

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
13 consent by this author to publication of this work has not been given. However, consent is
14 assumed from prior discussions we had.

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20 ROS

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](https://flybase.org/FASTA/unc-104/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*⁸, *unc-104*¹⁷⁰ and *Dhc64C*⁴⁻¹⁹ 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*⁸ 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*²⁷ and *Khc*⁸ 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*^{8/Df} 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*^{1ts} 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*^{mutA} 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*^{mutA}
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*^{robin} and *Pat1*^{grive} 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*⁹², *Miro*^{Sd32} or *Miro*^{B682}, 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*⁹² 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*⁹², *Miro*^{Sd32} or *Miro*^{B682} 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 μ M 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*ⁿ¹), an enzyme removing hydrogen-peroxide (H_2O_2 ;
201 Walker et al., 2018), and null alleles of two members of the Superoxide dismutase family, Sod1
202 (*Sod1*ⁿ¹ or *Sod1*ⁿ⁶⁴) and Sod2 (*Sod1*ⁿ²⁸³), which convert superoxide anions (O_2^-) into H_2O_2 (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_2^- levels, (b) of
209 *Sod1* to reduce O_2^- levels and enhance H_2O_2 , and (c) of *Duox* (Dual oxidase) to increase H_2O_2 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_2^- or H_2O_2 seems to cause this effect, although O_2^- may
214 elicit its effects through conversion into H_2O_2 (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 α -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*⁸ or *milt*⁹² were either pre-cultured for a day in the presence of
222 100 μ 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*⁸ and
224 *milt*⁹² 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 H₂O₂ 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 H₂O₂ levels down. To test this possibility, we
230 blocked peroxisome biogenesis using the *Pex3*² mutation (Faust et al., 2014). Abolishing
231 peroxisomes in this way did not induce any obvious MT curling phenotypes, but the *Pex3*² 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*^{T26}; Dynamin related
250 protein 1), of mammalian OPA1 (*Opa1*^{s3475}; Optic atrophy 1) and of mammalian MFN (*Marf*^B;
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*^{s3475} or *Marf*^B) mitochondrial
254 shapes were primarily short and dot-like (Fig.S5C,D), whereas loss of fission (*Drp1*^{T26}) 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*^{8ex94} or *Klc*^{8ex94/Df} 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*⁸, *Klc*^{8ex94} and
281 *milt*⁹² 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*^{8ex94}
284 mutant neurons. However, in contrast to *Khc*⁸ and *milt*⁹² 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*^{8ex94} and *syd*^{z4} 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*^{8ex94} 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*^{z4} or *syd*^{z4/Df} 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*⁸, *milt*⁹² and
314 *Klc*^{8ex94} (i.e. combining heterozygosity for two genes at a time in the same neurons). Of the three
315 constellations, only *Khc*^{8/+} *milt*^{92/+} 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*¹⁻⁸¹¹::GFP and *Khc*⁸²⁻⁷¹¹::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*¹⁻⁸⁵⁰::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*⁸²⁻⁷¹¹::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^{mutA}*), 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; Kevenaer 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^{1-850::GFP}, versus the strong phenotype
499 with genomically engineered *Khc*^{E177K} and *Khc*^{E177R, R947E} 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*^{92/92} *Klc*^{8ex94/8ex94} 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*^{mutA/mutA} *Klc*^{8ex94/8ex94} or *Pat1*^{robin/robin}
515 *Klc*^{8ex94/8ex94} 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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539 stocks as mentioned in Materials and Methods.

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 (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*¹⁷⁰ (Pack-Chung et al., 2007; Tom Schwarz)
- 550 • *Klp64D*^{K1} (Ray et al., 1999; hypomorphic allele; BL #5578)
- 551 • *Klp64D*ⁿ¹²³ (Perez and Steller, 1996; BL #5674)
- 552 • *Klp98A*^{A47} (Derivery et al., 2015; Marcos Gonzalez-Gaitan)
- 553 • *Dhc64C*⁴⁻¹⁹ (Gepner et al., 1996; BL #5274)
- 554 • *Khc*^B (Saxton et al., 1991; BL #1607)
- 555 • *Khc*²⁷ (Saxton et al., 1991; Isabel Palacios)
- 556 • *Klc*^{1ts} (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*^{mutA} (Winding et al., 2016; Vladimir Gelfand; confirmed by lethality of hetero-allelic
559 *Khc*^{mutA/B} animals)
- 560 • *Khc*^{E177K} and *Khc*^{E177K,R947E} (Kelliher et al., 2018; Jill Wildonger)
- 561 • *Df(Khc)* (*Df(2R)BSC309*; Cook et al., 2012; BL #23692)
- 562 • *milt*⁹² (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*^{Sd32} (Guo et al., 2005)
- 566 • *Miro*^{B682} (Guo et al., 2005; BL #52003)
- 567 • *Df(Miro)* (*Df(3R)Exel6197*; Parks et al., 2004; BL #7676)
- 568 • *Klc*^{8ex94} (Gindhart et al., 1998; BL #31997)

- 569 • *syd⁴* (Bowman et al., 2000; BL #32016)
570 • *Df(3L)syd^{A2}* (Bowman et al., 2000; deleting C-terminus; BL #32017)
571 • *Pat^{grive}* and *Pat^{robin}* (Loiseau et al., 2010; Isabel Palacios)
572 • *Sod1ⁿ¹* (Phillips et al., 1989; BL #24492)
573 • *Sod1ⁿ⁶⁴* (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^{T26}* (Verstreken et al., 2005; BL #3662)
576 • *Mar^B* (Sandoval et al., 2014; Hugo Bellen)
577 • *P{lacW}Opa1^{s3475}* (Spradling et al., 1999; BL #12188)
578 • *Catⁿ¹* (Mackay and Bewley, 1989; Matthias Landgraf)
579 • *Sod2ⁿ²⁸³* (Duttaroy et al., 2003; BL#34060)
580 • *Pex3²* (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^{FL}::GFP* (3rd, unpublished; Isabel Palacios)
586 • *UAS-Khc⁸²⁻⁷¹¹-GFP* (2nd, BL #9648; constitutively active Khc consisting of base pairs 248-
587 2134 / aa 82-711; fused with EGFP sequence; flybase.org: [FBF0198610](https://flybase.org/FAFB0198610))
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^{GD7467}*; 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) 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⁺-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 H₂O 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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1314

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** Endoplasmatic 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 *Khc⁸* 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 X² 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 (α helices in black, β 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*²⁷ 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*⁸ 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*^{8/Df} and *Klc*^{1ts} at 1DIV following 5d pre-culture; note that *Klc*^{1ts} 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 μ 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 *Ca¹*, 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 μ 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 *pex3²* mutant neurons. Quantification of
1431 phenotypes of wild-type (wt) and *Pex³* 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 μ 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 μ 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 μ 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 μ 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^{mutA}* 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^{mutA}* and
1513 *Pat1^{robin}* fail to suppress *Klc^{8ex94}*-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.

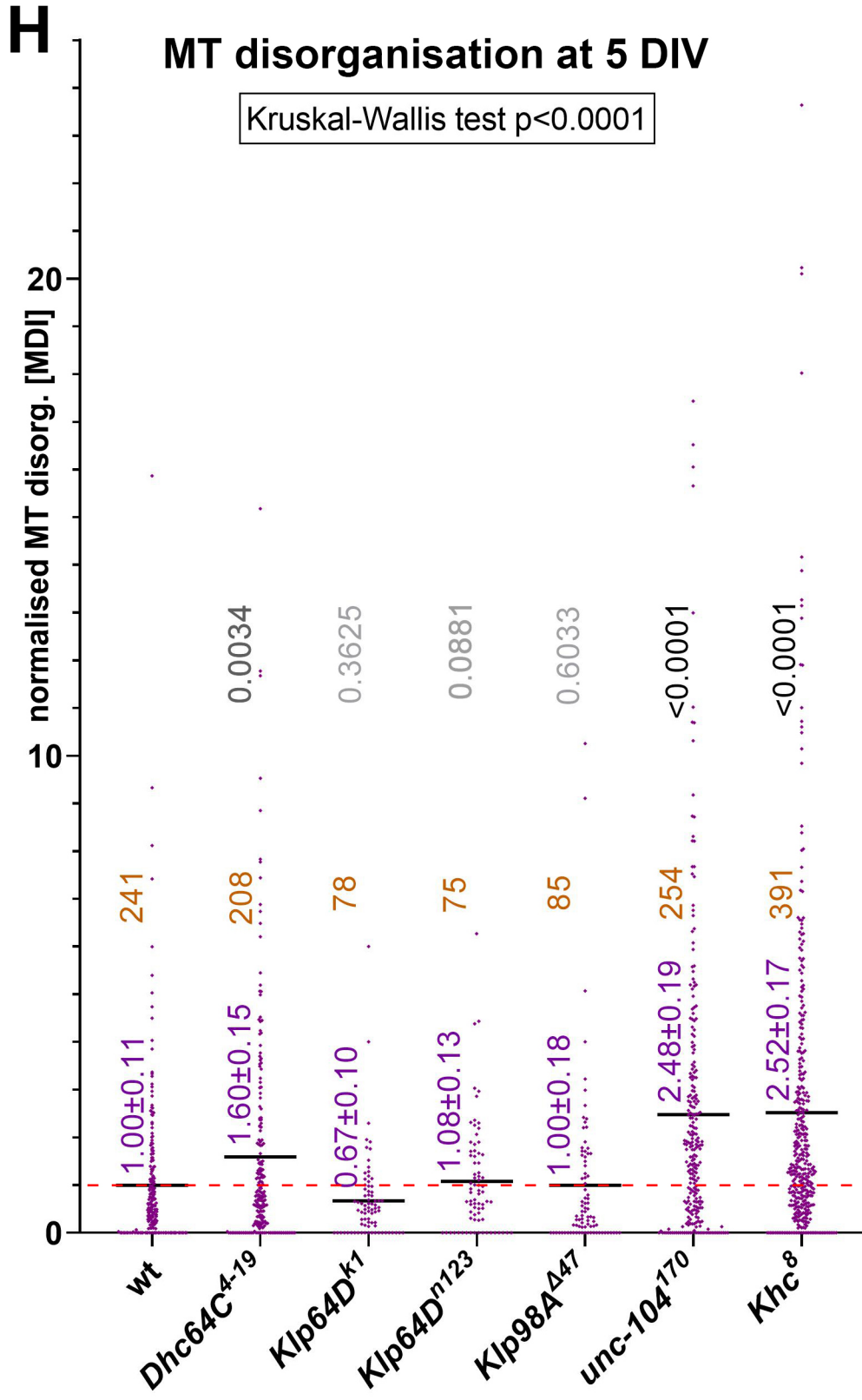
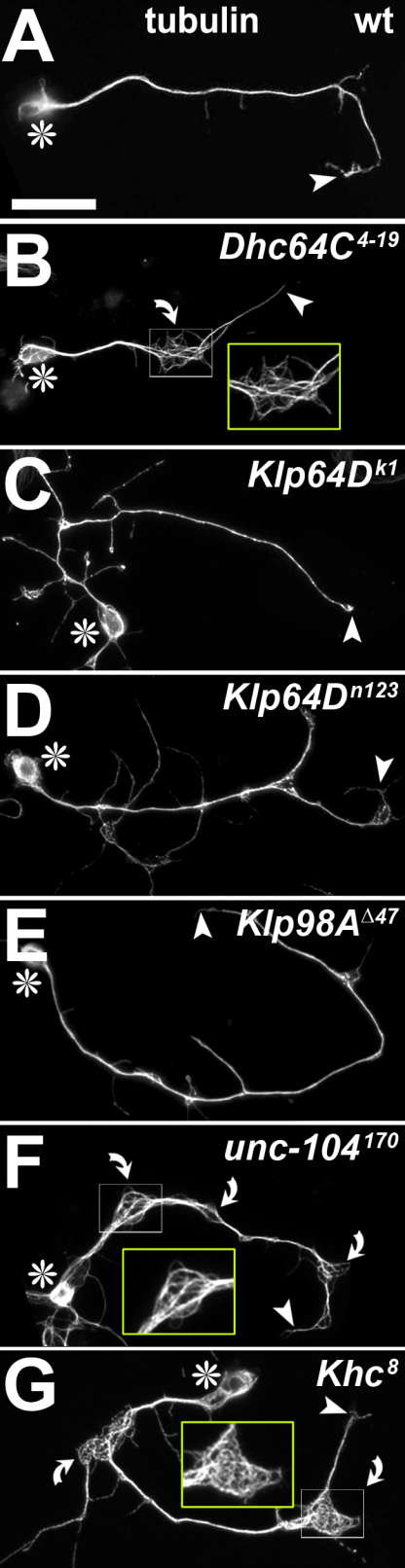


Fig. 1 Liew et al.

