Axonal transport of autophagosomes is regulated by dynein activators JIP3/JIP4 and ARF/RAB GTPases

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1   **Abstract**

2   Neuronal autophagosomes, "self-eating" degradative organelles, form at presynaptic sites in the distal

3   axon and are transported to the soma to recycle their cargo. During transit, autophagic vacuoles (AVs)

4   mature through fusion with lysosomes to acquire the enzymes necessary to breakdown their cargo. AV

5   transport is driven primarily by the microtubule motor cytoplasmic dynein in concert with dynactin and a

6   series of activating adaptors that change depending on organelle maturation state. The transport of

7   mature AVs is regulated by the scaffolding proteins JIP3 and JIP4, both of which activate dynein motility

8   in vitro. AV transport is also regulated by ARF6 in a GTP-dependent fashion. While GTP-bound ARF6

9   promotes the formation of the JIP3/4-dynein-dynactin complex, RAB10 competes with the activity of this

10 complex by increasing kinesin recruitment to axonal AVs and lysosomes. These interactions highlight the

11 complex coordination of motors regulating organelle transport in neurons.

12

13 **Summary**

14 Mature autophagosomes in the axon are transported by the microtubule motor dynein, activated by JNK-

15 interacting proteins 3 and 4 (JIP3/4). This motility is regulated by the small GTPases ARF6 and RAB10.

16 The tight regulation of autolysosomal transport is essential for intracellular recycling to maintain neuronal

17 homeostasis.

Introduction

Maintaining neuronal homeostasis across the lifespan requires the continuous turnover of dysfunctional or aged proteins and organelles (Eskelinen, 2019; Kulkarni et al., 2018). Autophagy is a process by which these components can be broken down and recycled (Stavoe and Holzbaur, 2019). Autophagic vacuoles (AVs), the "self-eating" organelle, engulf cargo proteins or organelles in a double-membrane and then fuse with late endosomes and lysosomes (collectively, endolysosomes), which provide the degradative enzymes necessary to breakdown the cargo (Yim and Mizushima, 2020). In neurons, AVs form preferentially at presynaptic sites and at the distal tip of the axon and must be actively transported to the soma, where the majority of protein and organelle biogenesis occurs (Maday et al., 2012; Maday and Holzbaur, 2014; Stavoe et al., 2016; Koltun et al., 2020; Farfel-Becker et al., 2019). The transport of AVs along the axon is primarily driven by the microtubule motor cytoplasmic dynein I, in coordination with its obligate partner complex dynactin (Kimura et al., 2008; Katsumata et al., 2010; Maday et al., 2012). The dynein-dynactin complex needs to be recruited to and activated locally on the AV by adaptor proteins (Fu et al., 2014; Cheng et al., 2015; Khobrekar et al., 2020; Cason et al., 2021). The opposing motor kinesin-1 also localizes to axonal AVs where it may compete with dynein (Wong and Holzbaur, 2014; Maday et al., 2012). Kinesin inactivation on AVs is essential for autophagic transport and flux, and its dysregulation can be observed in the context of neurodegenerative disease (Fu et al., 2014; Boecker et al., 2021; Dou et al., 2022).

The simultaneous activation of dynein-dynactin and inactivation of kinesin must therefore be coordinated locally at the AV membrane. This regulation is further complicated by autophagosomal maturation, during which the AV membrane and associated proteins are altered via fusion with endolysosomes (Cason et al., 2021). We previously found that different motor regulatory proteins drive the retrograde transit of AVs along the axon, dependent upon the sub-axonal location and maturation state of the AV (Cason et al., 2021). Specifically, JNK-interacting protein (JIP) 1 regulates the initial transit of nascent AVs in the distal axon by inactivating kinesin-1 (Fu et al., 2014). Huntingtin-associated protein 1 (HAP1) activates dynein on partially mature AVs in the mid-axon (Wong and Holzbaur, 2014; Cason et

44 al., 2021). Finally, the motility of the most mature population of axonal AVs is regulated by the motor-

45 interacting protein JIP3 (Cason et al., 2021).

46   Mutations in the JIP3 gene (*MAPK8IP3;* homolog of UNC-16 and Sunday Driver/Syd) result in a

47 rare neurodevelopmental disorder, and JIP3 expression is relatively limited to the brain; in contrast, the

48 related protein JIP4 (*SPAG9*) is expressed ubiquitously (Ito et al., 1999; Jagadish et al., 2005; Kelkar et

49 al., 2000; Platzer et al., 2019). Recent work has shown that JIP3 and JIP4 contain a structurally

50 conserved motif in their N-termini that mediates binding to dynein light intermediate chain (DLIC), a

51 feature common among dynein activating adaptors (Celestino et al., 2022). Further, JIP3/4 can bind to

52 the dynactin subunit p150^{Glued} (Fig. 1 A) and truncated JIP3 can activate dynein motility in a purified

53 system (Montagnac et al., 2009; Rao et al., 2022). However, JIP3 and JIP4 can also interact with the

54 kinesin-1 complex via interactions (Fig. 1 A) with kinesin heavy chain (KIF5) and kinesin light chain (KLC)

55 (Arimoto et al., 2011; Cavalli et al., 2005; Celestino et al., 2022; Cockburn et al., 2018; Montagnac et al.,

56 2009; Sun et al., 2011; Tuvshintugs et al., 2014; Vilela et al., 2019). Further, JIP3 has been reported to

57 induce kinesin activity *in vitro* (Sun et al., 2011; Watt et al., 2015).

58   We therefore asked how JIP3 and JIP4 are regulated to drive the highly processive retrograde

59 transit of AVs along the axon. JIP3 and JIP4 both associate with mature AVs as well as lysosomes, and

60 either full-length protein can activate dynein motility in an *in vitro* assay. We used proteomic databases

61 to identify potential regulators of JIP3/4-dependent motility, and found that the small GTPase ARF6 is

62 enriched in AVs isolated from brain (Goldsmith et al., 2022). In live neurons, we demonstrate that the

63 GTPase state of ARF6 is important for the regulation of both AV and lysosomal motility along axons.

64 Another JIP3/4-interacting GTPase, RAB10, enriched in a lysosomal fraction from brain

65 (Dumrongprechachan et al., 2022), also affected AV and lysosomal motility along axons, but in a distinct

66 fashion. We therefore propose that the recruitment and activation of JIP3/4 at organellar membranes is

67 differentially regulated by discrete small GTPases to generate unique motile behaviors. JIP3/4 represent

68 a growing group of motor-activating proteins that can bind both dynein and kinesin motors on organelle

69 cargos (Arimoto et al., 2011; Bielska et al., 2014; Canty et al., 2021; Cason et al., 2021; Celestino et al.,

70 2022; Colin et al., 2008; Fenton et al., 2021; Fu and Holzbaur, 2013; Kendrick et al., 2019; López-

71   Doménech et al., 2018; Twelvetrees et al., 2019; Vilela et al., 2019; Zhao et al., 2021), and must be tightly

72   regulated by additional binding partners to induce unidirectional transport in the cell.

73

74   **<u>Results</u>**

75   **JIP3 and JIP4 interact with dynein on autolysosomes**

76   Previous studies have implicated both JIP3 and JIP4 in the transport of a number of organelles, especially

77   degradative vesicles such as AVs, endosomes, and lysosomes (Abe et al., 2009; Boecker et al., 2021;

78   Brown et al., 2009; Cason et al., 2021; Choudhary et al., 2017; Drerup and Nechiporuk, 2013; Hill et al.,

79   2019; Kumar et al., 2022; Montagnac et al., 2009; Sun et al., 2017; Willett et al., 2017). Accordingly, both

80   proteins were recently identified via unbiased proteomics as enriched in lysosomal and AV fractions from

81   brain (Fig. 1 B) (Dumrongprechachan et al., 2022; Goldsmith et al., 2022). To assess comigration

82   between JIP3 or JIP4 and both AVs and lysosomes in neurons, we transfected low levels of HaloTag

83   (HT)-JIP3 or JIP4 into primary hippocampal neurons along with mScarlett (mSc)-light chain 3 (LC3)—an

84   autophagosomal marker—and lysosome-associated membrane protein 1 (LAMP1)-mNeonGreen

85   (mNeon). We imaged along the proximal axon, the closest 250 μm to the soma, where we previously

86   observed the strongest impact of JIP3 siRNA on AV motility (Cason et al., 2021). In this region, the vast

87   majority (80-100% depending on LAMP1 expression levels) of LC3 puncta colocalize with LAMP1,

88   indicating at least one fusion event has already occurred (Cason et al., 2022; Boecker et al., 2021; Maday

89   et al., 2012). By contrast, only about a quarter of LAMP1 puncta colocalize with LC3 (Farfel-Becker et

90   al., 2019; Cason et al., 2022). We observed both JIP3 and JIP4 comigrating with LC3 and LAMP1 puncta

91   (Fig. 1, C-D; S1, A-B). While high levels of JIP4 overexpression can disrupt the transport of AVs along

92   the axon (Boecker et al., 2021), we did not observe a change in motility under the conditions tested here

93   (Fig. S1, A-D) due to lower expression levels (see Methods for details). Likewise, overexpression of JIP3

94   or JIP4 did not affect LAMP1+ puncta motility, LC3 or LAMP1 density, nor colocalization between LC3

95   and LAMP1 in the axon (Fig. S1, A-I).

96   Looking specifically at the JIP3 or JIP4 puncta, we noticed that almost all of the motile puncta

97   (moving ≥ 10μm) were directed retrograde towards the soma (Fig. 1, E-H). Because microtubules in the

98   axon are uniformly polarized with their plus-ends out towards the axon tip and their minus ends pointing

99   towards the soma, the minus-end-directed motor dynein is responsible for retrograde transport (Schroer

100   et al., 1989; Schnapp and Reese, 1989; Heidemann et al., 1981). By contrast, plus-end-directed motors

101   including kinesin-1 are responsible for anterograde transport away from the soma (Vale et al., 1985b; a).

102   Because the vast majority of JIP3 or JIP4 puncta moved retrograde, we therefore asked whether we

103   could observe complex formation between JIP3 or JIP4 and dynein in the axon using proximity ligation

104   assays (PLA). PLA capitalizes on oligonucleotide complementation to identify and label proteins within

105   40 nm of one another in cells (Fig. 1 I) (Alam, 2018). We were indeed able to detect endogenous JIP3 or

106   JIP4 closely apposed to endogenous dynein intermediate chain (DIC) in the axon (Fig. S1, L-M).

107   In live cells, HT-JIP3 or HT-JIP4 colocalized mainly with puncta positive for both LC3 and LAMP1,

108   which can be referred to as mature AVs or autolysosomes. We therefore expressed low levels of HT-LC3

109   then fixed the cells and used antibodies to detect endogenous LAMP1 and assessed colocalization

110   between LC3, LAMP1, and PLA puncta. We observed a striking colocalization between JIP3- or JIP4-

111   DIC PLA puncta and puncta positive for both LC3 and LAMP1, with much less colocalization between

112   PLA puncta and LC3 only or LAMP1 only puncta (Fig. 1, J-M). This finding is consistent with our previous

113   discovery that JIP3 knockdown specifically affected the motility of mature AVs, as compared with other

114   axonal AVs (Cason et al., 2021). Thus, we conclude that JIP3 and JIP4 complex with dynein on mature

115   autolysosomes.

