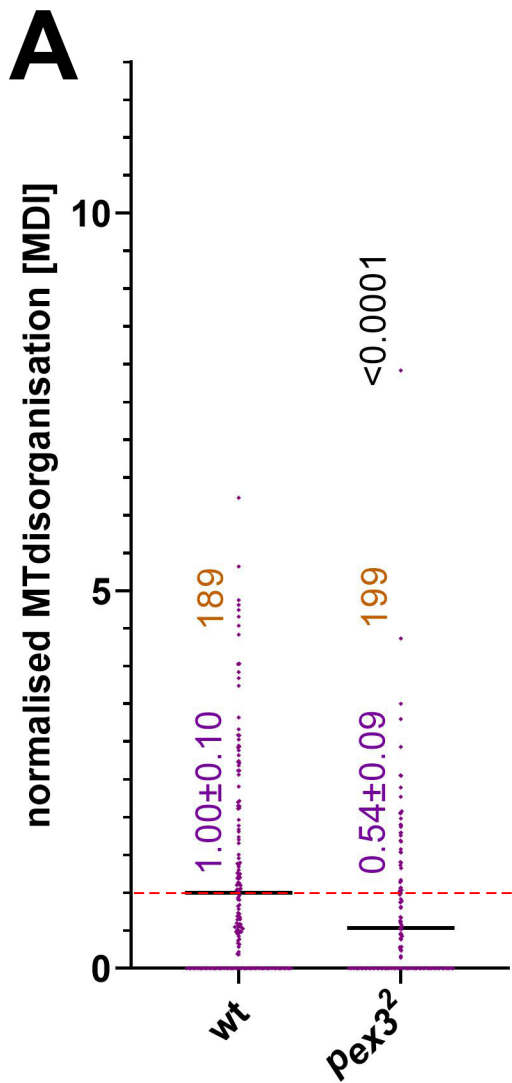
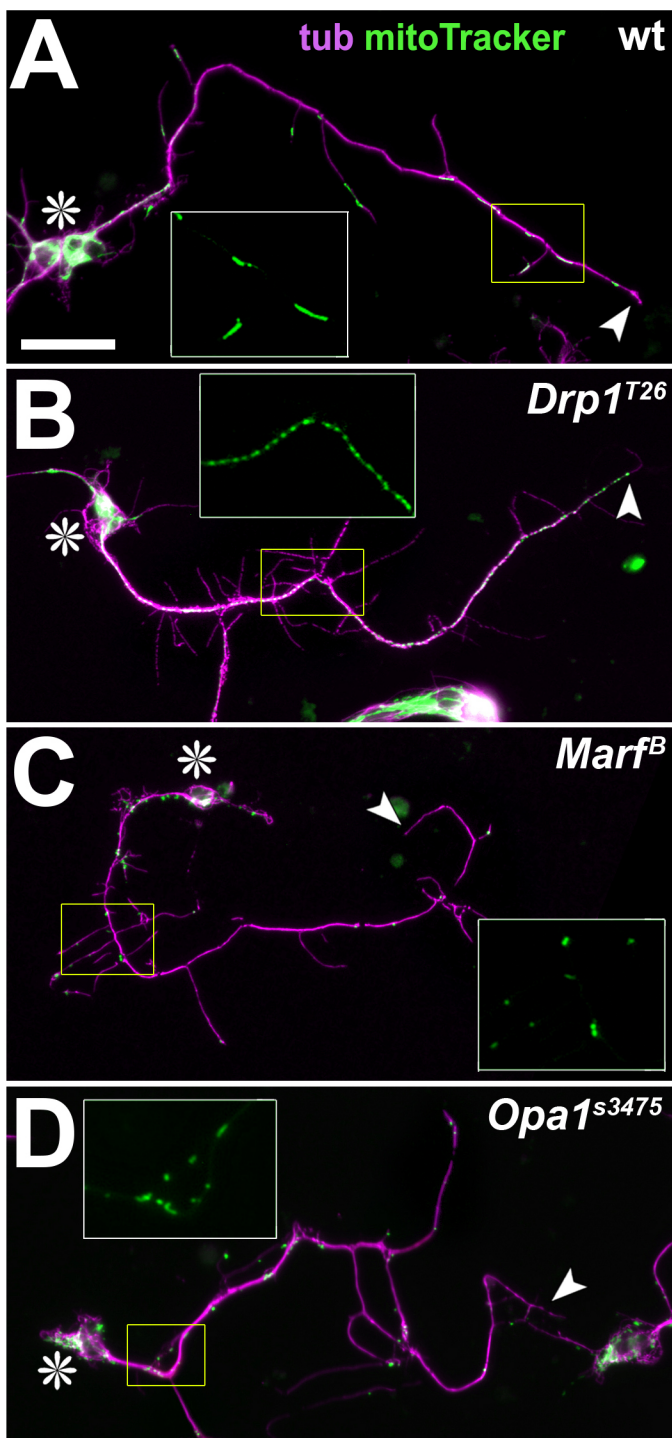
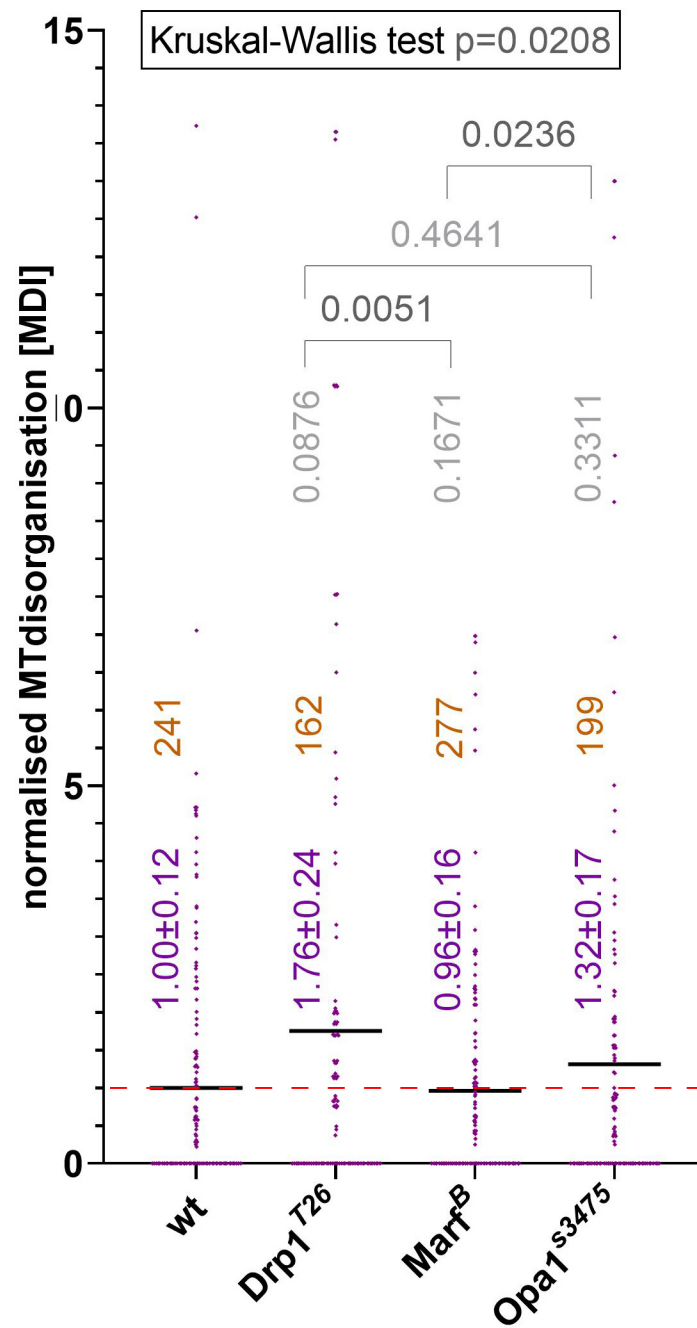