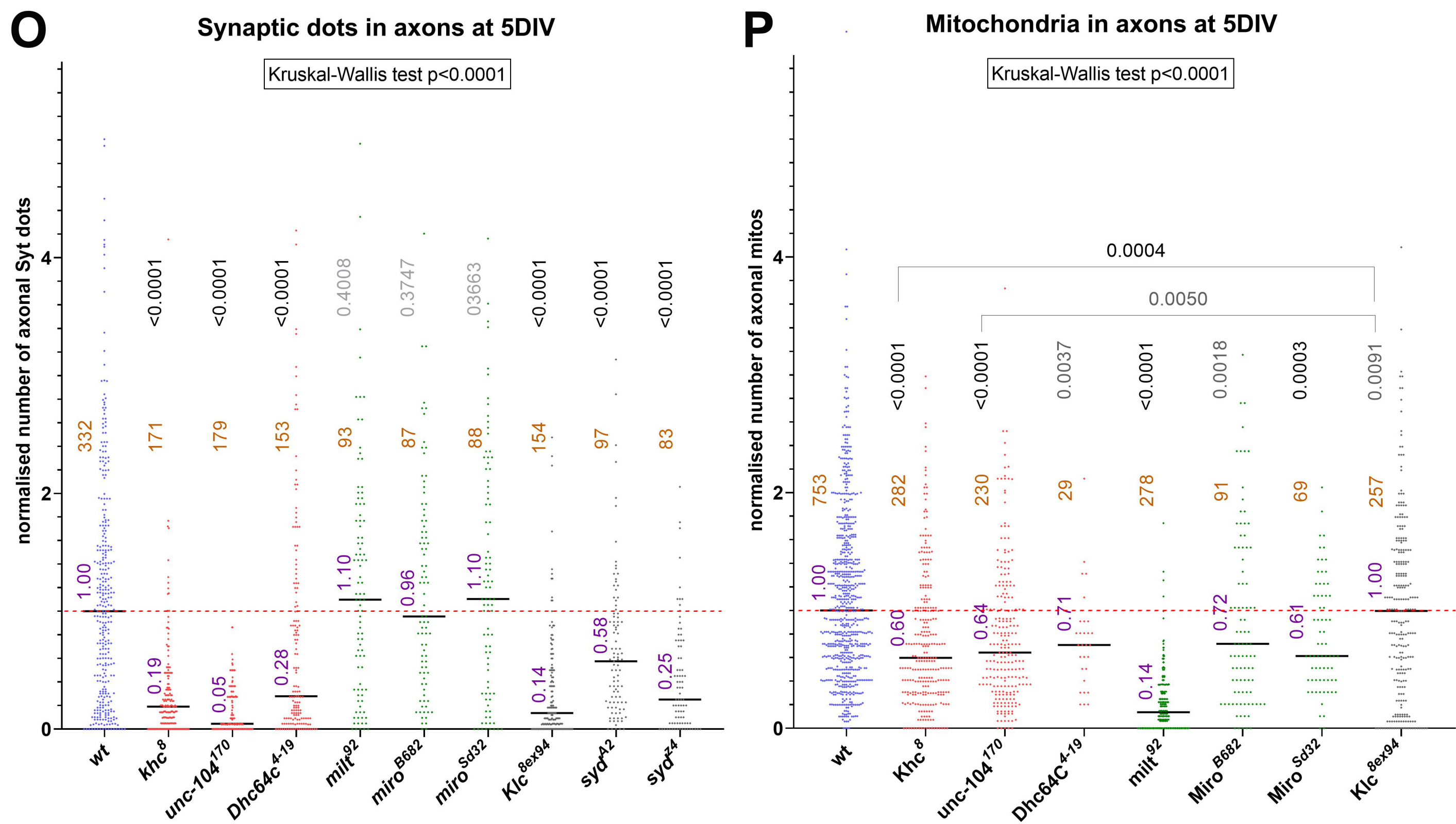
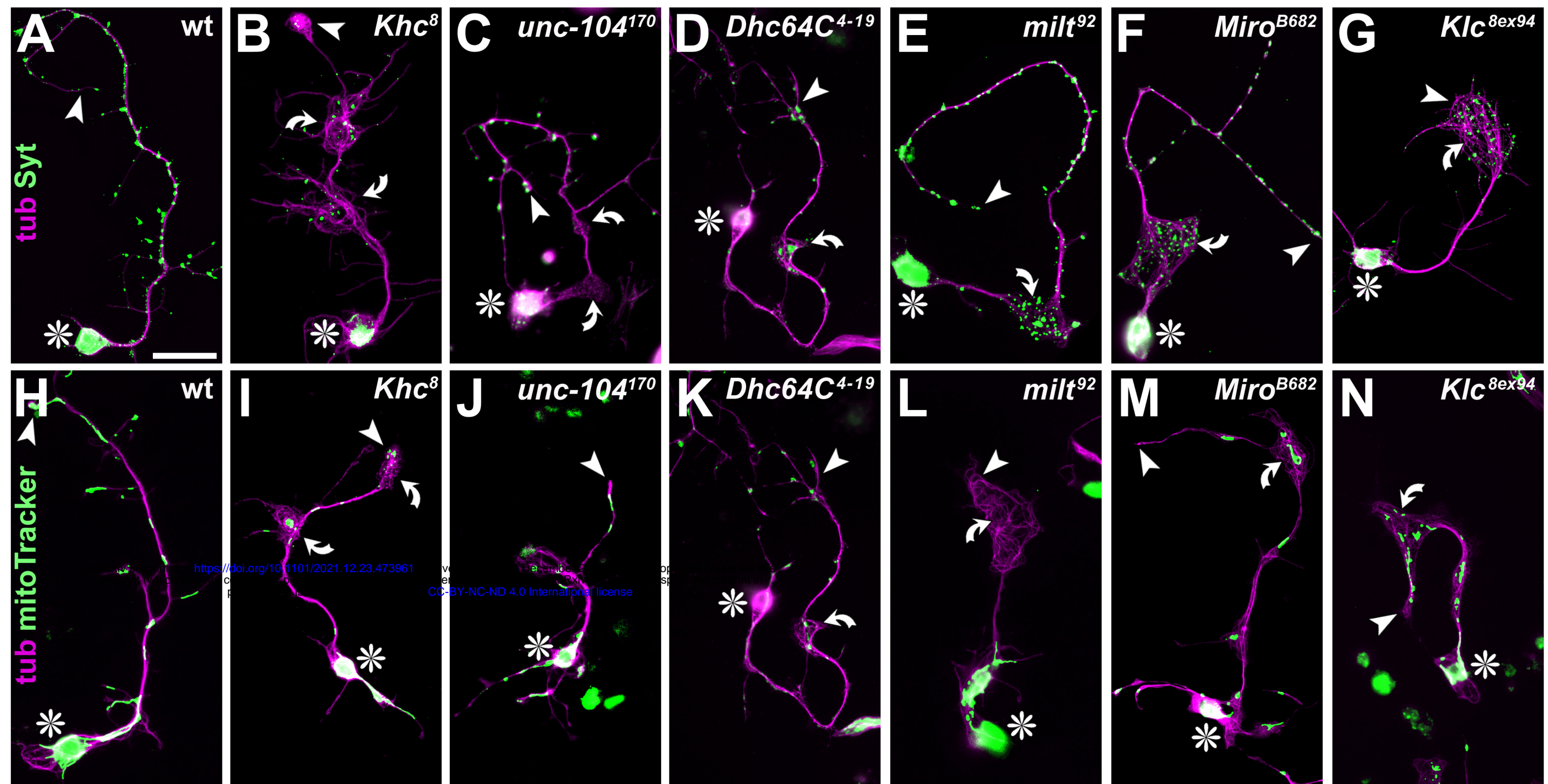


Fig. 2 Liew et al.

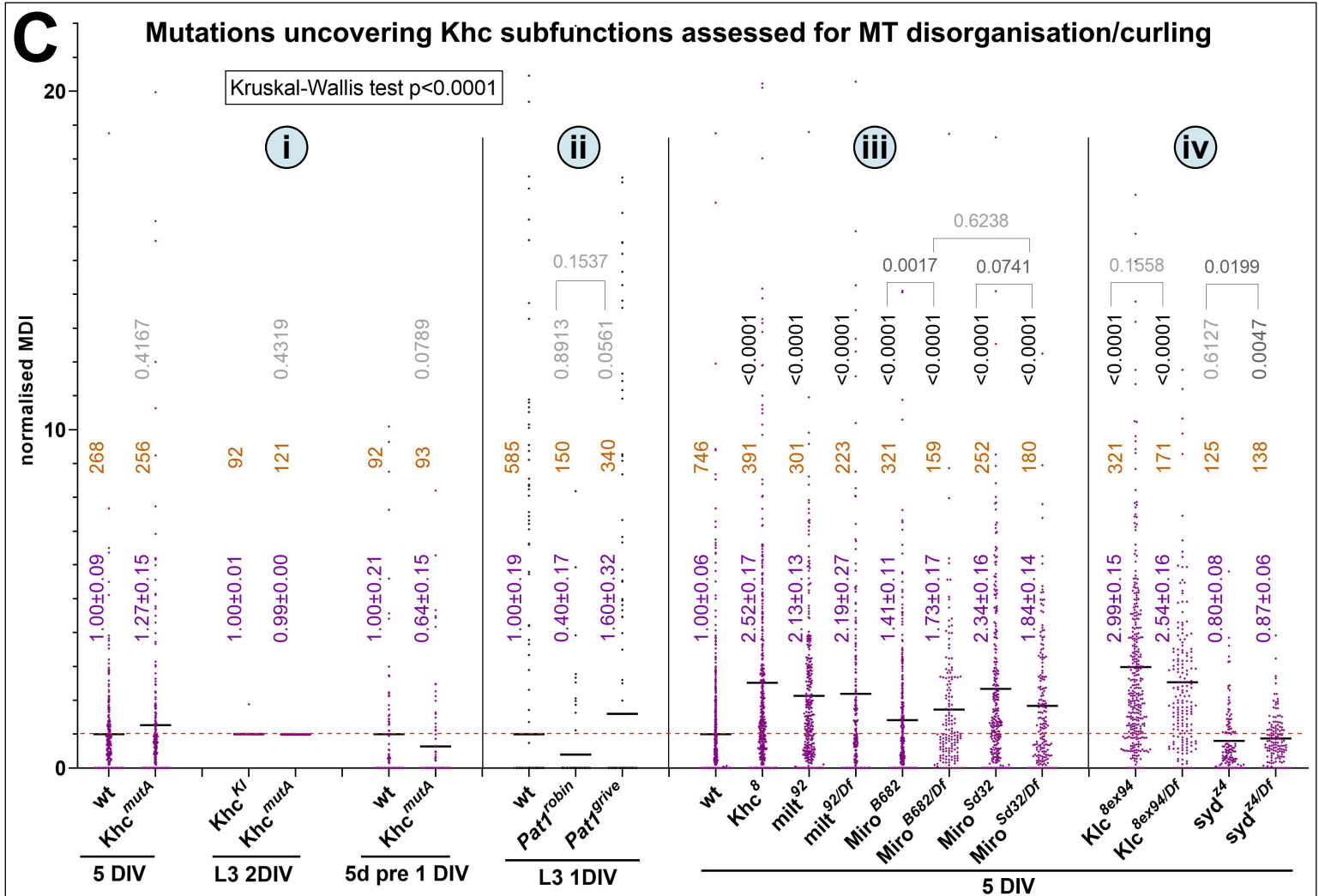
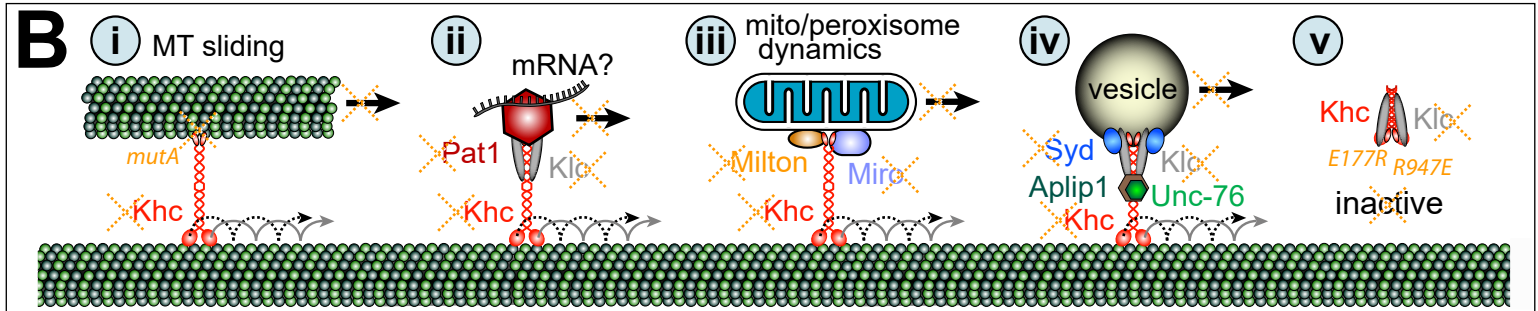
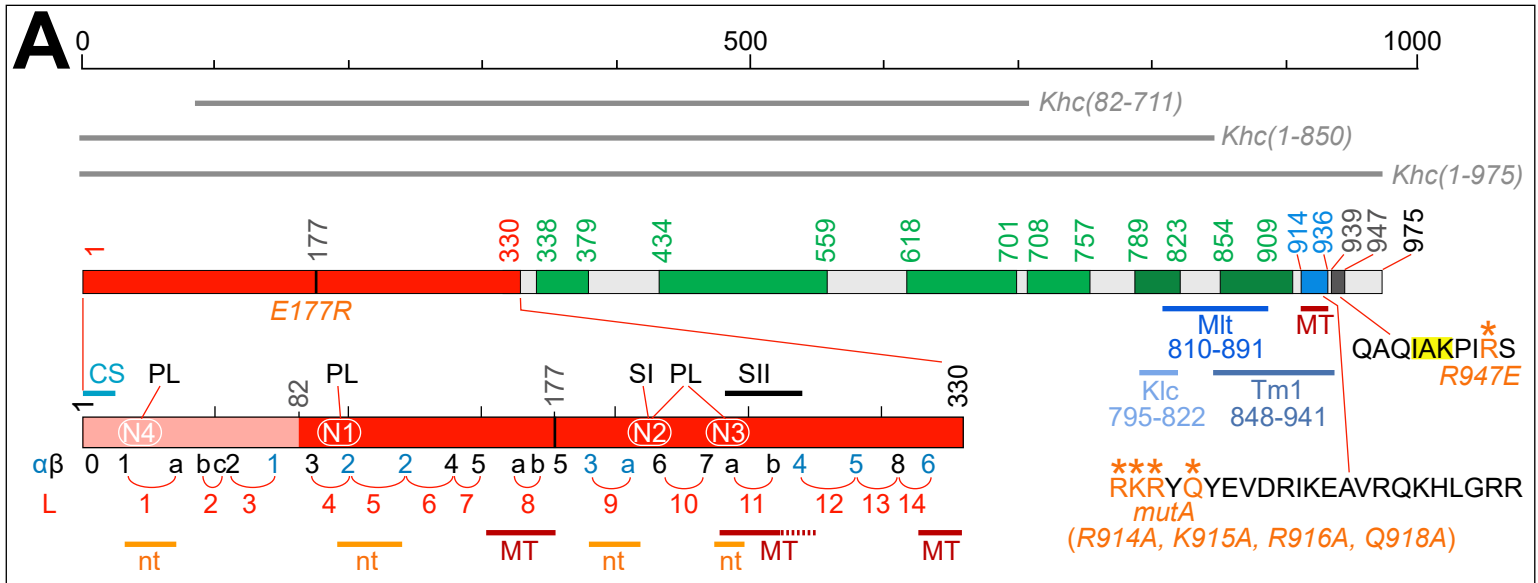