116

117   **JIP3 and JIP4 activate dynein *in vitro***

118   Given that JIP3 and JIP4 can each bind both dynein-dynactin and kinesin-1 (Fig. 1 A), we found it

119   surprising that almost all of the JIP3 or JIP4 puncta in axons moved in the retrograde direction (Fig. 1 H).

120   We therefore asked whether JIP3 or JIP4 preferentially activates dynein or kinesin motors using an *in*

121   *vitro* lysate-based assay with cellular extracts prepared from COS-7 cells (Fig. 2 A). We performed all

122   assays on dynamically growing microtubules; this allowed us to readily differentiate between the faster

123   growing plus-end (Fig. 2 B) and the slower growing minus-end. As positive controls for dynein and kinesin

124   activity respectively, we used BICD2[1-572] (BICD2N), a truncated form of the known dynein activating

125   adaptor BICD2 that lacks the autoinhibitory domain (Fig. 2, D-F); and KIF5C$^{1-560}$ (K560), the constitutively

126   active truncated version of KIF5C (Fig. S2, A-C). We also co-expressed HA-LIS1 to maximize the

127   assembly of dynein complexes (Elshenawy et al., 2020; Fenton et al., 2021; Htet et al., 2020; Marzo et

128   al., 2020). Note that we used full-length HT-JIP3 or JIP4 in our assays.

129   In the presence of 1mM ATP at a physiological temperature of 37ºC, the majority of runs by HT-

130   JIP3- or HT-JIP4-containing motor complexes were towards the minus-end of the microtubule (~90%,

131   Fig. 2 C), with velocities (~2μm/s) and run lengths (~3.2μm) very similar to that of BICD2N (Fig. 2, D-L).

132   The total number of events—both runs (≥ 0.8μm net displacement) and stationary landing events (≤ 0.8

133   s duration with < 0.8μm net displacement)—was also similar among BICD2N (Fig. S2 B), JIP3 (Fig. 2 K),

134   and JIP4 (Fig. 2, E, H, K). Our buffer conditions were sufficient to produce kinesin activity, as assessed

135   using the positive control K560 (Fig. S2, A-C), yet plus-end-directed runs were rare. The few plus-end-

136   directed events we did observe for JIP3- and JIP4-containing complexes moved slightly faster or with

137   shorter run lengths than K560, respectively; however the low *n* for these observations prevents a direct

138   comparison (Fig. S2, C-E).

139   We were surprised by the low number of plus-end-directed runs, as previous studies have

140   reported kinesin-1 activation by JIP3 and its homolog Sunday driver (Sun et al., 2011; Watt et al., 2015).

141   We tested whether omission of HA-LIS1 would increase the frequency of runs moving toward the

142   microtubule plus-end, but we did not observe more kinesin runs under these conditions (Fig. S2 E). Based

143   upon previous work, we tried combining lysate from cells expressing HT-JIP3 or JIP4 with cells

144   expressing full-length KIF5C-HT (labelled in a different color) and GFP-KLC2. However, these

145   experiments also failed to show substantial transport towards the plus-end of the microtubule (Fig. S2 F).

146   One possible explanation is that previous work did not use polarity-marked microtubules (Sun et al.,

147   2011; Watt et al., 2015); thus, it is possible that the motility they detected was in fact minus-end-directed

148   and dynein-driven. Based upon our data, and consistent with the recent reports on the activation of dynein

149   by JIP3 (Rao et al., 2022; Singh et al., 2022) we conclude that JIP3 and JIP4 robustly activate dynein

150   motility, with only marginal activation of kinesin under the conditions tested. Consistent with these

151   observations, labeled JIP3 and JIP4 both move almost exclusively retrograde in neuronal axons (Fig. 1

152   H).

153

154   **RAB10 overexpression differentially affects the transport of AVs and lysosomes**

155   Many RAB GTPases have been shown to regulate motor complexes at organellar membranes (Guo et

156   al., 2016; Amaya et al., 2016; Horgan et al., 2010; Johansson et al., 2007). In particular, RAB10 was

157   detected in proteomics from brain-derived AVs and lysosomes and is a known JIP3/4 interactor

158   (Waschbüsch et al., 2020; Dumrongprechachan et al., 2022; Goldsmith et al., 2022). To validate this

159   finding, we blotted for RAB10 in fractions from total brain lysate, isolated AVs, and the AV fraction treated

160   with Proteinase K (PK) to digest proteins specifically bound to the outer membrane as described in

161   Goldsmith et al. (2022). RAB10 was not highly enriched in the AV fraction and, additionally, there was no

162   significant difference between RAB10 inside (PK-protected) and outside the AV (Fig. 3 A).

163   Surprisingly, given this lack of enrichment of RAB10 on AVs, when we expressed EGFP-RAB10

164   in hippocampal neurons we noted a potent dominant negative effect on LC3 motility, with EGFP-RAB10

165   expression leading to significantly more stationary/bidirectional AVs as compared with EGFP-tag alone

166   (Tag; Fig. 3, B-C). In the RAB10-expressing condition, examination of RAB10 colocalization revealed

167   that the RAB10+ AVs were almost exclusively stationary, while the few motile AVs did not colocalize with

168   RAB10 (Fig. 3 D). RAB10 expression did not affect LC3 puncta density or colocalization with LAMP1

169   (Fig. S3, A-B).

170   Because the majority (~85%) of the AVs in the proximal axon have fused with a lysosome and

171   are LAMP1+ (Fig. S3 B), we assessed colocalization between EGFP-RAB10, HT-LC3, and endogenous

172   LAMP1 in fixed neurons. We found that RAB10 predominantly colocalized with LAMP1+ organelles,

173   including both autolysosomes and lysosomes (Fig. 3, E-F). Hence, we asked whether LAMP1+ puncta

174   motility is also affected by RAB10 expression. There was no gross effect on LAMP1 puncta motility (Fig.

175   4, A-B). However, when we specifically quantified the motile puncta (moving ≤ 10μm in either direction

176   during a 2 min video), we found a significant shift from a mild retrograde bias (~60%) in the GFP

177   expressing cells to a mild anterograde bias (~57%) in the RAB10 expressing cells (Fig. 4 C). Oddly,

178   however, when we examined the LAMP1 puncta colocalized with RAB10, we found the majority to be

179   stationary/bidirectional (Fig. 4 D), like for LC3 (Fig. 3 D). RAB10 expression had no effect on LAMP1

180   colocalization with LC3, but was associated with a mild decrease in LAMP1 puncta in the axon (Fig. S3,

181   C-D).

182   We used PLA (Fig. 1 I) to determine whether the changes in motility were due to a disruption in

183   the formation of JIP3/4-dynein complexes. We found that the number of JIP3/4-DIC PLA puncta did not

184   change upon RAB10 expression (Fig. S3, E-F), nor did the colocalization between JIP3/4-DIC PLA

185   puncta and autophagosomes, autolysosomes, or lysosomes (Fig. S3, G-H). Hence, the decrease in AV

186   motility and the increase in LAMP1 anterograde motility is not due to loss of JIP3/4-dynein complexes.

187   Together, these results suggest that RAB10 can modulate the motility of AVs and lysosomes, potentially

188   by affecting kinesin recruitment and/or activation, rather than by decreasing dynein recruitment or

189   activation.

190

191   **ARF6 GDP-locked mutant disrupts autophagosomal transport**

192   To better understand the regulation of JIP3/4-dependent AV motility, we probed the proteomic data for

193   other possible small GTPases that might affect motor activity or motor coordination. We identified the

194   candidate ARF6, which is present in both the AV and lysosomal proteomic datasets

195   (Dumrongprechachan et al., 2022; Goldsmith et al., 2022) and whose enrichment on the outside of the

196   AV we could validate via immunoblot (Fig. 5 A). ARF6 is a known JIP3/4 interactor and has been

197   previously shown to modulate JIP3/4 motor binding based upon its GTP-binding state: ARF6-GTP

198   increases the binding between JIP3/4 and the dynactin subunit p150^{Glued} (p150) while ARF6-GDP

199   increases binding between JIP3/4 and kinesin light chain (KLC; Fig. 5 B) (Montagnac et al., 2009). Hence,

200   we transfected CFP-ARF6 GTP-locked (Q67L) and GDP-locked (T27N) mutants into our primary

201   hippocampal neurons and assessed the motility of LC3 puncta. While expression of wildtype (ARF6^{WT})

202   and ARF6^{QL} did not have an obvious effect on LC3 motility, ARF6^{TN} expression induced a robust loss of

203   AV motility and a significant increase in the pausing of LC3 puncta (Fig. 5, C-E). However, there was no

204   effect on either LC3 puncta density or AV maturation, measured either by LAMP1 colocalization (Fig. S4,

205   A-B).

206

ARF6 GTP-locked mutant decreases retrograde lysosome pausing

208   We next assessed motility of LAMP1 puncta in the axon upon ARF6 expression. Here, we observed a

209   very different effect. While gross LAMP1 motility was not significantly different in neurons expressing

210   ARF6^{WT} or ARF6^{TN} expression, expression of the GTP-locked ARF6^{QL} mutant led to a decrease in the

211   stationary/bidirectional fraction of LAMP1 puncta (Fig. 6, A-B). Expression of either locked mutant led to

212   decreased pausing time, although the effect was much bigger in ARF6^{QL}-expressing cells (Fig. 6 C). We

213   evaluated pausing time for LAMP1 puncta colocalized with LC3 (autolysosomes) and those not

214   colocalized (lysosomes) and saw that both organelle subgroups were affected by ARF6^{QL} expression

215   (Fig. 6 D). Neither the LAMP1 puncta colocalized with LC3 nor the LAMP1 density was affected (Fig. S4

216   D-E). However, when we split the LAMP1 puncta into motile fractions, we saw that the pausing effect

217   was limited to the retrograde LAMP1 fraction, with no significant effect on anterograde-moving LAMP1

218   puncta (Fig. 6, E-F). Thus, GTP-bound but not GDP-bound ARF6 increases the efficiency of the dynein

219   complex on LAMP1-positive organelles.

220

ARF6 GTPase status is locally regulated by GAPs and GEFs on the membrane

222   We next asked whether wildtype ARF6 may be converted locally at AVs and lysosomes into a GTP-

223   bound and GDP-bound state respectively. The nucleotide state of small GTPases is regulated by GTPase

224   activating proteins (GAPs), which induce GTP-to-GDP hydrolysis, and guanine exchange factors (GEFs),

225   which induce release of GDP and binding of a new GTP molecule (Fig. 7 A). There are 10 known ARF6

226   GEFs and 20 GAPs. 60% of ARF6 GEFs and 60% of ARF6 GAPs were detected in lysosomal proteomics

227   (Fig. 7 B) (Dumrongprechachan et al., 2022). Because LC3+ puncta in cells expressing ARF6^{QL} behaved

228   similarly to wildtype-expressing cells, we hypothesized that AVs would be enriched for ARF6 GEFs.