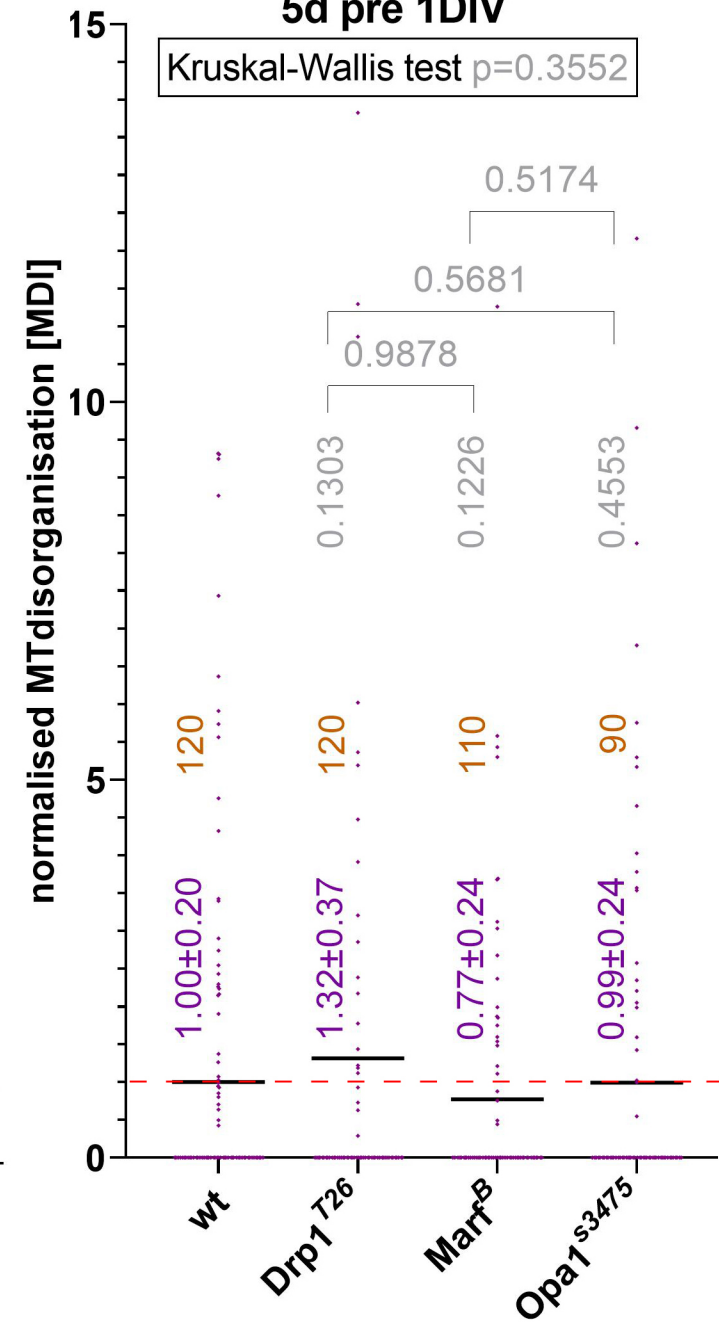
Fig. S4 Liew et al.