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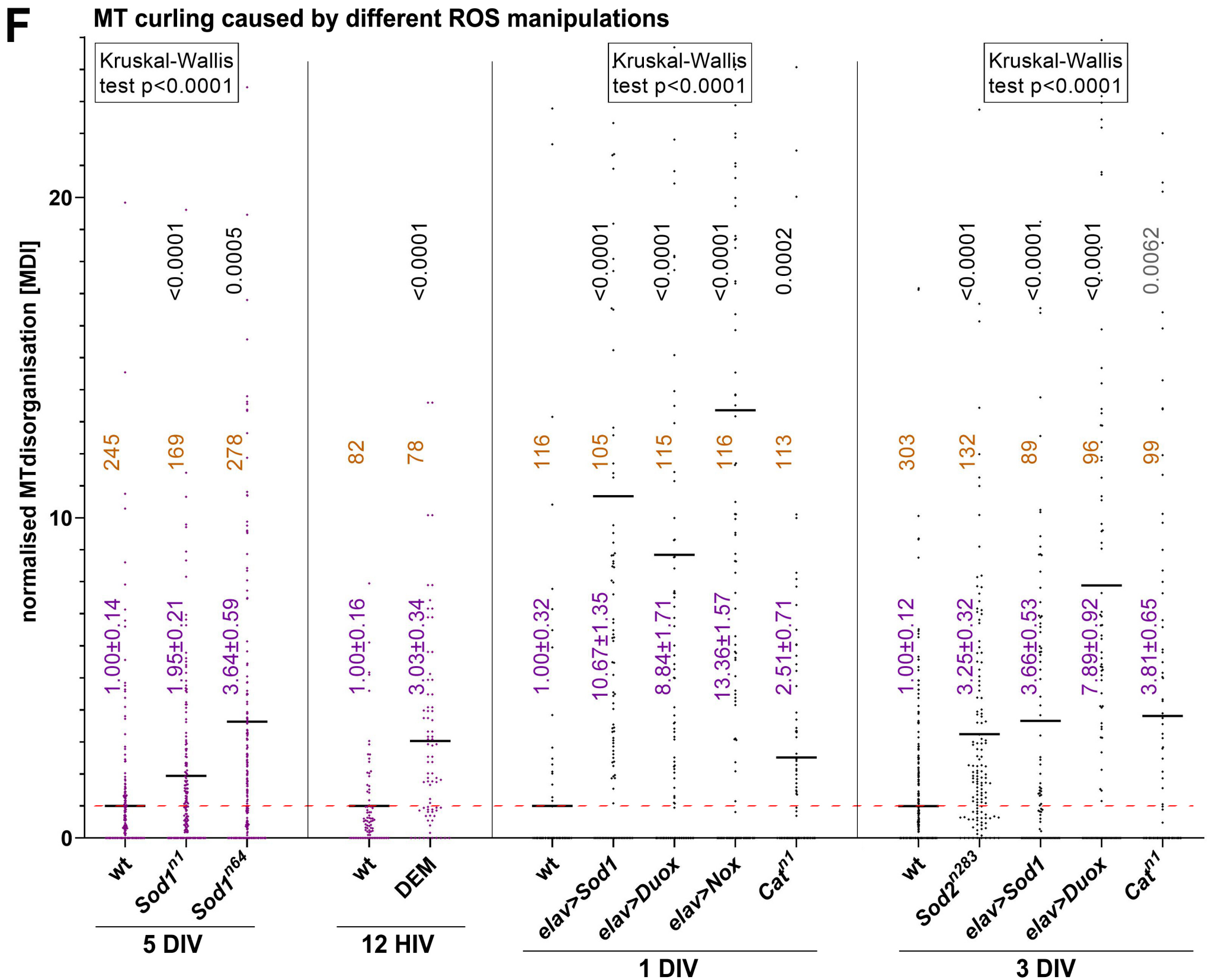
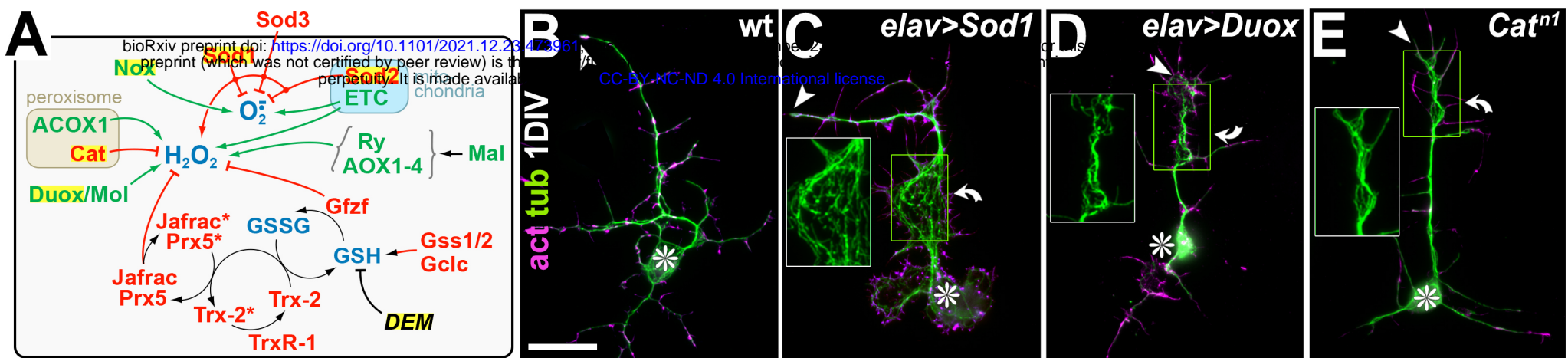


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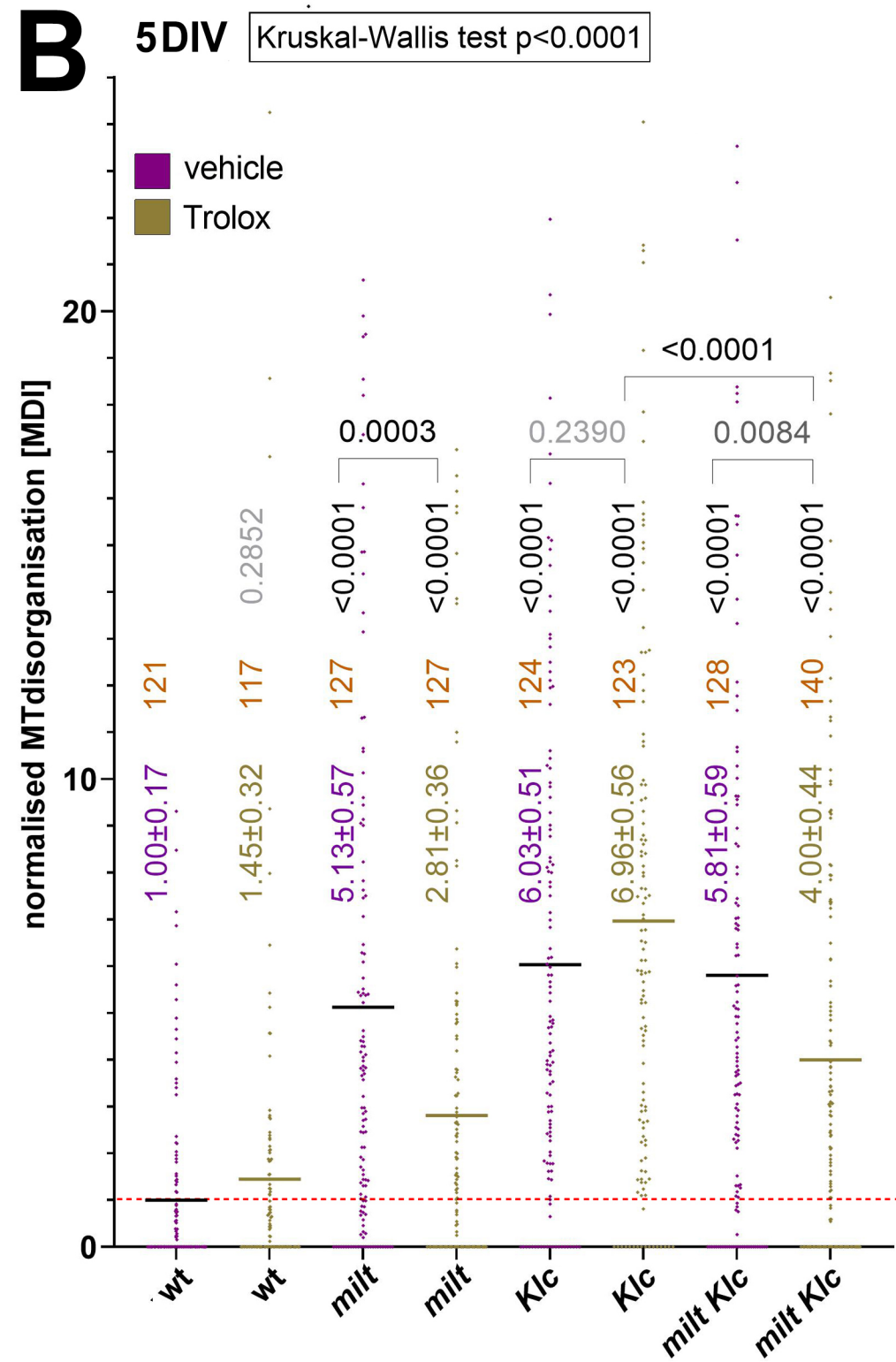
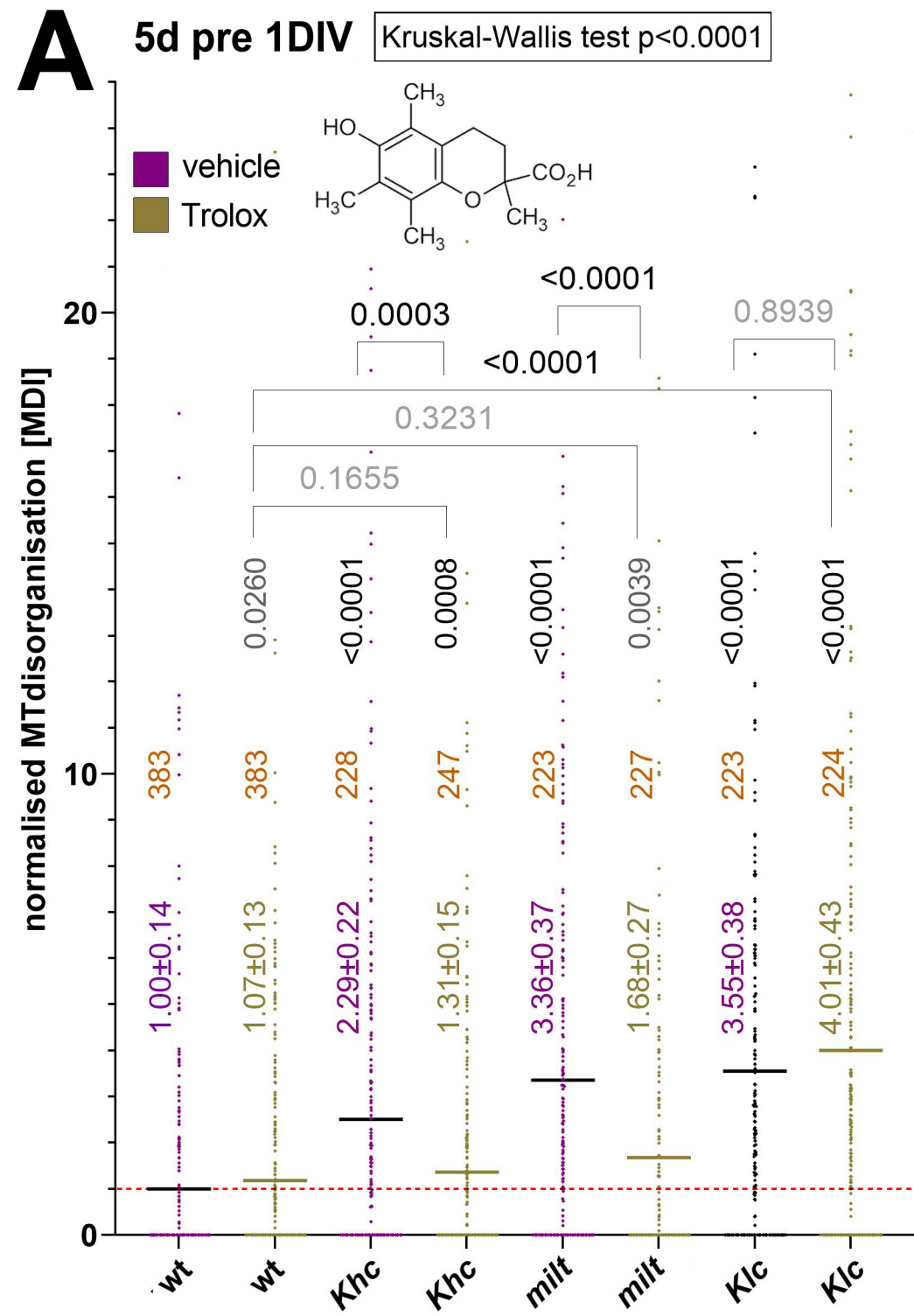