229   Indeed, 50% of ARF6 GEFs and only 20% of ARF6 GAPs were detected in AV proteomics (Fig. 7 B)

230   (Goldsmith et al., 2022). We used immunoblotting to validate the GAPs and GEFs detected in an AV-

231 enriched fraction from brain, and found that the GEFs were highly enriched in the AV fraction and

232 significantly localized to the outer membrane of the AV as judged by protease sensitivity (Fig. 7, C-G).

233 By comparison, the ARF6 GAPs were less enriched and not significantly localized to the outer membrane

234 (Fig. 7, H-J). These observations support a model in which ARF6 GEFs are localize to the AV membrane

235 to locally enrich for ARF6-GTP, which can in turn can recruit and/or activate JIP3/4-containing dynein

236 complexes.

237

238 **ARF6 increases the interaction of the JIP3/4-dynein complex with microtubules**

239 Finally, we asked how ARF6 might affect the behavior of JIP3/4-containing motor complexes using *in*

240 *vitro* motility assays. We found that overexpression of GTP-locked ARF6 significantly increased the

241 microtubule landing events of JIP3- or JIP4-containing motor complexes (Fig. 8, A-C), although the

242 frequency of plus- and minus-end-directed events did not change (Fig. 8, D-E). Interestingly, ARF6^{TN}

243 induced the same effect, a significant increase in microtubule-binding events (Fig. S5, A-E). There was

244 no effect on the velocity of motile complexes in either ARF6^{QL} or ARF6^{TN} conditions, but there was a mild

245 reduction in run length upon ARF6 inclusion (~28%; Fig. S5, F-G).

246 Because JIP3 and JIP4 do not directly bind microtubules, the increased landing events must be

247 due to increased interaction with a microtubule-binding protein. The dynactin subunit p150 interacts

248 directly with JIP3/4 and with microtubules via its CAP-Gly domain; additionally, ARF6 binding modulates

249 the interaction between p150 and JIP3/4 (Peris et al., 2006; Ayloo et al., 2014; Moughamian and

250 Holzbaur, 2012; Montagnac et al., 2009). Finally, p150 binding to the microtubule in the absence of dynein

251 induces statically bound and/or diffusive behaviors (Ayloo et al., 2014; Feng et al., 2020), similar to what

252 we observe. Therefore, we propose that ARF6 increases the efficiency of JIP3/4-containing motor

253 complexes in cells by increasing the microtubule association of JIP3/4 through p150.

254

255 **Discussion**

256 Here, we demonstrate that two related scaffolding proteins, JIP3 and JIP4, both activate dynein in vitro,

257 and form a complex with dynein on mature AVs (autolysosomes) in neuronal axons (Fig. 1, 2). We identify

258   two small GTPases that interact with JIP3/4 and affect the axonal transport of AVs and other LAMP1+

259   organelles. RAB10 overexpression halts the retrograde transit of AVs and increases the anterograde bias

260   observed for LAMP1-positive puncta in the axon (Fig. 3, 4). ARF6 also regulates AV motility, in a GTP-

261   binding-dependent fashion: GTP-locked ARF6 decreases the pausing of retrograde-moving LAMP1

262   puncta, while GDP-locked ARF6 increases the fraction of stationary AVs (Fig. 5, 6). Further, ARF6 GEFs

263   are enriched on the outer AV membrane (Fig. 7), meaning ARF6-GTP can be locally upregulated. We

264   propose that locally generated ARF6-GTP recruits the JIP3/4-dynein-dynactin complex and also

265   enhances the association of the complex with microtubules (Fig. 8), leading to more efficient transport of

266   AVs toward the soma.

267   Concurrent with our study, two other groups have shown that purified recombinant truncated JIP3

268   is sufficient to activate dynein-mediated motility *in vitro* (Rao et al., 2022; Singh et al., 2022). Our study

269   adds to this growing body of work, as the approaches used here (1) include endogenous binding partners,

270   which negates the need to use truncated constructs; (2) is performed at physiological temperature on

271   dynamically growing microtubules; and (3) includes competing kinesin complexes. Our average JIP3

272   velocity (~1.8 μms^{-1}) is higher than that observed by the other groups [0.7 μms^{-1} (Rao et al., 2022); 1 μms^{-}

273   ^{1} (Singh et al., 2022)], most likely due to the more physiological assay temperature (37ºC vs. room

274   temperature). It is, however, consistent with previous observations of dynein activation using lysate

275   assays performed at 37º (Fenton et al., 2021).

276   Previous work has reported that the Drosophila JIP3 ortholog Sunday driver (syd) activates

277   kinesin-1 (Sun et al., 2011). However, these assays were performed using mammalian cell lysate at room

278   temperature on stabilized microtubules without polarity labelling, and the average velocities (0.6-1.0 μms^{-}

279   ^{1}) and run lengths (3-5.5 µm) observed suggest that minus end-directed motility may have dominated in

280   their assays, as these values are more consistent with dynein-mediated transport (Olenick et al., 2016;

281   Urnavicius et al., 2018; Fenton et al., 2021; Canty et al., 2021; Fu et al., 2014; Fu and Holzbaur, 2013;

282   Rao et al., 2022; Singh et al., 2022; Sun et al., 2011). Similar assays performed with mammalian JIP3

283   (Watt et al., 2015) resulted in velocities (~0.25 μms^{-1}) more consistent with kinesin-1 activation; however,

284   the relatively short run lengths (~0.75 µm) suggest that JIP3 may require additional effectors to fully

285   activate kinesin-1 motility. Consistent with this conclusion, binding assays suggest that JIP3 and the

286   unrelated motor effector protein JNK-interacting protein 1 (JIP1) cooperatively activate kinesin-1 (Sun et

287   al., 2017). JIP1 alone is unlikely to be sufficient for kinesin-1 activation (Blasius et al., 2007; Sun et al.,

288   2017), but in single molecule assays using cell lysates, JIP1 overexpression increases the number of

289   kinesin-1-driven motility events (Fu and Holzbaur, 2013; Fu et al., 2014). Binding assays suggest that

290   the binding of JIP1 to KHC and KLC, concurrent with the binding of JIP3 to KLC, is necessary to fully

291   relieve kinesin-1 autoinhibition (Sun et al., 2017). Further, overexpression of either JIP1 or JIP3 leads to

292   the accumulation of the other adaptor at microtubule plus-ends in cells, suggesting cotransport with

293   kinesin (Hammond et al., 2008). While JIP3 does not oligomerize with JIP4, JIP1 and JIP3 interact both

294   directly and indirectly through KLC, where they bind distinct residues in the tetratricopeptide repeat (TPR)

295   domain (Hammond et al., 2008; Kelkar et al., 2005). Of note, JIP1 and JIP3 have been implicated in the

296   anterograde transport of many of the same organelles, including synaptic vesicle proteins, TrkB receptor,

297   amyloid precursor protein (APP), mitochondria, and signaling proteins such as JNK (Choudhary et al.,

298   2017; Horiuchi et al., 2005; Sun et al., 2017; Fu and Holzbaur, 2013; Drerup and Nechiporuk, 2013; Sato

299   et al., 2015), further supporting a cooperative interaction between these motor activators.

300   Notably, both JIP1 and JIP3/4 have been previously implicated in the transport of RAB10+

301   vesicles (Kluss et al., 2022; Bonet-Ponce et al., 2020; Deng et al., 2014). In our study, RAB10 appears

302   to increase the recruitment and/or activation of kinesin on LC3+ and LAMP1+ puncta. RAB10, like many

303   other RABs, is regulated by phosphorylation with phospho-RAB10 (especially T73) being generally more

304   active and membrane-associated (Yan et al., 2018; Lara Ordóñez et al., 2022; Kluss et al., 2022; Wauters

305   et al., 2020; Waschbüsch et al., 2020; Homma et al., 2021). RAB10, especially phospho-RAB10,

306   regulates the motility of multiple kinesin-1 and kinesin-3 cargoes within cells (Etoh and Fukuda, 2019;

307   Deng et al., 2014; Taylor et al., 2015; Zajac and Horne-Badovinac, 2022). RAB10 can directly complex

308   with kinesin-3 (KIF13) and regulate its activity (Etoh and Fukuda, 2019; Zajac and Horne-Badovinac,

309   2022). However, its interaction with kinesin-1 (KIF5) must be mediated by adaptor proteins. We therefore

310   propose an integrated model (Fig. 9 A) in which JIP3 or JIP4, together with JIP1, mediate the anterograde

311   transport of RAB10+ cargo, including some populations of lysosomes. Phospho-RAB10 recruits JIP3/4

312 and JIP1 to LAMP1+ organelles; upon RAB10 binding, JIP3/4 and JIP1 bind to kinesin-1 to induce the

313 anterograde transit of the organelle (Fig. 3, 4). RAB10 can also directly bind kinesin-3 to induce

314 anterograde transport, circumventing the JIP3/4-JIP1 complex.

315 RAB10 is a known target of the kinase LRRK2, which is hyperactive in some genetic forms of

316 Parkinson's disease (Yan et al., 2018; Wauters et al., 2020; Bonet-Ponce et al., 2020). Our group

317 previously showed that hyperactive, disease-associated LRRK2 increased the level of phospho-RABs

318 and kinesin present on the outer membrane of AVs (Boecker et al., 2021). Additionally, hyperactive

319 LRRK2 increased the recruitment of JIP4 and, to a lesser degree, JIP3 to the AV membrane (Boecker et

320 al., 2021). We therefore hypothesize that phospho-RAB10 forms a complex with JIP4 to recruit and

321 activate kinesin (Fig. 9 B). If exogenously expressed at high levels, JIP4 can block AV motility (Boecker

322 et al., 2021), but when expressed at more modest levels, JIP4 comigrates with autolysosomes (Fig. S2),

323 like JIP3. It is unknown whether JIP4 can also interact with JIP1 to activate kinesin-1. However, based

324 upon our work and others, we suggest that JIP3 and JIP4 are functionally redundant with their primary

325 difference being expression in different tissues (Gowrishankar et al., 2021; Tuvshintugs et al., 2014; Sato

326 et al., 2015). Thus, in non-neuronal cells where JIP3 is not expressed, JIP4 may replace JIP3 in the

327 kinesin activation complex.

328 JIP3 and JIP4 can also induce retrograde transport of LAMP1+ organelles, including

329 autolysosomes. ARF6 was previously shown to block JIP3 or JIP4 binding to kinesin, possibly through

330 steric hindrance: the KLC and ARF6 binding sites are highly overlapping (Vilela et al., 2019; Cockburn et

331 al., 2018; Hammond et al., 2008; Llinas et al., 2016; Isabet et al., 2009; Montagnac et al., 2009) (Fig. 1

332 B). Instead, ARF6 enhances the interaction between JIP3/4 and the dynactin subunit p150[Glued]

333 (Montagnac et al., 2009). The increased frequency of microtubule landing events for JIP3/4—in the

334 absence of changes to other motility parameters—induced by addition of ARF6 to our *in vitro* assays

335 (Fig. 8) is consistent with an increased interaction with p150[Glued], which contains a microtubule-binding

336 domain but no motor activity (Ayloo et al., 2014; Feng et al., 2020). Microtubule binding, especially

337 through dynactin and general dynein effectors like CLIP-170, has previously been shown to be important

338 for dynein recruitment and initiation of motility (Moughamian and Holzbaur, 2012; Moughamian et al.,

339   2013; Nirschl et al., 2016; McKenney et al., 2016). Stepwise recruitment of dynactin and then dynein to

340   the microtubule has not previously been shown for cargo-specific activating activators for dynein.