**E** Fission/fusion mutants at 5DIV



**F** Fission/fusion mutants 5d pre 1DIV



**Fig. S5** Liew et al.

# Double-mutant constellations with *Klc*<sup>8ex94</sup>

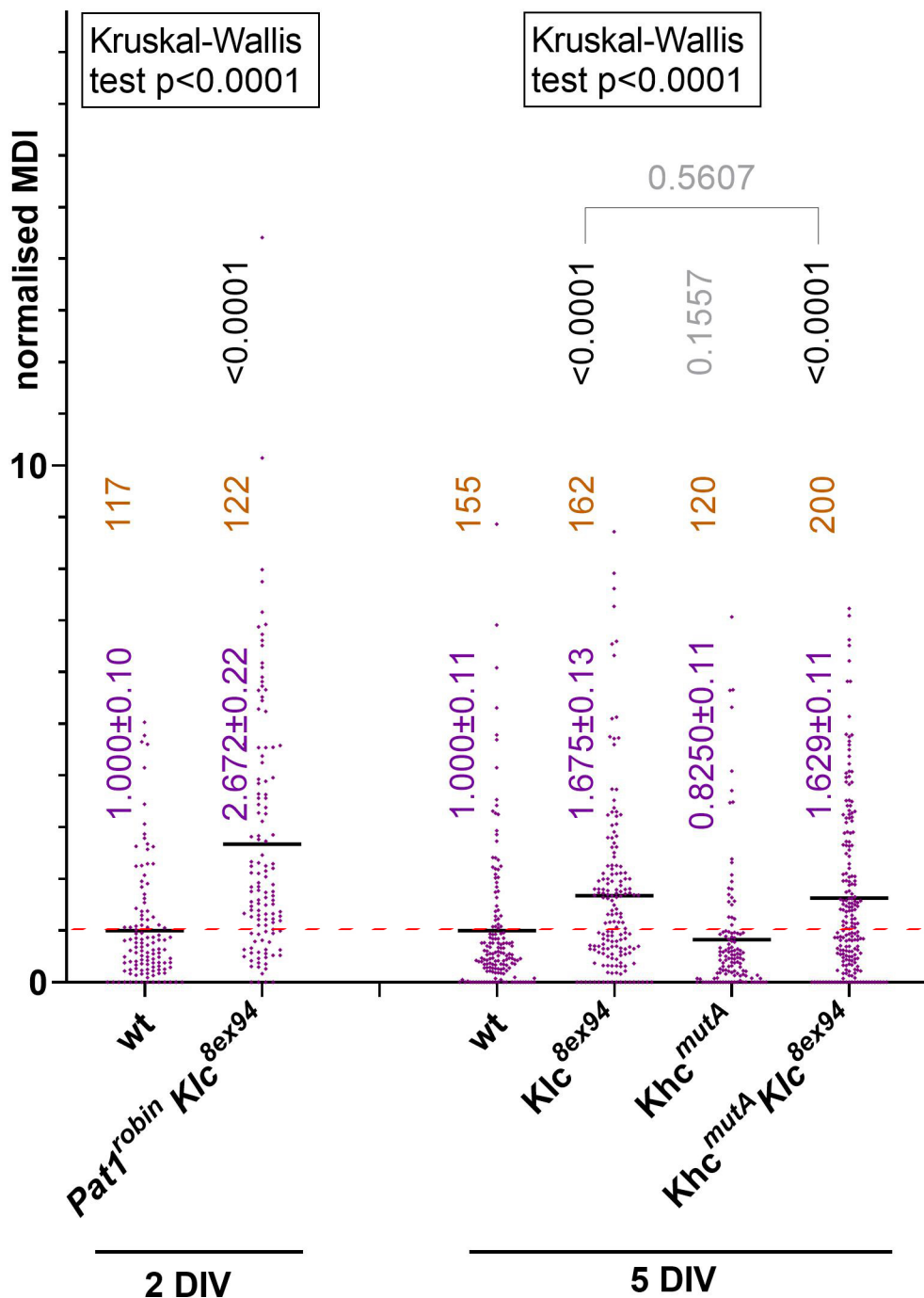


Fig. S6 Liew et al.