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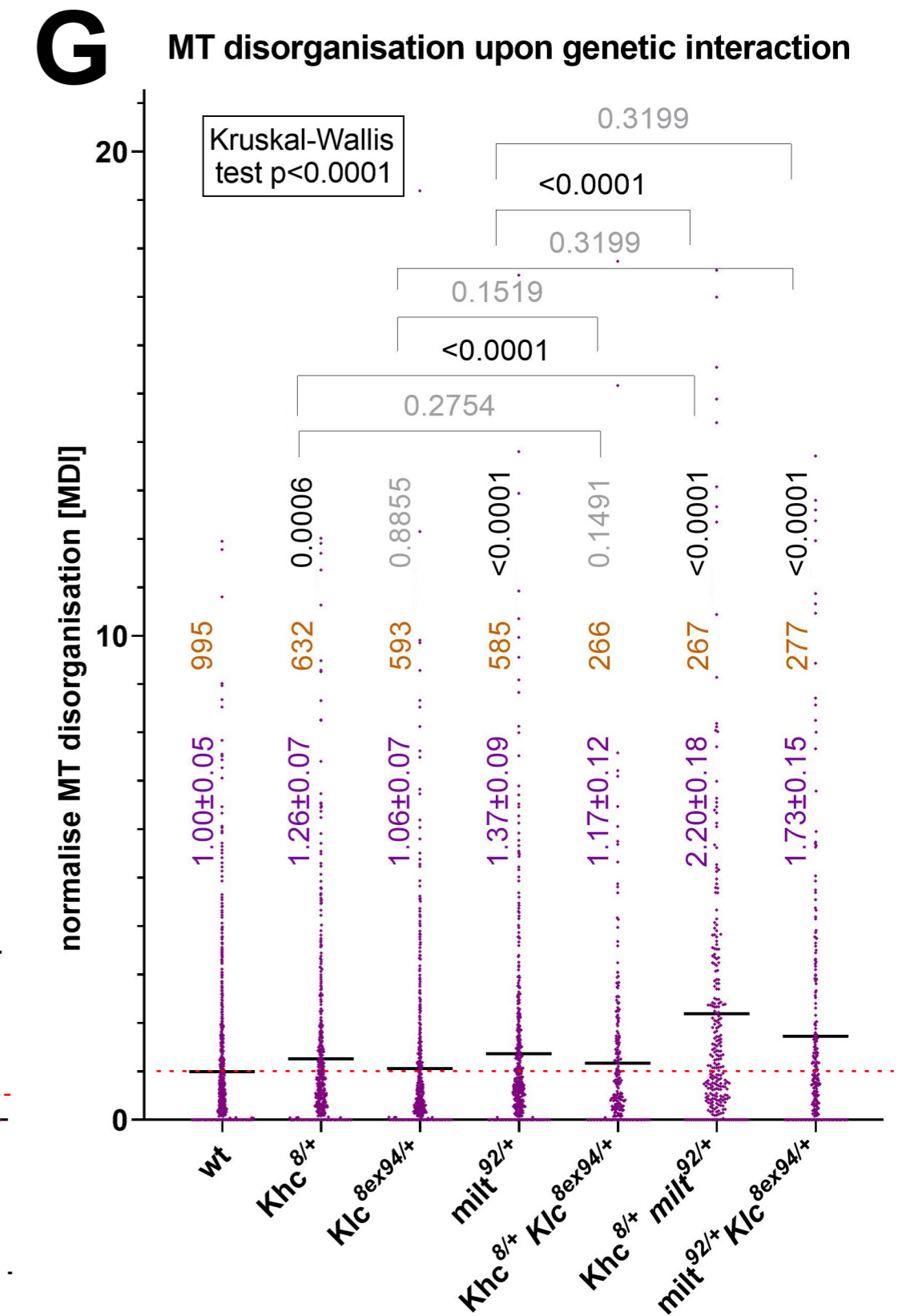
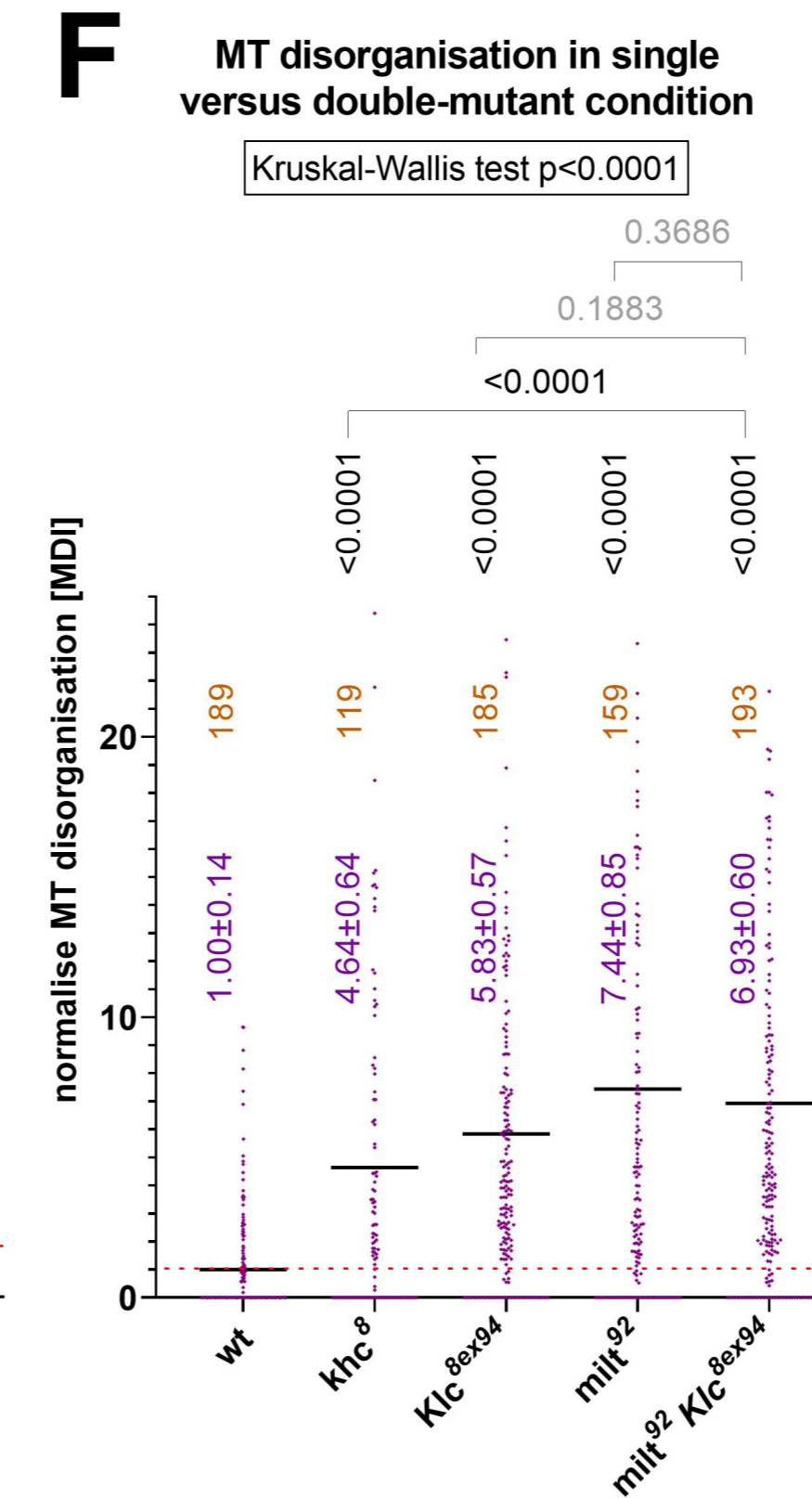
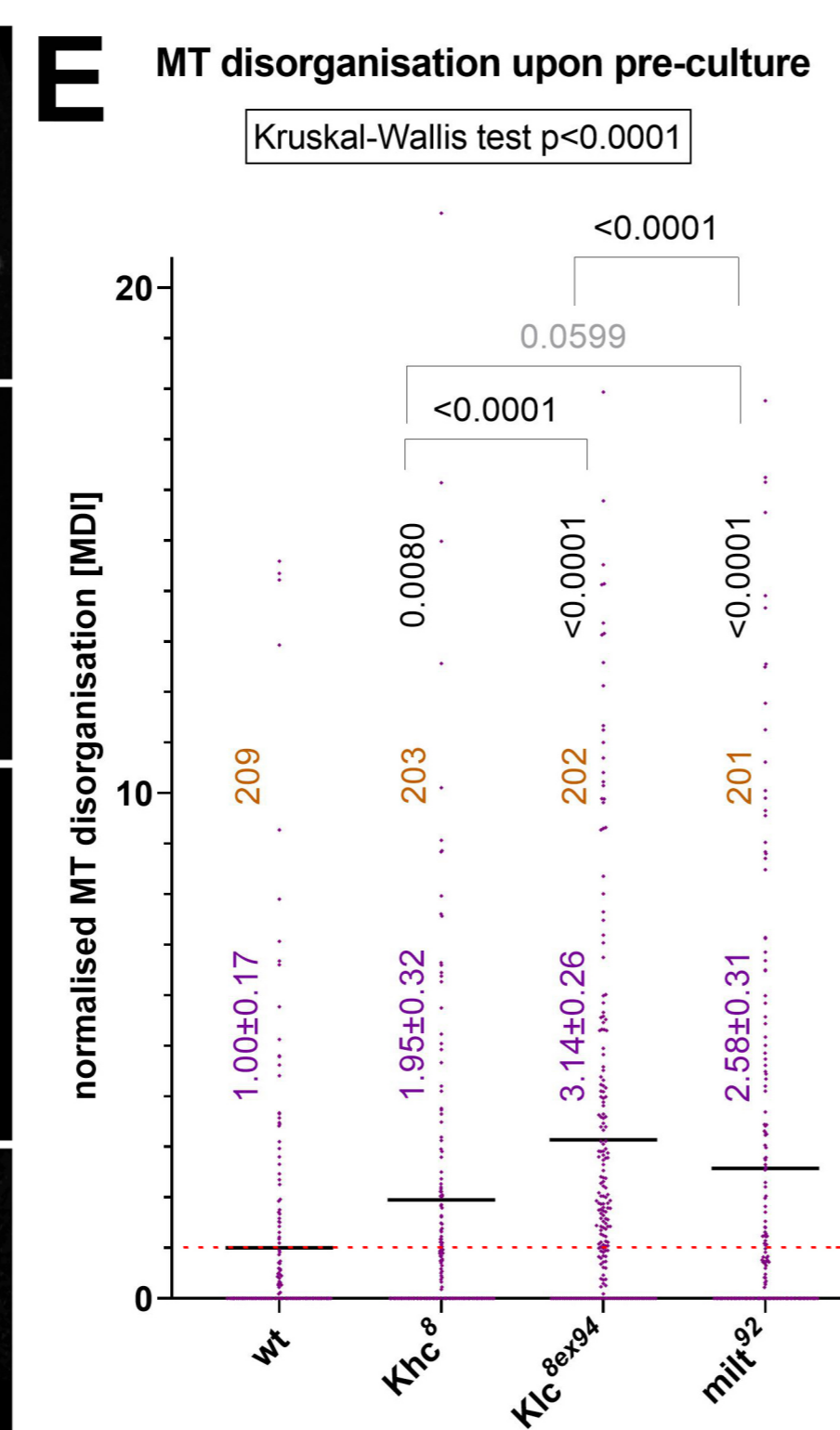
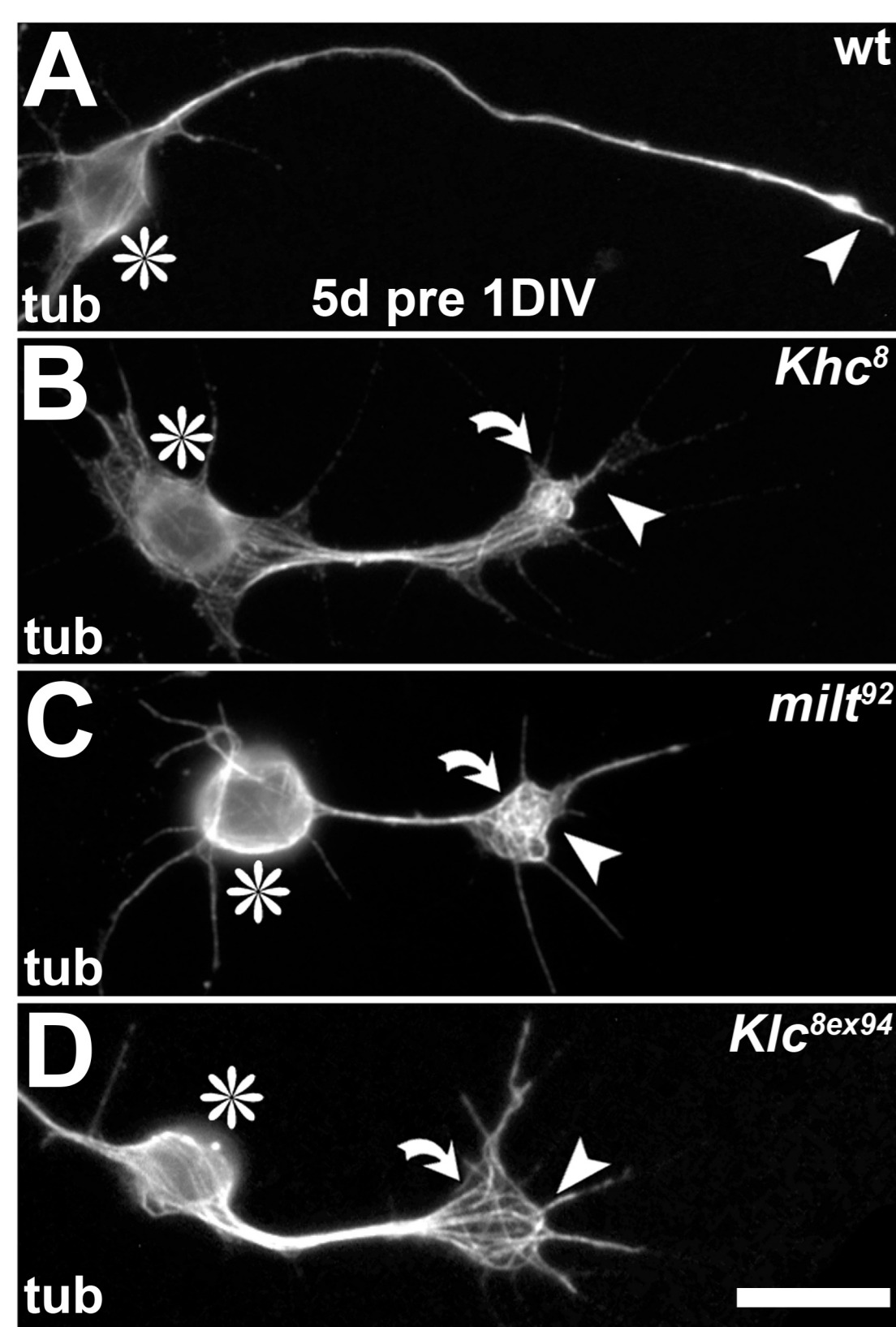