341   However, the JIP3/4-related dynein effector Rab7-interacting protein (RILP) was previously shown to

342   bind to dynactin prior to the initiation of dynein activity, suggesting a similar mechanism (Johansson et

343   al., 2007). While RILP has not yet been shown to activate motor activity *in vitro*, its N-terminus—including

344   the motor binding domains—is very similar to that of JIP3 and JIP4 (Celestino et al., 2022; Vilela et al.,

345   2019; Matsui et al., 2012). We therefore hypothesize that the formation of an initial microtubule-bound

346   cargo-binding protein-dynein activator-dynactin complex, such as the ARF6-JIP3/4-dynactin complex, is

347   a common mechanism in the initiation of dynein-mediated transport of diverse cargos.

348   In single molecule motility assays, we observed no difference between GTP- and GDP-locked

349   ARF6 (Fig. 8, S9). However, in neurons, expression of GTP- and GDP-locked ARF6 induced significant

350   changes in organelle transport (Fig. 5, 6). We hypothesize that this difference may be due to a key role

351   for bound nucleotide in regulating the membrane association of ARF6. ARF6 contains a myristoyl anchor

352   and binds more tightly to membranes in its GTP-bound state than in its GDP-bound state (Ménétrey et

353   al., 2000; Duellberg et al., 2021). In our *in vitro* assays, membranes are first removed via centrifugation

354   of cell lysates, making this assay less sensitive to effects of the nucleotide state of ARF6. Therefore, we

355   propose that the differential effects observed in cells and in vitro when comparing GTP- and GDP-locked

356   ARF6 are likely due to increased membrane interaction and the subsequent stepwise recruitment of JIP3

357   or JIP4, dynactin, and dynein (Fig. 9 A).

358   Interestingly, overexpression of GTP-locked ARF6 is sufficient to ameliorate the AV motility

359   phenotype observed in hyperactive LRRK2 mutant conditions (Dou et al., 2022). Under increased LRRK2

360   activity, JIP3/4 seem to exhibit enhanced interaction with kinesin and phosphorylated RABs (Dou et al.,

361   2022; Boecker et al., 2021; Kluss et al., 2022; Bonet-Ponce et al., 2020). In these conditions, GTP-locked

362   ARF6 expression presumably scaffolds the formation of more JIP3/4-dynein-dynactin complexes to

363   compete with the RAB-JIP3/4-kinesin-1 complexes (Dou et al., 2022). ARF6 and phospho-RAB10 (or

364   RAB35) may compete to bind the same limited pool of JIP3/4, or excess JIP3/4 may be available for

365   recruitment into multiple motor complexes concurrently (Bonet-Ponce et al., 2020; Miyamoto et al., 2014;

366   Kobayashi and Fukuda, 2012). While ARF6 and RAB10 bind different regions of JIP3/4, the ARF6 binding

367   site and the KLC binding site are mutually exclusive (Isabet et al., 2009); thus it is likely that these are

368   two completely discrete complexes.

369   It is possible that crosstalk occurs locally between ARF6 GAPs and GEFs and RAB kinases and

370   phosphatases to prevent local activation of both dynein and kinesin, which would result in non-processive

371   tug-of-war. For example, the RAB10/RAB35 effector ACAP2 is known to also function as a GAP for ARF6

372   (Shi and Grant, 2013; Shi et al., 2012; Kobayashi and Fukuda, 2012; Miyamoto et al., 2014). ACAP2 is

373   present in both the AV and lysosome proteomics datasets from brain (Goldsmith et al., 2022;

374   Dumrongprechachan et al., 2022); however, we found that ACAP2 was primarily an AV cargo, not on the

375   outer membrane, consistent with local enrichment for ARF6-GTP on the AV membrane. However,

376   ACAP2 enrichment on lysosomes may locally promote GTP hydrolysis by ARF6, leading to dissociation

377   of the GTPase from the membrane. Interestingly, analysis of the RAB35-ACAP2 structure indicates that

378   ACAP2 specifically binds to the LRRK2 phosphorylation site within RAB35 (Lin et al., 2019). Thus, LRRK2

379   kinase activity may locally enhance the formation of RAB-JIP3/4-kinesin-1 complexes, and also prevent

380   local accumulation of ARF6-GTP and the resultant activation of dynein-dynactin activity on lysosomes.

381   Hyperphosphorylated RAB10 is induced by Parkinson's disease-causing mutations in LRRK2,

382   and is also a hallmark pathological feature of Alzheimer's disease (Yan et al., 2018). Mutations in the

383   JIP1-JIP3 cargo APP cause familial Alzheimer's disease; further, both axonal transport and autophagy

384   are disrupted in a multitude of neurodegenerative diseases (Wong and Holzbaur, 2015; Kins et al., 2006;

385   Guillaud et al., 2020; Goldstein, 2012). Mutations in JIP3 cause a rare neurodevelopmental disorder

386   (Platzer et al., 2019) and double-knockout of JIP3 and JIP4 leads to robust neurodegeneration (Sato et

387   al., 2015; Gowrishankar et al., 2021). Additionally, ARF6 knockout in neurons leads to defects in axonal

388   development (Akiyama et al., 2014). Because these proteins and the processes they regulate are all

389   dysfunctional in neurodevelopmental and/or neurodegenerative disease, it is essential that we continue

390   to tease apart the detailed mechanisms involved in order to better inform therapy development.

391

392   **Acknowledgements**

397   **Materials and methods**

398   **Plasmids and reagents**

399   Constructs, all of which were verified by DNA sequencing, include the following:

Construct	Source
CFP-ARF6 WT, QL, and TN	Gift from J. Swanson (Addgene # 11382, 11386, 11387)
BICD2N-Halo	Full-length mouse BICD2 in the pEGFP vector (gift from A. Akhmanova, Utrecht University) was used to generate a truncated construct spanning residues 1–572 fused to the HaloTag and cloned into pcDNA3.1
Halo-JIP3 (EGFP vector)	HaloTag fused to the N-terminus of hJIP3 from cDNA Clone (GE: MGC9053013) fused in pEGFP vector backbone
Halo-JIP4 (CMV vector)	Subcloned from pGEXP1-JIP4 #DU27651, acquired from MRC PPU Reagents and services, University of Dundee
Halo-JIP3 (CMV vector)	Halo-JIP3 sequence (from EGFP vector construct) subcloned into (Halo-JIP4) CMV vector
Halo-JIP4 (EGFP vector)	Halo-JIP4 sequence (from CMV vector construct) subcloned into (Halo-JIP3) EGFP vector
K560-Halo	First 560 aa of human KIF5B from pET17: K560 GFP ST (gift from R. Vale, University of California San Francisco) subcloned into pHTC-HaloTag CMVneo vector (Promega)
KIF5C-Halo	Full-length mouse kinesin-1 heavy chain (KIF5C) in pRK5 myc plasmid (gift from J. Kittler, University of Surrey) with HaloTag fused to C-terminus
GFP-KLC2	Full-length WT mouse KLC2 in CB6 driven expression vector
LAMP1-mNEON	Gift from D. Gadella (Addgene plasmid # 98882).
mCherry-EGFP-LC3B	Gift from T. Johansen, University of Troms
mScarlet-LC3B	Subcloned from Addgene #21073 and Addgene #85054
HA-LIS1	Gift from D. Smith, University of South Carolina
EGFP-RAB10	Gift from M. Scidmore (Addgene plasmid # 49472)

400

401   We previously used a HT-JIP4 construct in a CMVNeo backbone that expressed in cells at extremely

402   high levels (Fig. S1, J-K). We subcloned the HT-JIP4 into an EGFP backbone (the EGFP was previously

403   removed via subcloning), which we were already using for our HT-JIP3. This new HT-JIP4 expresses at

404   more modest levels and does not affect AV or LAMP1 motility (Fig. S1, A-E). Therefore, we report that

405   our previous finding was an overexpression artifact. In all neuronal experiments, we used the EGFP

406   backbone JIP3/4; in the TIRF assays, we used the CMV backbone JIP3/4.

407

408   Antibodies include the following:

Antibody	Source	Dilution
anti- ACAP2/CENTB2	ThermoFisher, PA5-18209	WB 1:1000
anti- ARF6	Cell Signaling, D12G6	WB 1:800
anti- CYTH (pan)	ThermoFisher, MA1-062	WB 1:1000
anti- DIC	EMD Millipore, MAB1618	PLA@1:200
anti- GIT1	Biorbyt, orb99082	WB 1:1000
anti- Halo (polyclonal)	Promega, G9281	WB 1:500
anti- Halo (monoclonal)	Promega, G9211	WB 1:500
anti- IQSEC1/BRAG2	ThermoFisher, PA5-38019	WB 1:500
anti- IQSEC2/BRAG1	ThermoFisher, PA5-72831	WB 1:500
anti- IQSEC3/BRAG3	Biorbyt, orb317640	WB 1:500
anti- JIP3/MAPK8IP3	Abcam, ab196761	PLA 1:25
anti- JIP4/SPAG9	Cell Signaling, 5519	PLA 1:25
anti- LAMP1	R&D Systems, AF4800	IF 1:50
anti- RAB10	Abcam, ab237703	WB 1:500
anti- SMAP2	Biorbyt, orb1142130	WB 1:2000
anti-Sheep (2º) 405	Abcam, ab175676	IF 1:1000
anti-Sheep (2º) 647	Abcam, ab150179	IF 1:1000

409

Primary hippocampal culture

Sprague Dawley rat hippocampal neurons at embryonic day 18 were obtained from the Neurons R Us

Culture Service Center at the University of Pennsylvania. Cells (proximity ligation assay, 40,000 cells on

7mm glass; live imaging, 200,000 cells on 20 mm glass) were plated in glass-bottom 35 mm dishes

(MatTek) that were precoated with 0.5 mg/ml poly-L-lysine (Sigma Aldrich). Cells were initially plated in

Attachment Media (MEM supplemented with 10% horse serum, 33 mM D-glucose, and 1 mM sodium

pyruvate) which was replaced with Maintenance Media (Neurobasal [Gibco] supplemented with 33 mM

D-glucose, 2 mM GlutaMAX (Invitrogen), 100 units/ml penicillin, 100 mg/ml streptomycin, and 2% B-27

[ThermoFisher]) after 5-20 h. Neurons were maintained at 37 C in a 5% CO_2 incubator; cytosine

arabinoside (Ara-C; final conc. 1 μM) was added the day after plating to prevent glia cell proliferation. For

transfections, neurons (7-10 DIV) were transfected with 0.35–1.5 μg of total plasmid DNA using

Lipofectamine 2000 Transfection Reagent (ThermoFisher, 11668030) and incubated for 18-48 h.