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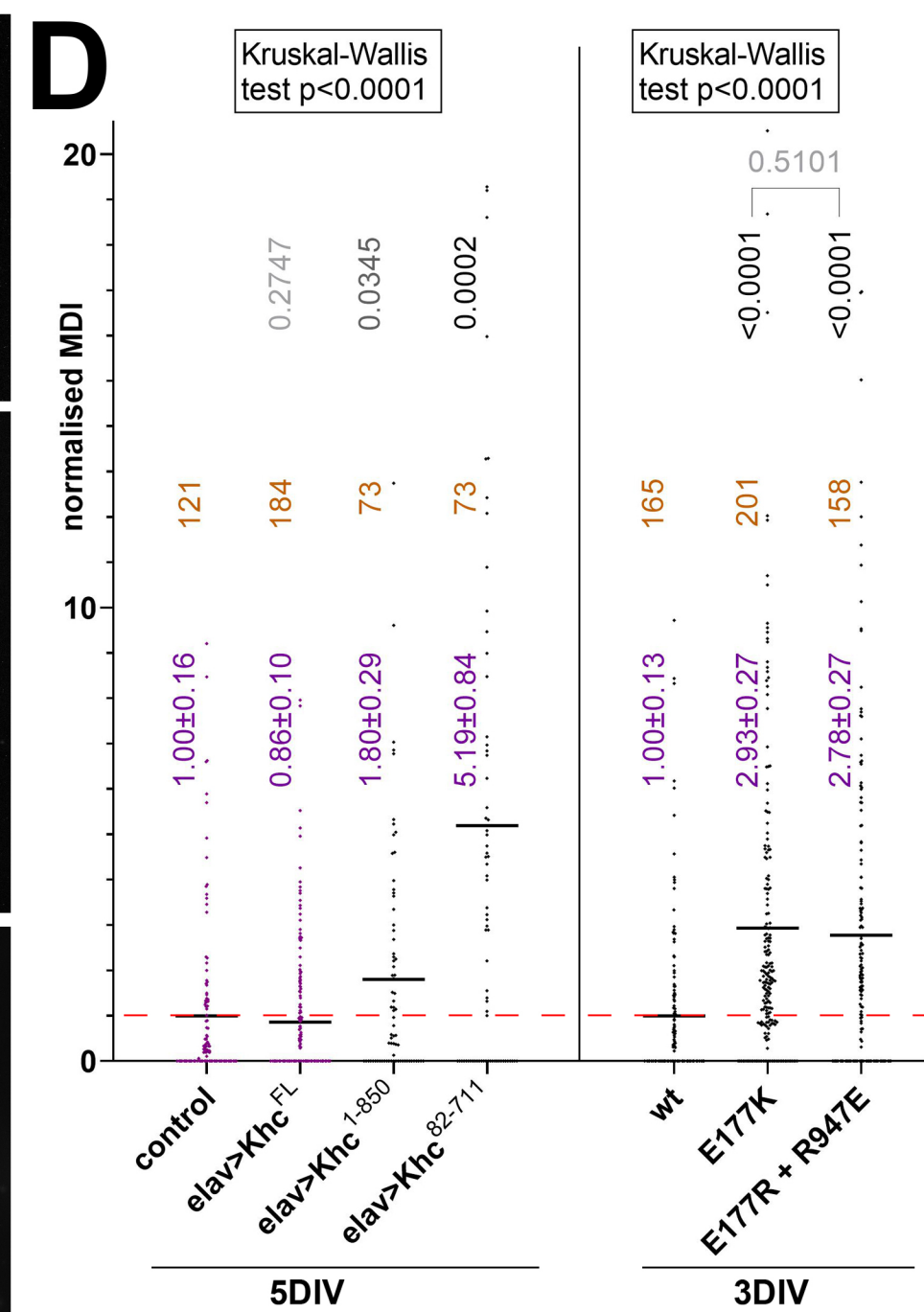
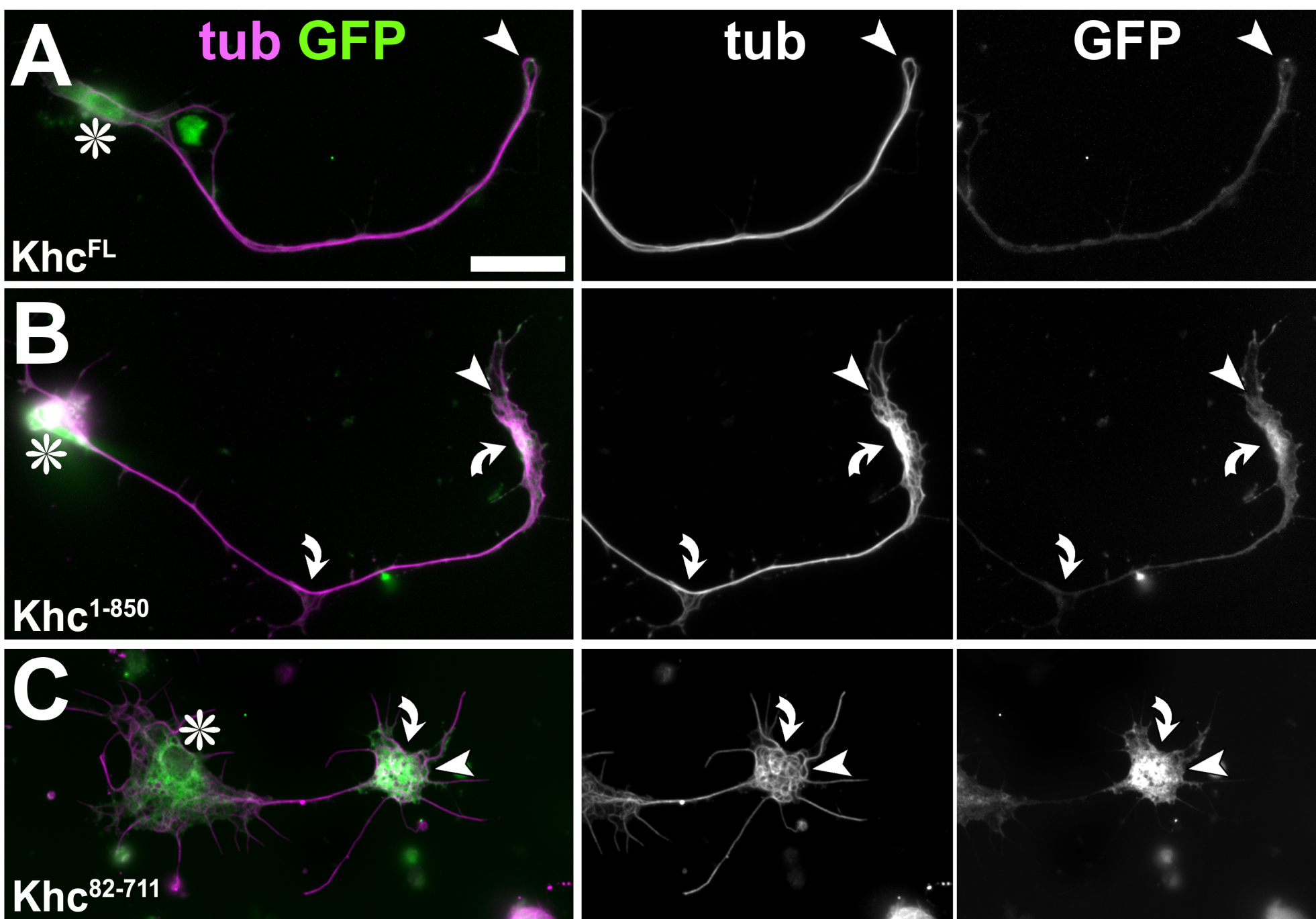