422

Live neuron imaging and analysis

424 Neurons were imaged in Imaging Media (HibernateE [Brain Bits] supplemented with 2% B27 and 33 mM

425 D-glucose). Autophagosome (1-1.3 frames/sec) and lysosome (0.5-0.65 frames/sec) behavior was

426 monitored in the proximal axon (<200 µm from the soma) of 8-12 DIV neurons for 2-3 min. Neurons were

427 imaged in an environmental chamber at 37°C on a Perkin Elmer UltraView Vox spinning disk confocal

428 on a Nikon Eclipse Ti Microscope with an Apochromat 100 x 1.49 numerical aperture (NA) oil-immersion

429 objective and a Hamamatsu EMCCD C9100-50 camera driven by Volocity (PerkinElmer). Only cells

430 expressing moderate levels of fluorescent proteins were imaged to avoid overexpression artifacts or

431 aggregation. It should be noted that the quality of the primary neuron dissections affected

432 autophagosomal motility, but compared conditions were always collectected from the same dissections

433 and imaging sessions.

434 Kymographs were generated in ImageJ (https://imagej.net/ImageJ2) using the MultiKymograph

435 plugin (line width, 1-5) and analyzed in ImageJ. Puncta were classified as either anterograde (moving

436 ≥10µm towards the axon tip), retrograde (moving ≥10µm towards the soma), or stationary/bidirectional

437 (net movement <10µm during the video). Because fluorescent LC3 is cytosolic (as well as punctate) and

438 neurites occasionally crossed in culture, raw videos were referenced throughout kymograph analysis to

439 ensure only real puncta (≥ 1.5 SD from the axon mean) were included in analyses. All comigration

440 analyses were performed using kymographs.

441

442 **Proximity ligation assay**

443 Neurons were transfected (Lipofectamine 2000) with 0.3 µg EGFP plasmid (for GFP fill) and 0.5 µg Halo-

444 tagged effector following above protocol then 24 h later (DIV 7–8) fixed in PBS containing 4%

445 paraformaldehyde and 4% sucrose for 8 min. Duolink™ In Situ PLA Mouse/Rabbit kit with red detection

446 reagents (Sigma-Aldrich, DUO92101-1KT) was used according to manufacturer's protocol. We used

447 dynein intermediate chain antibody (Mouse MAB1618) plus JIP3 antibody (Rabbit ab196761), JIP4

448 antibody (Rabbit Cell Signalling, 5519), or no second 1° antibody (negative control). Both 2° antibodies

449 (Mouse and Rabbit) were added for all experiments (including negative control). Z-stacks (0.25 µm steps)

450 were acquired on an inverted epifluorescence microscope (DMI6000B; Leica) with an Apochromat 63 x

451   1.4 NA oil-immersion objective and a charge-coupled device camera (ORCA-R2; Hamamatsu Photonics)

452   using LAS-AF software (Leica). Puncta were counted manually using ImageJ.

453

454   **Cell line culture**

455   COS-7 (ATCC) cells were maintained in DMEM (Corning) supplemented with 1% GlutaMAX and 10%

456   FBS. Cells were maintained at 37 C in a 5% CO_2 incubator. For motility assays and co-

457   immunoprecipitation experiments, COS-7 cells were plated on 10 cm plates and transfected 24h prior to

458   lysis using FuGENE 6 (Promega; 6-12 µg total DNA). Cells were routinely tested for mycoplasma using

459   a MycoAlert detection kit (Lonza, LT07). COS-7 cells were authenticated by ATCC.

460

461   **Motility assay**

462   The movement of JIP3-, JIP4-, BICD2N-, or K560-containing complexes from cell extracts was tracked

463   using TIRF microscopy. Motility assays were performed in flow chambers constructed with a glass slide

464   and a coverslip silanized with PlusOne Repel-Silane ES (GE Healthcare), held together with vacuum

465   grease to form a ~10 µl chamber. Rigor kinesin-1E236A (0.5 µM) was non-specifically absorbed to the

466   coverslip 73 and the chamber was then blocked with 5% pluronic F-127 (Sigma-Aldrich). 250 nM

467   GMPCPP microtubule (MT) seeds, labeled at a 1:40 ratio with HiLyte Fluor 488 tubulin (Cytoskeleton,

468   Denver, CO), were flowed into the chamber and immobilized by interaction with rigor kinesin-1E236A.

469   11.25 µM free tubulin (labeled at a 1:20 ratio with HiLyte Fluor 488 tubulin) was added with the lysate to

470   grow dynamic microtubules from the seeds. COS-7 cells grown in 10 cm plates to 70–80% confluence

471   expressing full-length Halo-tagged HAP1, BICD2N or HaloTag alone were labeled with TMR 18–24 h

472   post-transfection then lysed in 100 µl lysis buffer [40 mM Hepes (pH 7.4), 120 mM NaCl, 1 mM EDTA, 1

473   mM ATP, 0.1% Triton X-100, 1 mM PMSF, 0.01 mg ml-1 TAME, 0.01 mg ml-1 leupeptin, and 1 µg ml-1

474   pepstatin-A]. Cell lysates were clarified by centrifugation (17,000g) and diluted in P12 motility buffer [12

475   mM Pipes (pH 6.8), 1 mM EGTA, and 2 mM $MgCl_2$] supplemented with 1 mM Mg-ATP, 1 mM GTP, 0.08

476   mg ml-1 casein, 0.08 mg ml-1 bovine serum albumin, 2.55 mM DTT, 0.05% methylcellulose, and an

477   oxygen scavenging system (0.5 mg ml-1 glucose oxidase, 470 U ml-1 catalase, and 3.8 mg ml-1 glucose).

478   All the videos (2 min, 4-5 frames s-1) were acquired at 37°C using a Nikon TIRF microscopy system

479   (Perkin Elmer, Waltham, MA) on an inverted Ti microscope equipped with a 100× objective and an

480   ImageEM C9100-13 camera (Hamamatsu Photonics, Hamamatsu, Japan) with a pixel size of 0.158 μm

481   and controlled with the program Volocity (Improvision, Coventry, England).

482

483   **Motility assay analysis**

484   At least 5 microtubules per video were analyzed by generating kymographs using the MultiKymograph

485   plugin of ImageJ and analyzed in Excel (Microsoft, Redmond, WA). MY polarity was determined one of

486   two ways. (1) MT were imaged at 10 sec intervals during the entire acquisition (2 min). (2) MT were

487   imaged for 30 sec at 4-5 frames sec -1 before and after motor imaging. In this case, only MT present in

488   both the before and after videos were analyzed. The MT length (to which the number of events was

489   normalized) was either (1) the final length at the end of the entire (2 min) acquisition; or (2) the initial

490   length at the beginning of the "after" video, respectively. In either case, only non-bundled MT that could

491   be clearly seen both growing and catastrophing regularly were analyzed.

492   At least 5 microtubules were analyzed per replicate; 3 biological and technical replicates were

493   performed for a final n = 20 microtubules per condition. Kymographs were generated using the

494   MultiKymograph plugin (line width, 1) in ImageJ (https://imagej.net/ImageJ2). Analysis was performed

495   using KymoButler 74 with manual post-hoc curation, as described here. To be classified as an event, the

496   duration must be greater than 0.8 seconds or the run length greater than 1.6μm, and at least 1.5 SD

497   above the local background (surrounding ~100μm2). To be classified as plus-end- or minus-end-directed

498   run, the punctum must travel greater than 5 pixels (0.8μm) in that direction.

499

500   **Autophagosome fractionation**

501   Enriched autophagosome fractions were isolated from mouse brain via sequential ultracentrifugation,

502   adding Gly-Phe-β-naphthylamide to inactivate and deplete lysosomal vesicles and thus enhance the

503   integrity of autophagosome-associated proteins 75; detailed protocols and validations can be found in

504   60. Briefly, brains were collected from wildtype mice on the C57BL/6J background (Ref 14699058) and

505 homogenized in a tissue grinder in an ice cold buffered 10mM Hepes, 1mM EDTA, 250 mM sucrose

506 solution, then subjected to three differential centrifugations through Nycodenz and Percoll discontinuous

507 gradients to isolate vesicles of the appropriate size and density. The autophagosome enriched fraction

508 was then divided and either immediately lysed for the identification of all internal and externally-

509 associated proteins on autophagosomes (A fraction), treated with 10 μg proteinase-K for 45min at 37ºC

510 to degrade externally associated proteins and enrich for membrane-protected autophagosome cargo (P

511 fraction), or membrane permeabilized by the addition of 0.2% triton x-100 prior to proteinase K treatment

512 to confirm proteinase K efficacy (T fraction). The lysis buffer used contained a final concentration of 0.5%

513 NP-40 with 1x protease and phosphatase inhibitors, PMSF and Pepstatin A. Protein concentration was

514 measured by Bradford assay and equal amounts of protein in denaturing buffer were run on SDS-PAGE

515 gels.

516

517 **Immunoblotting**

518 For fluorescence Western blotting, samples were analyzed by SDS- PAGE and transferred onto PDVF

519 Immobilon FL (Millipore). Membranes were dried for at least 1 h, rehydrated in methanol, and stained for

520 total protein (LI-COR REVERT Total Protein Stain). Following imaging of the total protein, membranes

521 were destained, blocked for 5min in EveryBlot Blocking Buffer (BioRad #12010021), and incubated

522 overnight at 4ºC with primary antibodies diluted in EveryBlot Blocking Buffer. Membranes were washed

523 four times for 5 min in 1xTBS Washing Solution (50 mM Tris-HCl pH 7.4, 274 mM NaCl, 9 mM KCl, 0.1%

524 Tween-20), incubated in secondary antibodies diluted in EveryBlot Blocking Buffer with 0.01% SDS for 1

525 hr, and again washed four times for 5 min in the washing solution. Membranes were immediately imaged

526 using an Odyssey CLx Infrared Imaging System (LI-COR). Band intensity was measured in the LI-COR

527 Image Studio application.

528

529 **Analysis of organelle enrichment publications**

530 Goldsmith et al., (2022) and Dumrongprechachan et al., (2022) performed AV and lysosomal

531 enrichments, respectively, and performed mass spectrometry on the resultant proteins to determine

532 proteins associated with the organelles. To compare these datasets, which pulled down different amounts

533 of total protein, we normalized the number of peptides from each to the average number of peptides

534 detected for ARF-related proteins (normalization factor) in each preparation. The number of peptides for

535 each detected protein was divided by the normalization factor (13.5 for lysosomes, 2.6 for AVs). Note

536 that for the AVs, we used the number of peptides in the fraction not treated with proteinase K (AV fraction).

537

538 **Statistics**

539 All statistical analyses were performed in Prism (GraphPad, San Diego, CA). Bars represent mean ±

540 S.E.M. unless otherwise indicated. n indicates the number of events or cells pooled across at least 3

541 trials per experiment. Parametric or nonparametric tests were used where appropriate, but formal testing

542 was not performed. Statistical measures are described in the legends.