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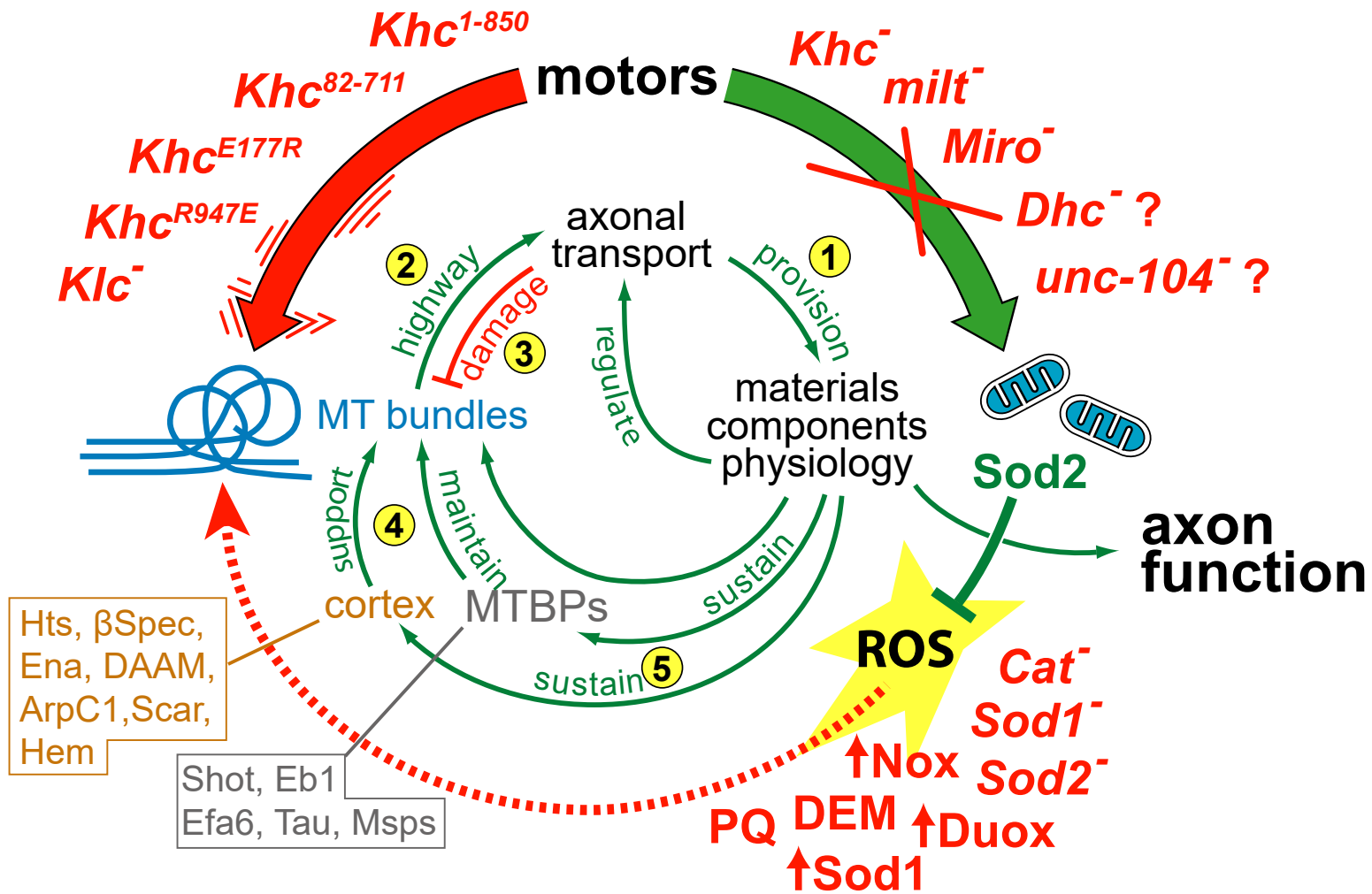


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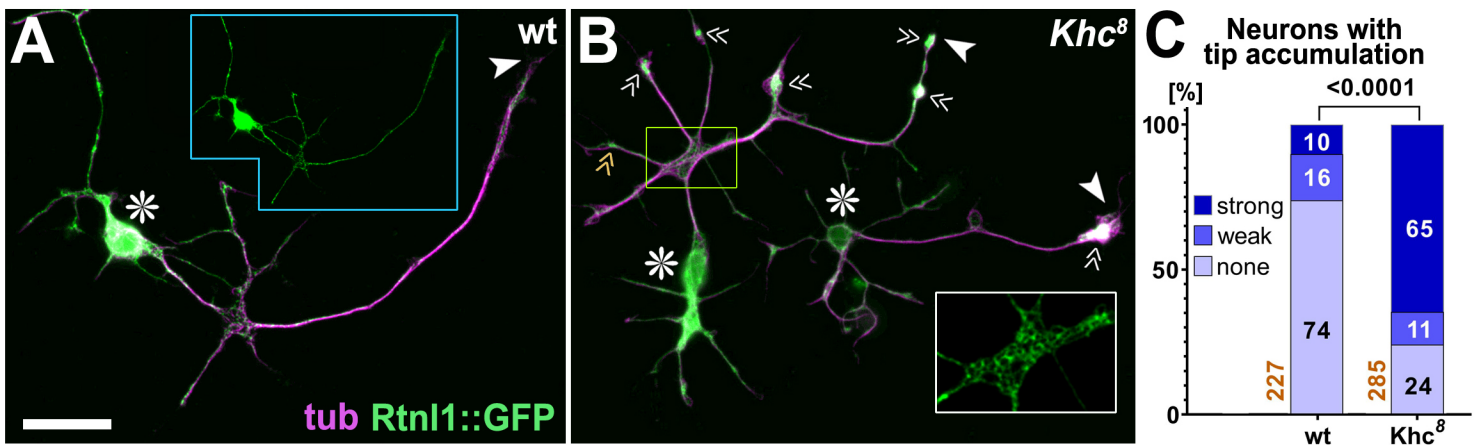


Fig. S1 Liew et al.

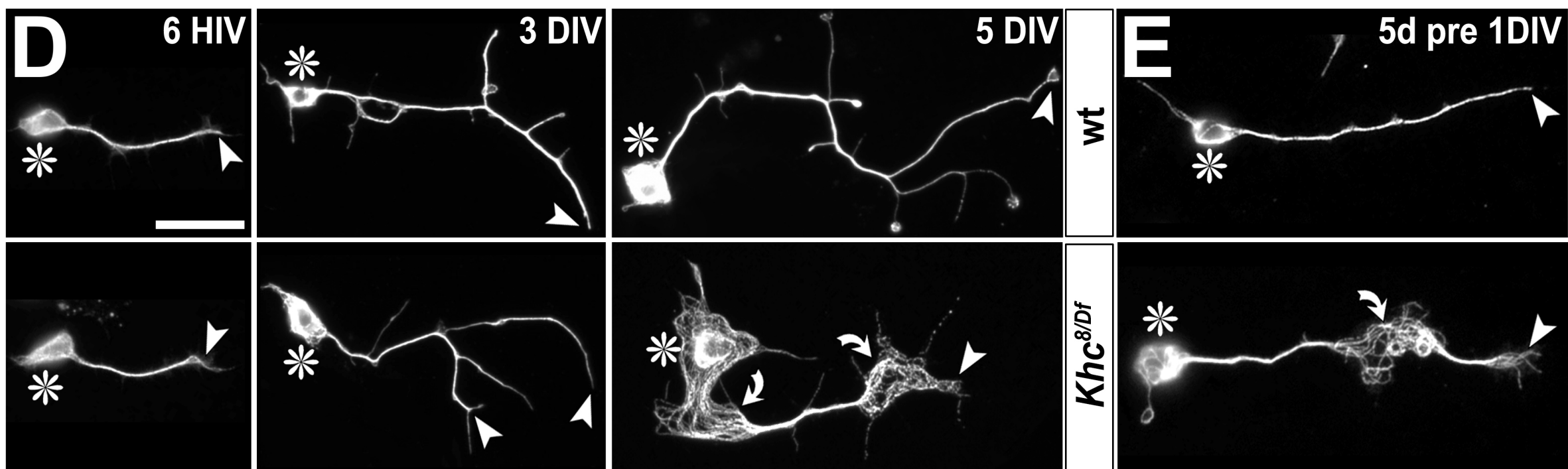
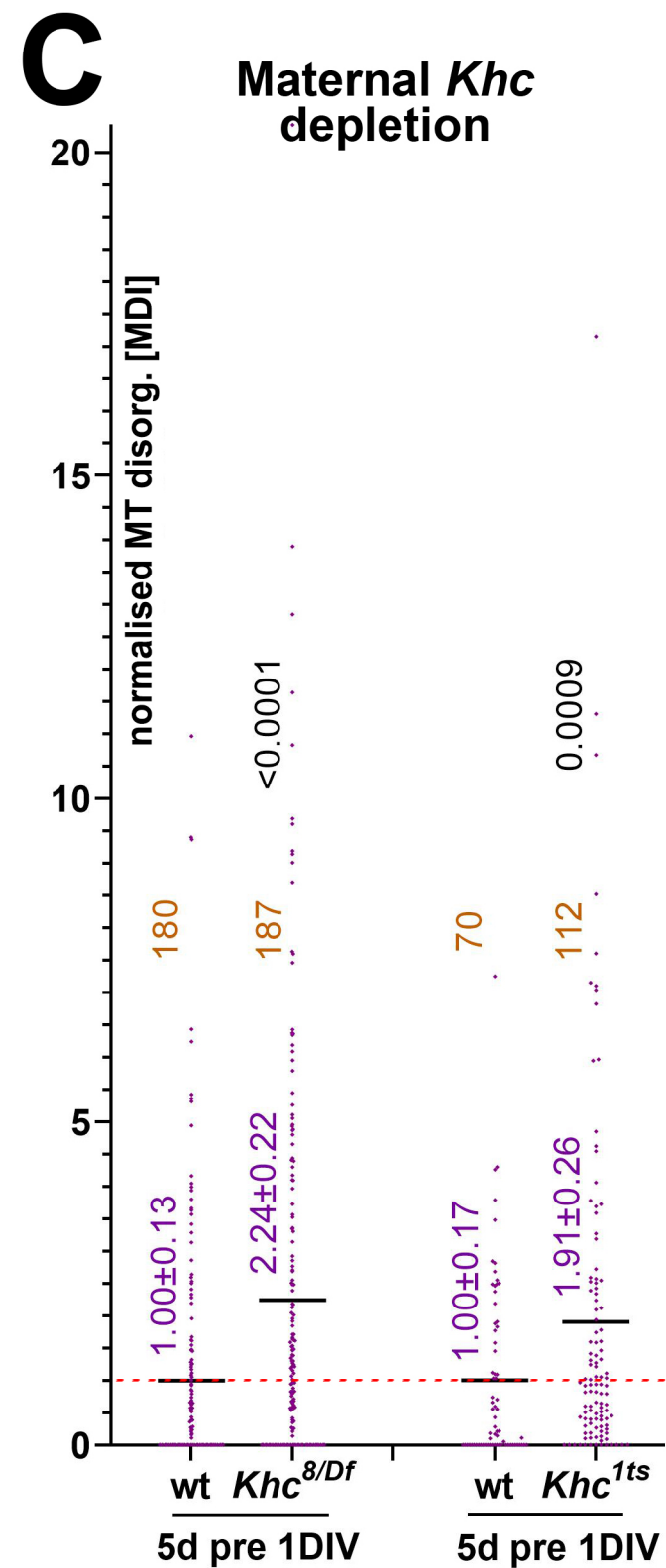
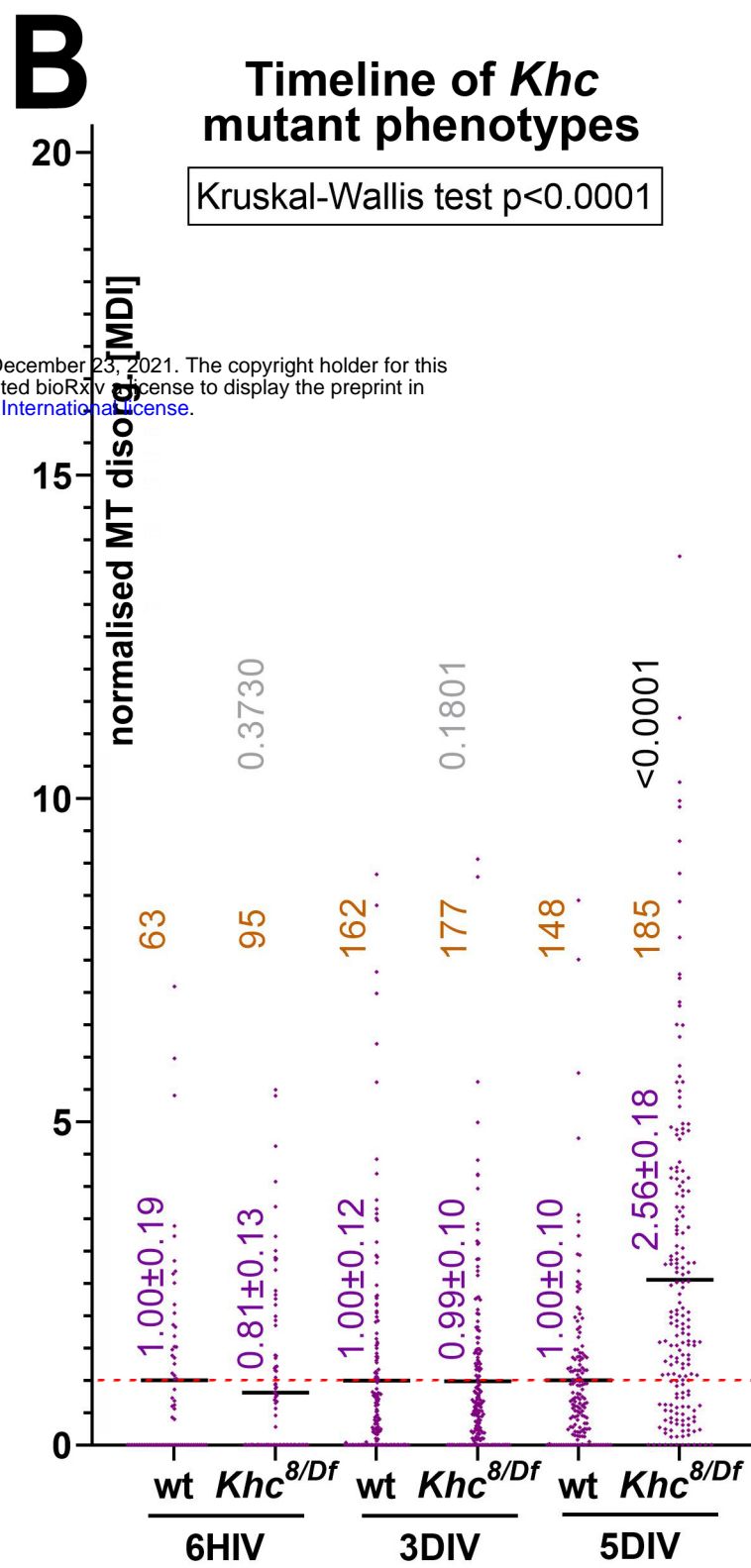
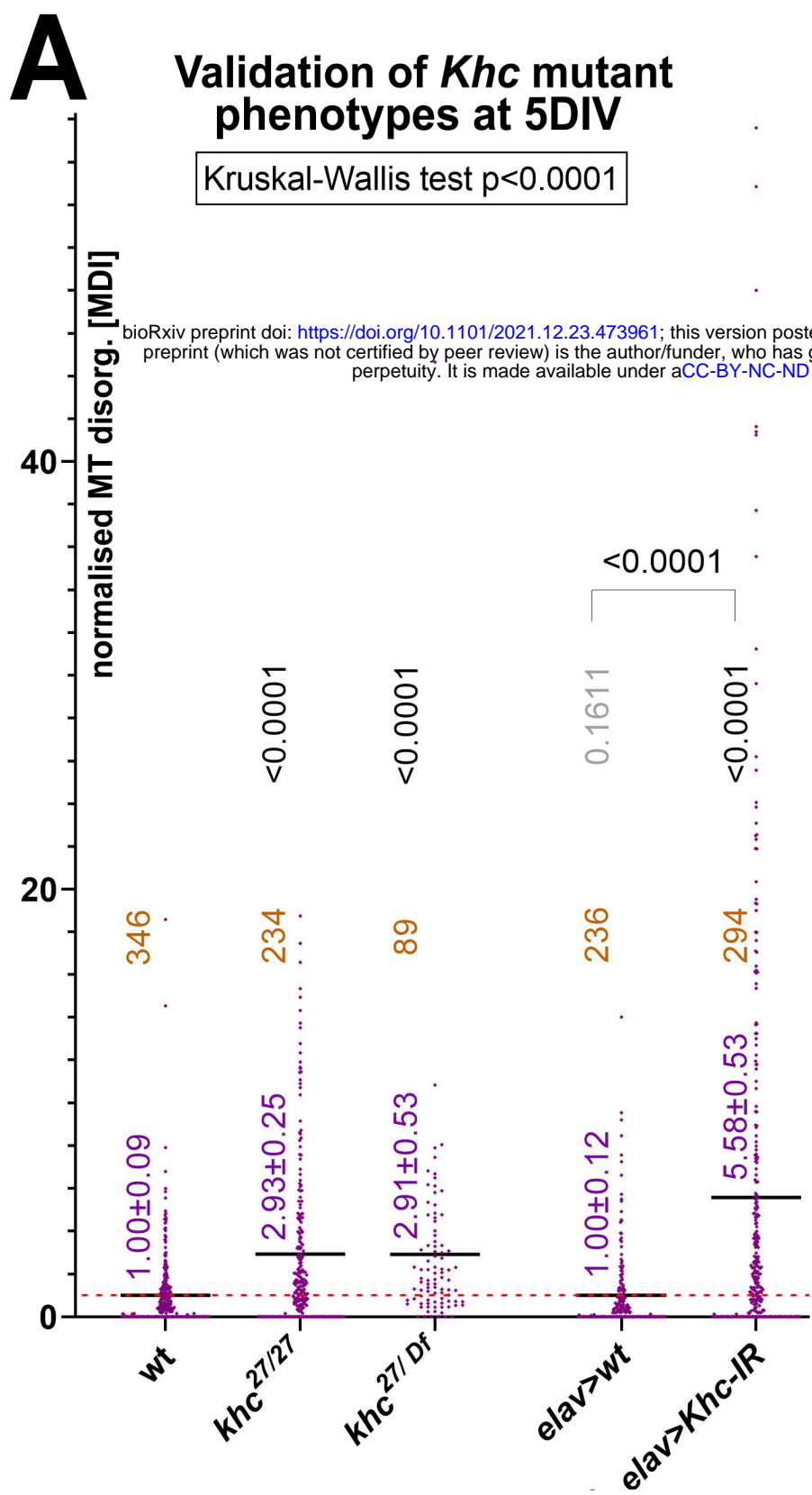


Fig. S2 Liew et al.

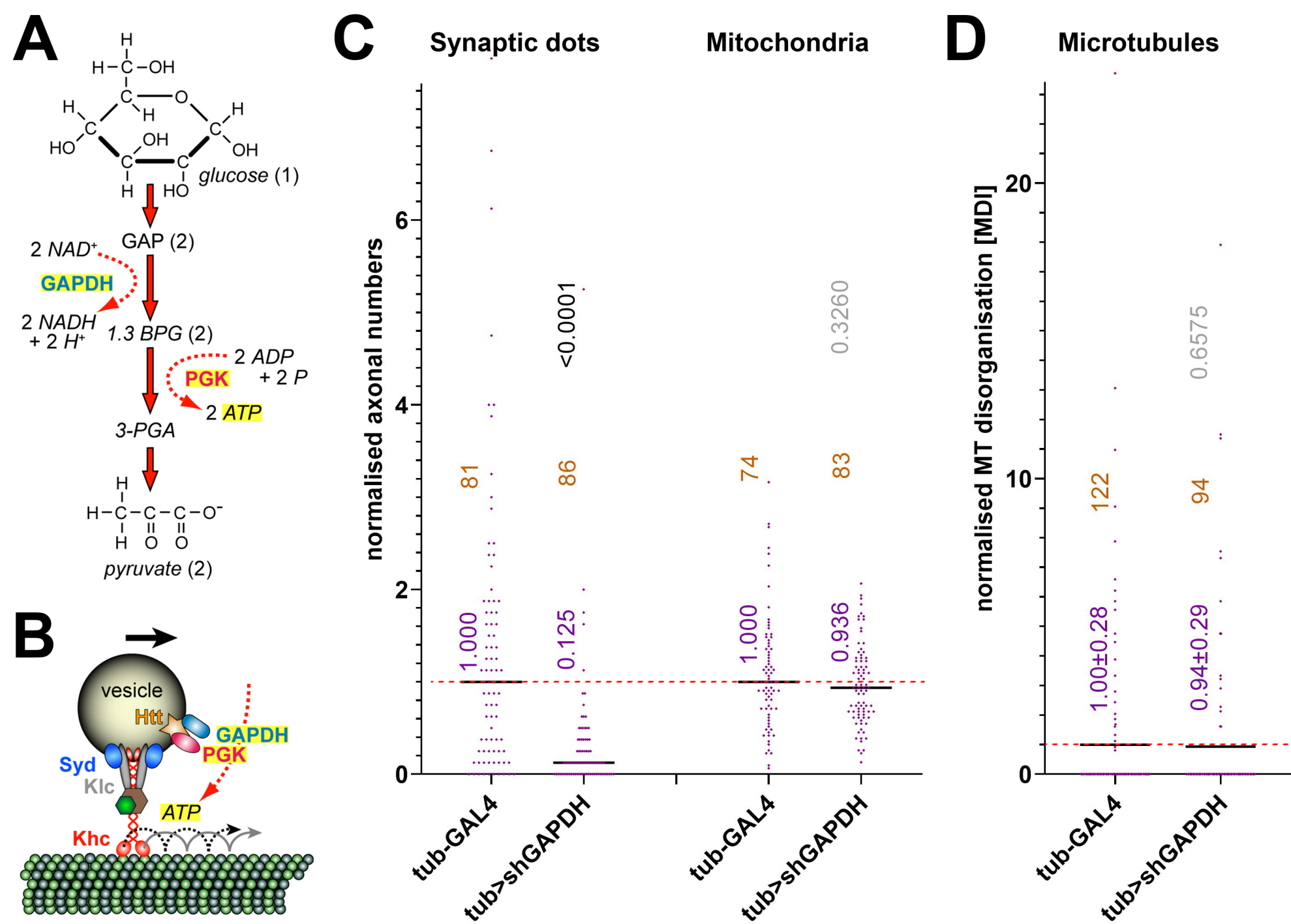


Fig. S3 Liew et al.