543

544 **Author contributions**

545 Sydney E. Cason, Conceptualization, Resources, Data curation, Formal analysis, Validation,

546 Investigation, Visualization, Methodology, Project administration, Writing—original draft and

547 review/editing; Erika L.F. Holzbaur, Conceptualization, Supervision, Funding acquisition, Project

548 administration, Writing—review/editing

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837

838 **Legends**

839 **Figure 1. JIP3/4 comigrate with and interact with dynein on autolysosomes. (A)** N-terminal region

840 of JIP3 and JIP4, scaled to the primary sequence. Arrows: green, JIP-DIC PLA only; magenta, LC3 only;

841 cyan, LAMP1 only; ochre, LC3 + LAMP1; white, LC3 + LAMP1 + JIP-DIC PLA. **(B)** Relative enrichment

842 (normalized number of peptides, see Methods for details) for JIP3 (Mapk8ip3) and JIP4 (Spag9) in the

843 proteomics performed by Goldsmith et al., (2022) and Dumrongprechachan et al., (2022). **(C-D)** Time

844 series demonstrating JIP3 and JIP4 comigration with LC3 (AV marker) and LAMP1 (lysosome marker).

845 **(E-F)** Example kymographs from the proximal axons of neurons transfected with JIP3 or JIP4.

846 Kymographs depict distance on the x-axis and time on the y-axis. Annotated kymographs (annot.) mirror

847 the above kymographs with the JIP3/4+ puncta paths pseudo-colored for visualization. **(G)** Quantification

848 of JIP3/4+ puncta moving retrograde (≥10µm towards the soma), anterograde (≥10µm towards the axon

849 tip), or exhibiting bidirectional/stationary motility (moving <10µm). n = 10 neurons; two-way ANOVA with

850 Sidak's multiple comparisons test (anterograde, P = 0.8976; stationary/bidirectional, P = 0.0767;

851 retrograde, P = 0.2792). **(H)** Fraction of motile events (≥ 10 µm either direction) moving retrograde. n =

852 10 neurons; unpaired t test (P = 0.9776). **(I)** Schematic illustrating the proximity ligation assay (PLA). **(J-**

853 **M)** Example micrographs and quantifications showing colocalization between LC3, LAMP1, and JIP3-

854 DIC (J-K) or JIP4-DIC (L-M) puncta. n = 20 neurons; one-way ANOVA with Tukey's multiple comparisons

855 test; JIP3 (LC3 v. LC3 + LAMP1, P < 0.0001; LC3 v. LAMP1, P = 0.7236; LAMP1 v. LC3 + LAMP1, P <

856 0.0001); JIP4 (LC3 v. LC3 + LAMP1, P < 0.0001; LC3 v. LAMP1, P = 0.9879; LAMP1 v. LC3 + LAMP1,

857 P < 0.0001).

858

859 **Figure 2. JIP3 and JIP4 induce dynein activity *in vitro*. (A)** Schematic illustrating our single-molecule

860 motility assay. **(B)** Example kymographs showing the growth and catastrophe dynamics used to

861 differentiate the plus-end of the microtubule from the more stable minus-end. **(C)** Quantification of the

862 directionality of runs on each microtubule. Runs were defined as events ≥ 0.8 µm in length towards either

863 the minus- or plus-end of the microtubule (MT). Symbols indicate comparison to the BICD2N dynein

864 positive control. Kruskal-Wallis test with Dunn's multiple comparisons. n = 20 MT each. K560 v. BICD2N,

865   P < 0.0001; K560 v. JIP3, P < 0.0001; K560 v. JIP4, P < 0.0001; BICD2N v. JIP3, P > 0.9999; BICD2N

866   v. JIP4, P > 0.9999; JIP3 v. JIP4, P > 0.9999. **(D-F)** Example kymograph and quantification showing the

867   activity of BICD2N-containing dynein complexes. **(G-I)** Example kymograph and quantification showing

868   the activity of JIP3-containing dynein complexes. **(J-L)** Example kymograph and quantification showing

869   the activity of JIP4-containing dynein complexes. All velocity histograms were fit to a Gaussian curve and

870   all run length histograms (1– cumulative distribution frequency) were fit to a one phase decay. Listed

871   values are median (25th percentile-75th percentile). n = 97-192 events. Complexes with a net direction of

872   "0" were stationary landing events, while complexes with a net direction of "–" or "+" moved ≥ 0.8 µm

873   towards the minus- or plus-end of the microtubule respectively. n = 20 MT each; Kruskal-Wallis test with

874   Dunn's multiple comparisons; JIP3 (0 v. –, P = 0.6412; 0 v. +, P = 0.0051; – v. +, P < 0.0001); JIP4 (0 v.

875   –, P > 0.9999; 0 v. +, P = 0.0004; – v. +, P < 0.0001).

876

877   **Figure 3. RAB10 overexpression inhibits retrograde autophagosomal transport. (A)** Example

878   western blot and quantification showing RAB10 in the AV fraction. n = 4 preparations; unpaired t test, P

879   = 0.1308. **(B-C)** Example kymograph and quantification showing the fraction of LC3 moving retrograde,

880   anterograde, or exhibiting bidirectional/stationary motion in the presence of EGFP alone (Control) or

881   EGFP-RAB10. n = 9 neurons; two-way ANOVA with Sidak's multiple comparisons test (anterograde, P

882   = 0.7646; stationary/bidirectional, P = 0.0166; retrograde, P = 0.0013). **(D)** Within the RAB10-expressing

883   cells, motility of the LC3+ puncta either colocalized with RAB10 (+ RAB10) or not (– RAB10). n = 9

884   neurons; two-way ANOVA with Sidak's multiple comparisons test (anterograde, P = 0.0897;

885   stationary/bidirectional, P = 0.0016; retrograde, P = 0.3700). **(E)** Fraction of autophagosomes (HT-LC3

886   only), autolysosomes (LC3+ LAMP1), or lysosomes (endogenous LAMP1 only) in fixed cells colocalized

887   with EGFP-RAB10. n = 14-16 neurons; one-way ANOVA with Tukey's multiple comparisons test (LC3 v.

888   LC3 + LAMP1, P = 0.0181; LC3 v. LAMP1, P = 0.0179; LAMP1 v. LC3 + LAMP1, P > 0.9999). **(F)** Of the

889   EGFP-RAB10 that was colocalized with LC3 and/or LAMP1, fraction colocalized with each organelle

890   type. n = 14-16 neurons; one-way ANOVA with Tukey's multiple comparisons test (LC3 v. LC3 + LAMP1,

891   P = 0.0070; LC3 v. LAMP1, P < 0.0001; LAMP1 v. LC3 + LAMP1, P = 0.0030).

892

Figure 4. RAB10 overexpression shifts the lysosomal population towards anterograde motility.

(A-B) Quantification and example kymographs showing the fraction of LC3 moving retrograde, anterograde, or exhibiting bidirectional/stationary motion in the presence of EGFP alone (Control) or EGFP-RAB10. n = 14 neurons; two-way ANOVA with Sidak's multiple comparisons test (anterograde, P = 0.8445; stationary/bidirectional, P = 0.7328; retrograde, P = 0.2696). **(C)** Fraction of the motile LAMP1 puncta (moving ≤ 10μm in either direction during a 2 min video) moving anterograde. n = 14 neurons; unpaired t test (P = 0.0297). **(D)** Within the RAB10-expressing cells, motility of the LAMP1+ puncta either colocalized with RAB10 (+ RAB10) or not (– RAB10). n = 9 neurons; two-way ANOVA with Sidak's multiple comparisons test (anterograde, P = 0.5595; stationary/bidirectional, P = 0.3011; retrograde, P = 0.9651).

903

Figure 5. ARF6 regulates the motility of autophagosomes in the axon in a GTP-dependent fashion.
(A) Example western blot and quantification showing ARF6 in the AV fraction. n = 4 preparations; unpaired t test, P = 0.0159. **(B)** Schematic illustrating the general characteristics of GTP- or GDP-ARF6 and the locked point mutants. **(C-D)** Example kymographs and quantification of mCherry (mCh)-LC3 motile fractions under the expression of CFP-ARF6^{WT}, ARF6^{Q67L}, or ARF6^{T27N}. n = 15-18 neurons; two-way ANOVA with Tukey's multiple comparisons; anterograde (WT v. QL, P = 0.9333; WT v. TN, P = 0.8377; QL v. TN, P = 0.9795); stationary/bidirectional (WT v. QL, P = 0.0824; WT v. TN, P = 0.0021; QL v. TN, P < 0.0001); retrograde (WT v. QL, P = 0.1723; WT v. TN, P = 0.0003; QL v. TN, P < 0.0001). Symbols indicate comparison to ARF6^{WT}. **(E)** Number of seconds paused per min in each of the three conditions (for all AVs). n = 83-111 puncta; Kruskal-Wallis test with Dunn's multiple comparisons; WT v. QL, P = 0.1526; WT v. TN, P = 0.0327; QL v. TN, P < 0.0001.

915

Figure 6. ARF6 GTP-locked mutant decreases retrograde lysosome pausing. (A-B) Example kymographs and quantification of LAMP1-HT motile fractions under the expression of CFP-ARF6^{WT}, ARF6^{Q67L}, or ARF6^{T27N}. n = 10 neurons; two-way ANOVA with Tukey's multiple comparisons; anterograde

919   (WT v. QL, P = 0.0428; WT v. TN, P = 0.0314; QL v. TN, P = 0.9915); stationary/bidirectional (WT v. QL,

920   P = 0.0011; WT v. TN, P = 0.2466; QL v. TN, P = 0.0960); retrograde (WT v. QL, P = 0.4213; WT v. TN,

921   P = 0.6013; QL v. TN, P = 0.0729). Symbols indicate comparison to ARF6^{WT}. **(C-F)** Number of seconds

922   paused per min in each of the 3 conditions for all LAMP1 puncta (C; n = 223-274 puncta; WT v. QL, P <

923   0.0001; WT v. TN, P = 0.0059; QL v. TN, P = 0.0001), LAMP1 with (D; n = 49-67 puncta; WT v. QL, P =

924   0.0029; WT v. TN, P > 0.9999; QL v. TN, P = 0.0265) and without LC3 (D; n = 174-206 puncta; WT v.

925   QL, P < 0.0001; WT v. TN, P = 0.0198; QL v. TN, P = 0.0431), and all the retrograde- (E; n = 49-65

926   puncta; WT v. QL, P = 0.0004; WT v. TN, P = 0.6390; QL v. TN, P = 0.0574) or anterograde- (E; n = 27-

927   73 puncta; WT v. QL, P = 0.0507; WT v. TN, P > 0.9999; QL v. TN, P = 0.0846) moving LAMP1 puncta.

928   Kruskal-Wallis test with Dunn's multiple comparisons.

929

930   **Figure 7. ARF6 GEFs are enriched on the outer AV membrane and may act to locally enrich ARF6-**

931   **GTP. (A)** Schematic illustrating the GAP-GEF cycle for small GTPases. **(B)** Relative enrichment

932   (normalized number of peptides, see Methods for details) for ARF6 GEFs and GAPs in the proteomics

933   performed by Goldsmith et al., (2022) and Dumrongprechachan et al., (2022). All of the known ARF6

934   GEFs and GAPs that were detected in either organelle enrichment are listed in the figure. Note that some

935   ARF6 GAPs/GEFs were not found in either enrichment [EFA6A-D (GEFs), GIT2 (GAP), ADAP1 (GAP),

936   ACAP1,3 (GAP), ASAP3 (GAP), ARAP1,3 (GAP)]. **(C)** Example western blot and **(D-G)** quantification

937   showing GEF enrichment in the AV fraction. **(H)** Example western blot and **(I-K)** quantification showing

938   GAPs in the AV fraction. n = 4 preparations; unpaired t test; (D) P = 0.0139; (E) P = 0.0038; (F) P =

939   0.0701; (G) P = 0.0050; (I) P = 0.1549; (J) P = 0.3208; (K) P = 0.7078.

940

941   **Figure 8. ARF6 induces the recruitment of JIP3/4 to microtubules. (A)** Example kymographs showing

942   the activity of JIP3- and JIP4- containing complexes in the presence of CFP-ARF6^{Q67L}. **(B-C)**

943   Quantification of the number of total landing events for JIP3- and JIP4-containing complexes in the

944   presence or absence of CFP-Arf6^{Q67L}. n = 20 MT each; unpaired t test; JIP3, P < 0.0001; JIP4, P =

945   0.0011. **(D-E)** Number of events (per μm microtubule per min) observed for JIP3-containing and JIP4-

946   containing complexes, either in the presence or absence of ARF6^{Q67L}. Complexes with a net direction of

947   "0" were stationary landing events, while complexes with a net direction of "–" or "+" moved ≥ 0.8 μm

948   towards the minus- or plus-end of the microtubule respectively. Note that the –ARF6 data from is repeated

949   from Figure 2. Kruskal-Wallis test with Dunn's multiple comparisons. n = 20 MT each. JIP3 –ARF6 v. +

950   ARF6: 0, $P < 0.0001$; –, $P > 0.9999$; +, $P > 0.9999$. JIP4 –ARF6 v. + ARF6: 0, $P = 0.0033$; –, $P > 0.9999$;

951   +, $P > 0.9999$.

952

953   **Figure 9. Integrated model of autophagosome, autolysosome, and lysosome transport along**

954   **axons. (A)** We demonstrate that the GTP-bound ARF6 is enriched on AV membranes, where it can

955   recruit its interacting partners JIP3 or JIP4. JIP3/4 can then recruit dynactin and dynein and activate

956   minus-end-directed retrograde motility especially of autolysosomes. By contrast, we propose that ARF6

957   is removed from the lysosomal membrane by local ARF6 GAP activity, which may be promoted by the

958   presence of phosphorylated RABs, including RAB10. RAB10 plays an unknown role in transport, but

959   seems to induce anterograde transit, possibly through a JIP3- or JIP4-JIP1-kinesin-1 complex. These

960   motor complexes are not the only ones involved in AV or lysosome transport; we highlight a few

961   complementary complexes on the left. **(B)** One mechanism by which this pathway may be disrupted in

962   neurodegeneration is via hyperphosphorylation of RABs. The disease-causing mutations in LRRK2

963   kinase result in increased phospho-RABs and also increased recruitment of kinesin-1 to the AV

964   membrane. However, the resulting loss of AV motility can be rescued by expressing GTP-locked ARF6;

965   thus these motor-regulatory mechanisms are interconnected and possibly competitive.

966 **Supplement legends**

967 **Figure S1. JIP3 or JIP4 overexpression does not affect AV or lysosome transport. (A-C)** Example

968 kymographs showing LC3 and LAMP1 puncta motile behavior in the axons of neurons expressing

969 HaloTag (HT) alone (Tag), HT-JIP3, or HT-JIP4. Annotated kymographs (annot.) show paths pseudo-

970 colored for visualization; heavier weight lines represent paths with both LC3 and LAMP1 co-migrating.

971 **(D-E)** Quantification of the fraction of LC3 or LAMP1 puncta moving retrograde (≥10μm towards the

972 soma), anterograde (≥10μm towards the axon tip), or exhibiting bidirectional/stationary motility (moving

973 <10μm). Symbols indicate comparison to Tag; n = 15 neurons; two-way ANOVA with Tukey's multiple

974 comparisons test; LC3 [variation between motile fractions ($P < 0.0001$) but no variation between

975 conditions ($P > 0.9999$) nor interaction ($P = 0.4514$)]; LAMP1 [variation between motile fractions ($P <$

976 0.0001) and mild interaction ($P = 0.0032$) but no variation between conditions ($P > 0.9999$)]. **(F-G)** LC3

977 and LAMP1 puncta density (per μm in a 2 min video) in cells expression HT-JIP3, HT-JIP4, or Tag. n =

978 11-15 neurons; one-way ANOVA with Tukey's multiple comparisons test (LC3, $P = 0.9076$; LAMP1, $P =$

979 0.9397). **(H-I)** Colocalization between LC3 and LAMP1 puncta in cells expression HT-JIP3, HT-JIP4, or

980 Tag. n = 11-15 neurons; one-way ANOVA with Tukey's multiple comparisons test (LC3, $P = 0.3154$;

981 LAMP1, $P = 0.5569$). **(J-K)** Western blot and quantification demonstrating that HT-JIP3 or JIP4 in the

982 CMV backbone expresses far more highly than in the EGFP backbone (EGFP sequence has been

983 removed by subcloning). This experiment was performed using COS-7 cells, which we transfected at the

984 same confluence (~50%) with FuGene and equal DNA quantities. After lysis in RIPA buffer, we assessed

985 for protein concentration using BCA assay. Equal protein concentrations were loaded, which was

986 confirmed using Revert Total Protein Stain. Finally, a monoclonal HT antibody was used to assess

987 expression of the HT proteins. n = 3; one-way ANOVA with Sidak's multiple comparisons test (JIP3 EGFP

988 v. JIP3 CMV, $P = 0.0051$; JIP4 EGFP v. JIP4 CMV, $P = 0.0444$; JIP3 EGFP v. JIP4 EGFP, $P = 0.9118$;

989 JIP3 CMV v. JIP4 CMV, $P = 0.0857$). **(L)** Example PLA negative (Neg.) control (missing JIP3/4 antibody).

990 **(M)** Quantification of DIC PLA puncta either with JIP3, JIP4, or no second 1° antibody (Neg. control). n =

991 20-21 neurons; one-way ANOVA with Tukey's multiple comparisons test (JIP3 v. JIP4, $P = 0.6763$; JIP3

992 v. Neg., $P < 0.0001$; JIP4 v. Neg., $P < 0.0001$).

993

994   **Figure S2. JIP3 and JIP4 do not induce kinesin activity *in vitro*. (A)** Example kymograph of labeled

995   KIF5C$^{1-560}$ (K560) activity. **(B)** Number of events (per μm microtubule per min) observed for K560-

996   containing complexes. n = 20 MT each; Kruskal-Wallis test with Dunn's multiple comparisons; K560: 0 v.

997   –, P = 0.0004; 0 v. +, P = 0.3092; – v. +, P < 0.0001. **(C-E)** Quantification of velocities and run lengths

998   towards the MT plus end for K560-, JIP3-, or JIP4-containing complexes. All velocity histograms were fit

999   to a Gaussian curve and all run length histograms (1– cumulative distribution frequency) were fit to a one

1000   phase decay. Listed values are median (25th percentile-75th percentile). JIP3/JIP4, n = 19-22 events;

1001   K560, n = 160 events. **(F-G)** Quantification of the directionality of runs on each microtubule. Runs were

1002   defined as events ≥ 0.8 μm in length towards either the minus- or plus-end of the microtubule. Note that

1003   the + LIS1 and – KIF5C & KLC2 are repeated from Figure 2. n = 15-20 MT each; Kruskal-Wallis test with

1004   Dunn's multiple comparisons; LIS1: JIP3 P > 0.9999, JIP4 P > 0.9999; KIF5&KLC: JIP3 P = 0.3572, JIP4

1005   P > 0.9999.

1006

1007   **Figure S3. RAB10 expression does not affect the formation of JIP3/4-dynein complexes. (A-B)**

1008   Quantification of mSc-LC3 puncta linear density and colocalization with LAMP1-HT. n = 9 neurons;

1009   unpaired t test; density, P = 0.7068; colocalization, P = 0.3086. **(C-D)** Quantification of LAMP1-HT puncta

1010   linear density and colocalization with mSc-LC3. n = 9 neurons; unpaired t test; density, P = 0.0438;

1011   colocalization, P = 0.8564. **(E-F)** Total JIP3- or JIP4-DIC PLA puncta linear density, compared between

1012   cells expressing EGFP alone (Tag) and cells expressing EGFP-RAB10. Note that the Tag data is

1013   repeated from Fig. S1 M. n = 20 neurons; unpaired t test; JIP3, P = 0.3278; JIP4, P = 0.5541. **(G-H)**

1014   Colocalization between JIP3/4-DIC PLA puncta and AVs or lysosomes. Note that Note that the Tag data

1015   is repeated from Fig. 1 K, M. n = 20 neurons; one-way ANOVA with Tukey's multiple comparisons test;

1016   JIP3 (LC3 only, P = 0.7989; LC3 + LAMP1, P = 0.9971; LAMP1 only, P = 0.7486); JIP4 (LC3 only, P =

1017   0.9984; LC3 + LAMP1, P = 0.2160; LAMP1 only, P > 0.9999).

1018

1019 **Figure S4. ARF6 expression does not affect LC3 or LAMP1 density or colocalization. (A)**

1020 Quantification of mCh-LC3 puncta linear density. n = 13-16 neurons; one-way ANOVA with Sidak's

1021 multiple comparisons test; WT v. QL, P = 0.6382; WT v. TN, P = 0.8339; QL v. TN, P = 0.6382. **(B)**

1022 Quantification of mCh-LC3 colocalization with LAMP1-HT. one-way ANOVA with Sidak's multiple

1023 comparisons test; n = 9-11 neurons; WT v. QL, P = 0.4183; WT v. TN, P = 0.9791; QL v. TN, P = 0.4183.

1024 **(C)** Quantification of LC3 non-processive movement, as described by Δ run length (net run length of each

1025 vesicle subtracted from its total run displacement). n = 71-111 puncta; Kruskal-Wallis test with Dunn's

1026 multiple comparisons test; WT v. QL, P > 0.9999; WT v. TN, P > 0.9999; QL v. TN, P > 0.9999. **(D)**

1027 Quantification of LAMP1-HT puncta linear density. one-way ANOVA with Sidak's multiple comparisons

1028 test; n = 10 neurons; WT v. QL, P = 0.1665; WT v. TN, P = 0.6735; QL v. TN, P = 0.2495. **(E)**

1029 Quantification of LAMP1-HT colocalization with mCh-LC3. one-way ANOVA with Sidak's multiple

1030 comparisons test; n = 10 neurons; WT v. QL, P = 0.6020; WT v. TN, P = 0.5742; QL v. TN, P = 0.3846.