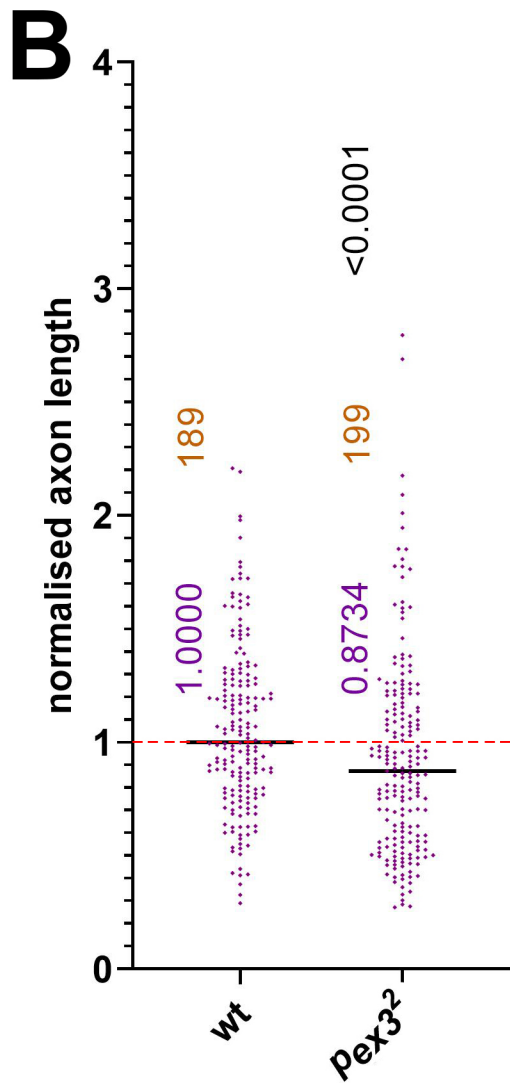
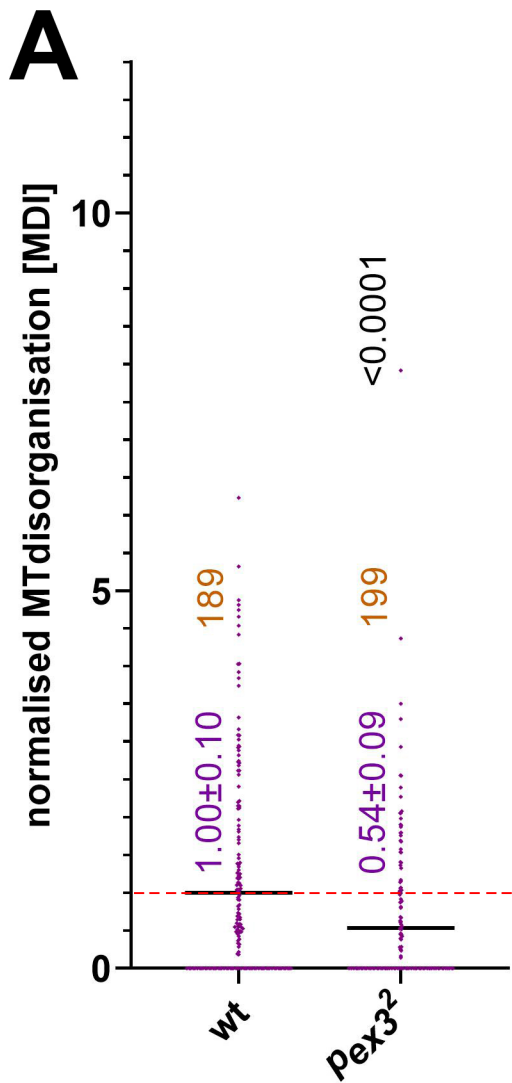
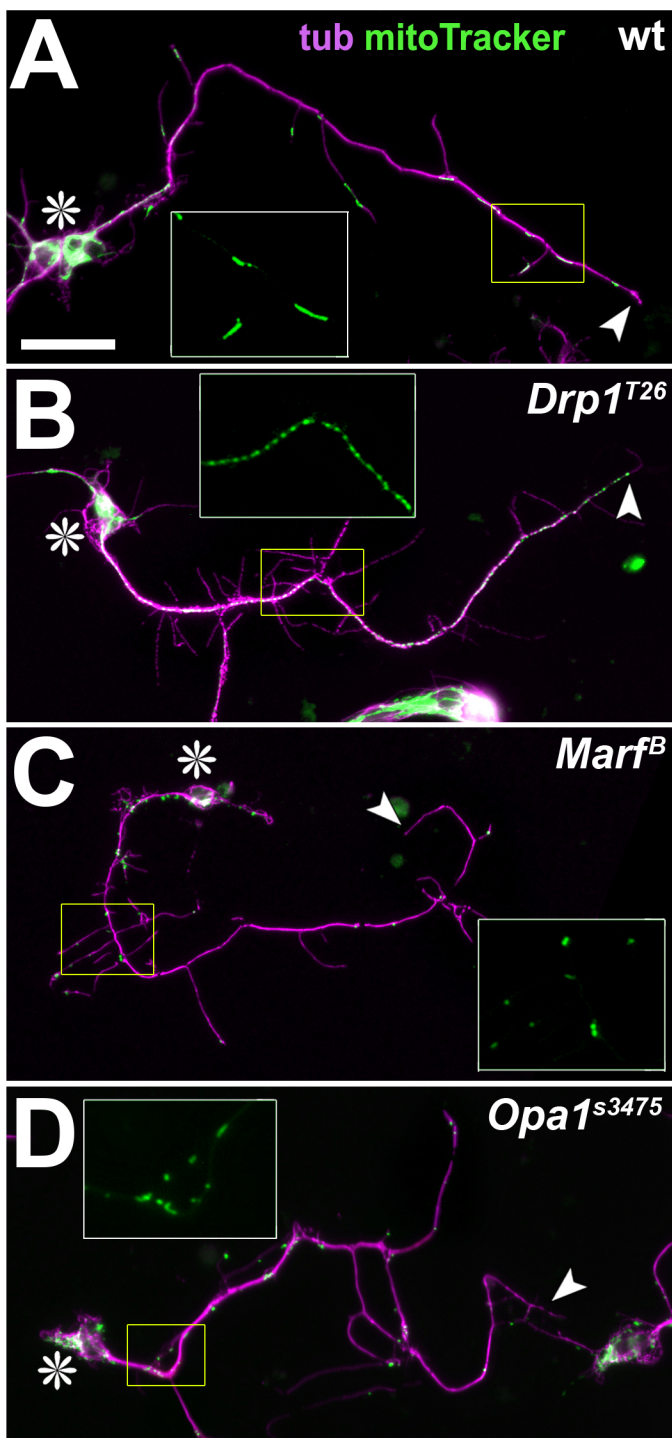
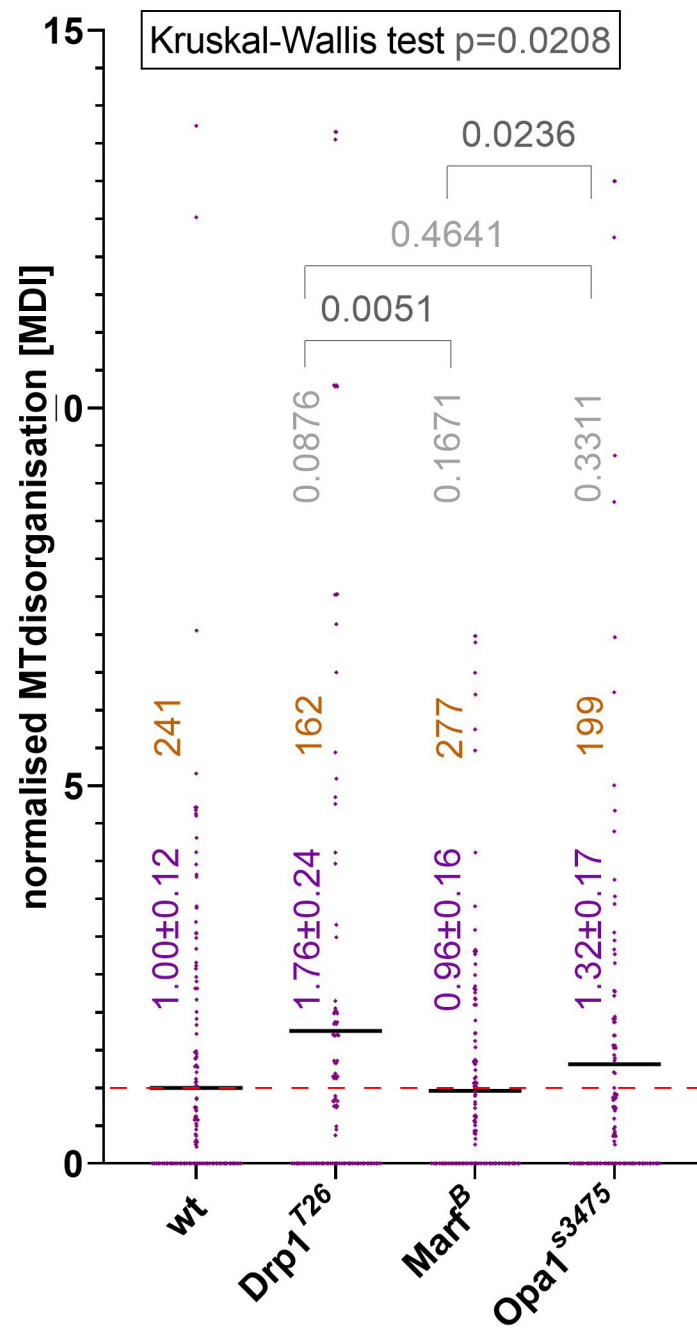


Fig. S4 Liew et al.



E Fission/fusion mutants at 5DIV



F Fission/fusion mutants 5d pre 1DIV

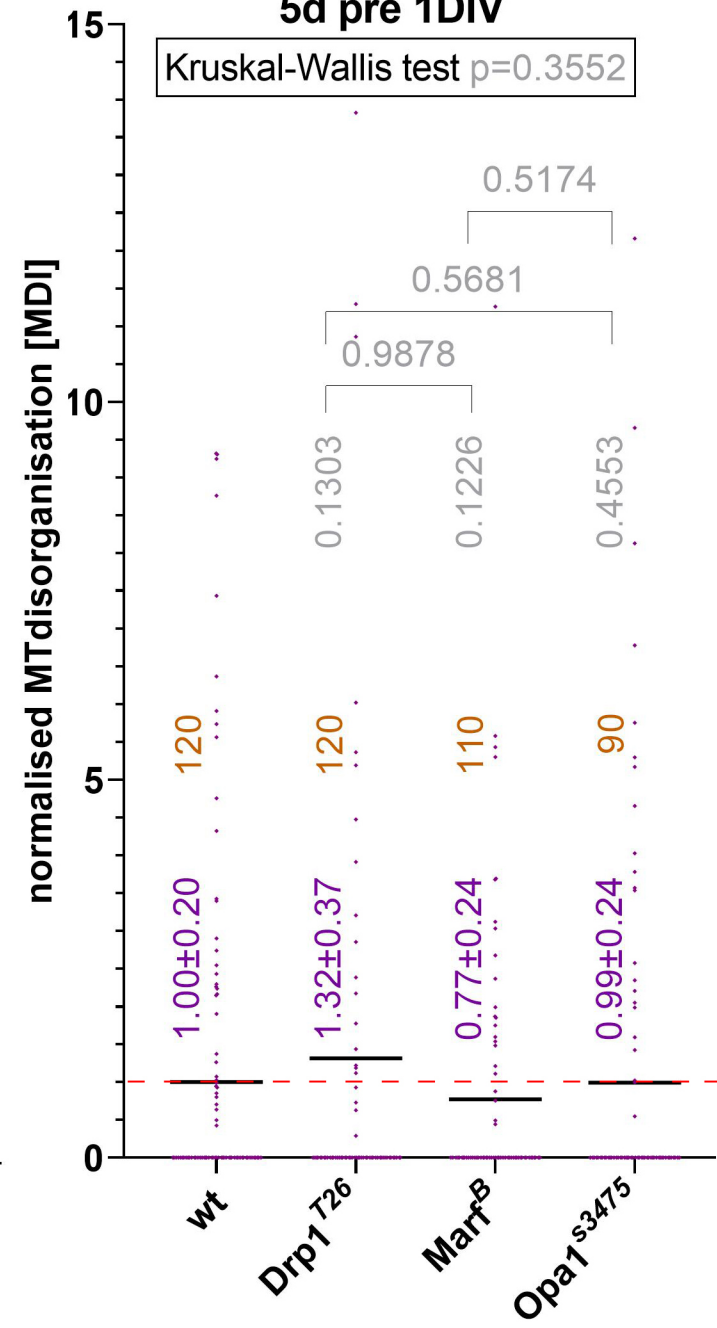


Fig. S5 Liew et al.

Double-mutant constellations with *Klc*^{8ex94}

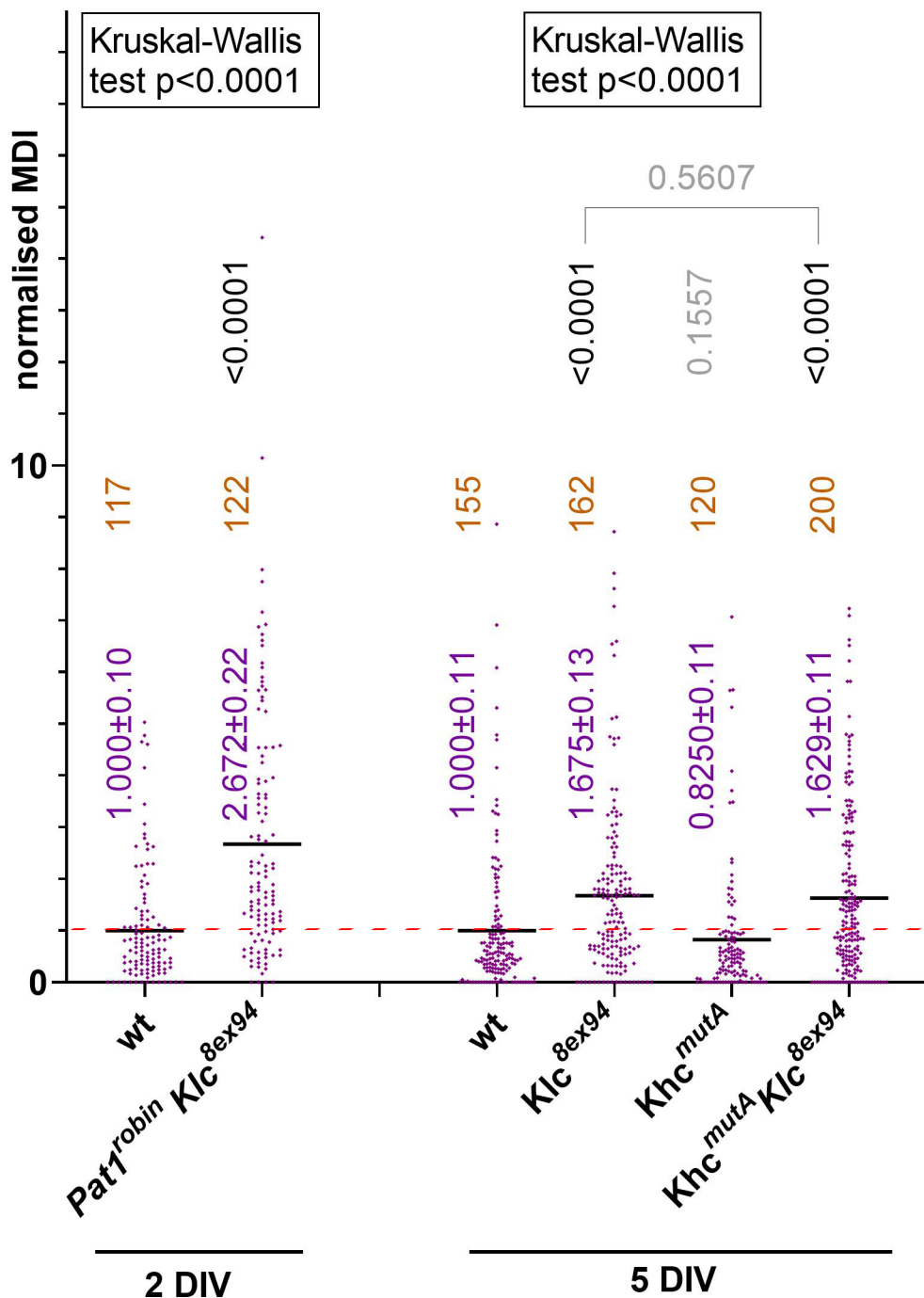


Fig. S6 Liew et al.