1031 **(F)** Quantification of LAMP1 non-processive movement, as described by Δ run length. n = 13-16 neurons;

1032 Kruskal-Wallis test with Dunn's multiple comparisons test; WT v. QL, P > 0.9999; WT v. TN, P > 0.9999;

1033 QL v. TN, P > 0.9999.

1034

1035 **Figure S5. ARF6 does not affect motile events *in vitro*. (A)** Example kymographs showing the activity

1036 of JIP3- and JIP4- containing complexes in the presence of CFP-ARF6^{T27N}. **(B-C)** Quantification of the

1037 number of total landing events for JIP3- and JIP4-containing complexes in the presence of CFP-Arf6^{Q67L}

1038 or CFP-Arf6^{T27N}. n = 20-23 MT each; unpaired t test; JIP3, P < 0.6856; JIP4, P = 0.1028. **(D-E)** Number

1039 of events (per μm microtubule per min) observed for JIP3-containing and JIP4-containing complexes in

1040 the presence of CFP-Arf6^{Q67L} or CFP-Arf6^{T27N}. Complexes with a net direction of "0" were stationary

1041 landing events, while complexes with a net direction of "−" or "+" moved ≥ 0.8 μm towards the minus- or

1042 plus-end of the microtubule respectively. Note that the Arf6^{QL} data from is repeated from Figure 8.

1043 Kruskal-Wallis test with Dunn's multiple comparisons. n = 20-23 MT each. JIP3 QL v. TN: 0, > 0.9999; −

1044 , P > 0.9999; +, P > 0.9999. JIP4 QL v. TN: 0, 0, > 0.9999; −, P = 0.9389; +, P > 0.9999. Dashed lines

1045 indicate mean without added ARF6. **(F)** Quantification of the activity of JIP3 or JIP4-containing dynein

1046   complexes in the presence of CFP-Arf6^{Q67L}. **(G)** Quantification of the activity of JIP3 or JIP4-containing

1047   kinesin complexes in the presence of CFP-Arf6^{Q67L}. All velocity histograms were fit to a Gaussian curve

1048   and all run length histograms (1– cumulative distribution frequency) were fit to a one phase decay. Listed

1049   values are median (25th percentile-75th percentile). n = 39-140 events.

A

JIP3

KIF5, DLIC, DHC, p150, KLC, ARF6, RAB10

N - 1 60 115 490 575 1336 - C

JIP4

N - 1 55 100 480 555 1321 - C

Dynein heavy chain (DHC) binding

p150^Glued (p150) binding

Kinesin light chain (KLC) binding

ARF6 binding

RAB10 binding

Dynein light intermediate chain (DLIC) binding

Kinesin heavy chain (KIF5) binding

B Relative enrichment

JIP3, JIP4 — Autophagic vacuole (AV)

JIP3, JIP4 — Lysosome

C

HT-JIP3, mSc-LC3, mNeon-LAMP1

2 μm

to soma →

0 s, 2 s, 4 s, 6 s

D

HT-JIP4, mSc-LC3, mNeon-LAMP1

2 μm

0 s, 2 s, 4 s, 6 s

Retrograde — Stationary / Bidirectional — Anterograde

E

HT-JIP3

annot.

to soma →

F

HT-JIP4

annot.

5 μm

1 min

G

Motile fractions

JIP3 JIP4

ns, ns, ns

H

Fraction of runs retrograde

JIP3 JIP4

ns

I

① Transfect fluorescent LC3

② Fix neurons

③ Add 1° antibodies (JIP3/4, DIC, & LAMP1)

④ Add PLA 2° antibodies conjugated to oligos

⑤ Polymerase connects & amplifies oligos (where DIC–JIP3/4 are within 40nm)

⑥ Add fluorescently-tagged oligo

⑦ Add fluorescent 2° antibody to anti-LAMP1

-LC3- -LAMP1

endogenous JIP3 or JIP4

endogenous dynein (DIC)

J JIP3

PLA, LC3, LAMP1, Merge

3 μm

L JIP4

PLA, LC3, LAMP1, Merge

3 μm

K

Colocalized with DIC PLA puncta

LC3 only, LC3 + LAMP1, LAMP1 only

ns, ****, ****

M

Colocalized with DIC PLA puncta

LC3 only, LC3 + LAMP1, LAMP1 only

ns, ****, ****

A COS-7 expressing HT - JIP3 / JIP4 / BICD2N + HA - LIS1 → Add HT ligand and lyse → dynamic microtubules growth/catastrophe

KLC2 KIF5C JIP3/4 Dynactin Dynein

rigor kinesin   coverslip

TIRF illumination

B Example MT dynamics

C Fraction of runs to the minus-end: BICD2N, JIP3 (ns), JIP4 (ns), K560 (****)

D BICD2N **G** JIP3 **J** JIP4

E BICD2N events (µm⁻¹min⁻¹)

F BICD2N: 1.7 µms⁻¹ (1.2-2.4 µms⁻¹) — BICD2N: 2.4 µm (1.8-3.7 µm)

H JIP3 events (µm⁻¹min⁻¹)

I JIP3: 2.0 µms⁻¹ (1.3-3.2 µms⁻¹) — JIP3: 2.7 µm (1.8-4.0 µm)

K JIP4 events (µm⁻¹min⁻¹)

L JIP4: 1.6 µms⁻¹ (1.0-2.0 µms⁻¹) — JIP4: 2.6 µm (1.9-3.8 µm)

A

25 —

RAB10 (normalized to brain lysate)

ns

AV   PK

B

to soma

5 μm

Control mSc-LC3

Control annot.

with EGFP-RAB10 mSc-LC3

with EGFP-RAB10 annot.

1 min

Retrograde
Stationary / Bidirectional
Anterograde

C

LC3+ puncta (fraction)

**
*
ns

Control   RAB10

D

LC3+ puncta (fraction)

ns
**
ns

−RAB10   +RAB10

in RAB10-expressing cell

E

Each organelle type colocalized with RAB10 (fraction)

*
*
ns

LC3 only   LC3 + LAMP1   LAMP1 only

F

RAB10 colocalized with each organelle type (fraction)

****
**
**

LC3 only   LC3 + LAMP1   LAMP1 only

A to soma   5 µm   1 min

Control | LAMP1-HT | annotated

with EGFP-RAB10 | LAMP1-HT | annotated

- Retrograde
- Stationary / Bidirectional
- Anterograde

B LAMP1+ puncta (fraction) — Control, RAB10 — ns, ns, ns

C Anterograde LAMP1+ (fraction of motile) — Control, RAB10 — *

D LAMP1+ puncta (fraction) — − RAB10, + RAB10 — ns, ns, ns — in RAB10-expressing cell

A

Brain lysate | Autophagic vacuole (AV) | AV + Proteinase K (PK)

25-

ARF6

ARF6 (normalized to brain lysate)

AV | PK

*

B

GTP-locked: ARF6^{Q67L} (QL)

GDP-locked: ARF6^{T27N} (TN)

GTP —— GDP

increased JIP3/4-p150^{Glued} binding

increased JIP3/4-KLC binding

C

to soma →

5 μm

1 min

with CFP-ARF6 WT / mCh-LC3

annotated

with CFP-ARF6 QL / mCh-LC3

annotated

with CFP-ARF6 TN / mCh-LC3

annotated

Retrograde

Stationary / Bidirectional

Anterograde

D

LC3+ puncta (fraction)

WT | QL | TN

ARF6

ns | ***
| **
ns | ns
ns | ns

E

Sec paused (min^{-1})

WT | QL | TN

ns | ****

*

A

to soma

5 µm

1 min

with CFP-ARF6 WT — LAMP1-HT

annotated

with CFP-ARF6 QL — LAMP1-HT

annotated

with CFP-ARF6 TN — LAMP1-HT

annotated

Retrograde

Stationary / Bidirectional

Anterograde

B

LAMP1+ puncta (fraction)

WT   QL   TN

ARF6

ns
ns
**
*
*

C

Sec paused (min^{-1})

WT   QL   TN

**
****
***

D

Sec paused (min^{-1})

ns
ns
ns

**   *
****   *

WT   QL   TN   WT   QL   TN

with LC3   without LC3

E

Sec paused (min^{-1})

ns
***   ns

WT   QL   TN

Retrograde

ns
ns   ns

WT   QL   TN

Anterograde

A GTPase Activating Protein (GAP) / GTP hydrolysis; Guanine Exchange Factor (GEF) / GDP release & GTP binding cycle diagram (GTP, GDP)

B Lysosome | Autophagic vacuole (AV) — Relative Enrichment bar chart

Lysosome: CYTH1, CYTH2, CYTH3, IQSEC1, IQSEC2, IQSEC3 (ARF6 GEFs); AGAP1, AGAP2, AGAP3, AGFG1, AGFG2, ARAP2, ASAP1, ASAP2, SMAP1, SMAP2, GIT1, ACAP2, ADAP2 (ARF6 GAPs)

Autophagic vacuole (AV): CYTH1, CYTH2, CYTH3, IQSEC1, IQSEC2, IQSEC3 (ARF6 GEFs); AGAP1, AGAP2, AGAP3, AGFG1, AGFG2, ARAP2, ASAP1, ASAP2, SMAP1, SMAP2, GIT1, ACAP2, ADAP2 (ARF6 GAPs)

C GEFs — Brain lysate | Autophagic vacuole (AV) | AV + Proteinase K (PK): pan-CYTH (50), IQSEC1 (90), IQSEC2 (90), IQSEC3 (125)

D Immunoblot signal (normalized to brain lysate) — AV, PK — pan-CYTH — *

E IQSEC1 — AV, PK — **

F IQSEC2 — AV, PK — p = 0.0701

G IQSEC3 — AV, PK — **

H GAPs — Brain lysate | Autophagic vacuole (AV) | AV + Proteinase K (PK): SMAP2 (50), GIT1 (90), ACAP2 (90)

I GIT1 — AV, PK — p = 0.1549

J SMAP2 — AV, PK — p = 0.3208

K ACAP2 — AV, PK — p = 0.7078

A JIP3 + ARF6^{QL} JIP4 + ARF6^{QL}

B
JIP3 landing events ($\mu m^{-1} min^{-1}$)
****
$-$ ARF6 $+$ ARF6

C
JIP4 landing events ($\mu m^{-1} min^{-1}$)
**
$-$ ARF6 $+$ ARF6

D
JIP3 events ($\mu m^{-1} min^{-1}$)
ns
ns
****

E
JIP4 events ($\mu m^{-1} min^{-1}$)
ns
ns
**
0 $-$ $+$ 0 $-$ $+$
Net direction $-$ ARF6   Net direction $+$ ARF6

A

autophagosome

LC3

other lysosomes

RUFY3/4 and/or TMEM55B

GTP

BORC, SKIP, and ARL8

JIP1

immature autolysosome

LC3

LAMP1

Huntingtin and HAP1

mature autolysosome

LC3

ARF6 GEFs

LAMP1

GTP

ARF6

JIP3 or JIP4

lysosome

LAMP1

RAB10

P

ARF6 GAPs

JIP3/JIP4? with JIP1?

+

−

Healthy neuron

B

Parkinson's disease-causing mutation

Hyperactive LRRK2 kinase

LAMP1

RAB10

P

RAB35

P

LC3

RAB10

P

GTP

+

−

Increased anterograde bias

Non-motile (tug-of-war)