TraB is a novel component of the ER-Mitochondria contact site (EMCS) with dual roles in ER-Mitochondrial tethering and mitophagy

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Abstract

ER-mitochondria contact sites (EMCSs) are important for mitochondrial function. Here, we have identified a novel eukaryotic EMCS complex, comprising a family of uncharacterised mitochondrial outer-membrane proteins, TraB1 and the ER protein, VAP27-1. In *Arabidopsis*, there are two TraB1 isoforms and the *trab1a/trab1b* double mutant exhibits abnormal mitochondrial morphology, strong starch accumulation and impaired energy metabolism, indicating that these proteins are essential for normal mitochondrial function. Moreover, TraB1 proteins also interact with ATG8 in order to regulate mitochondrial degradation (mitophagy). The turnover of depolarised mitochondria is significantly reduced in both *trab1a/b* and VAP27 mutants (*vap27-1/3/4/6*) under mitochondrial stress conditions, with an increased population of dysfunctional mitochondria present in the cytoplasm. Consequently, plant recovery after stress is significantly perturbed. A similar phenotype is found in both autophagy mutants (*atg5* and *atg7*), suggesting that TraB1 regulated mitophagy and ER-mitochondrial tethering are two closely related processes, necessary for normal mitochondrial function. Taken together, we ascribe a dual role to TraB1 which is a novel component of the EMCS complex in eukaryotes, regulating both tethering of the mitochondria to the ER and mitophagy.

Key words: Membrane contact sites, Endoplasmic Reticulum, Mitochondria, Autophagy, Mitophagy, *Arabidopsis*

35 Introduction

36 In plant cells, organelles interact with each other to regulate multiple physiological
37 activities^{1,2,3,4,5}. Such intimate contact between the different organelles generates an
38 interactive network which facilitates rapid material and signal exchange. The endoplasmic
39 reticulum (ER) is physically connected to other membrane structures through membrane
40 contact sites⁶ (MCS), including the ER-PM contact sites^{7,8} (EPCSs) and ER-mitochondria
41 contact sites^{4,9} (ERMCs). The ERMC interactions in particular are essential in multiple
42 physiological activities, for example Ca^{2+} transfer, lipid metabolism, autophagy,
43 mitochondrial dynamics and morphogenesis^{3,4,9,10,11,12}. However, the protein components
44 and biological relevance of EMCSs are poorly understood in plants compared to their
45 counterparts in animals and fungi.

46 In order to maintain cellular homeostasis, impaired mitochondria have to be removed
47 effectively through a process called mitophagy, which is a highly selective autophagic
48 process. The successful removal of damaged mitochondria is able to reduce the production
49 of excessive ROS, maintaining the oxidation-reduction environment and the stability of
50 mitochondrial membrane potential^{13,14,15,16}. Interestingly, the formation of autophagosomes
51 (mitophagosomes) can also occur at ERMCs and it has been shown that altering the
52 structure of ERMCs can reduce the number of autophagosomes produced.^{3,14,17} Thus, it is
53 likely that the establishment of ER-mitochondrial connections and the initiation of
54 mitophagy are two inter-related processes.

55 Previous studies have shown that VAP27-1 localize to the ER-PM contact sites (EPCS) and the
56 entire ER membrane^{18,19,20,21,22,23}. VAP27-1 interacts with proteins involved in cytoskeleton
57 interaction (e.g NET3C), membrane trafficking (e.g AtEH1/Pan, Clathrin), lipid transport (e.g
58 ORP3a) and lipid-droplet biogenesis (e.g SEIPIN), regulating multiple subcellular activities.
59 Here, we identify a novel mitochondrial membrane protein TraB1a, which also interacts with
60 VAP27-1 to regulate ER-mitochondrial tethering. In parallel, TraB1 also interacts with ATG8
61 to regulate mitochondrial degradation functioning as putative mitophagy receptors. From a
62 combination of experimental approaches, our data indicate that TraB1-mediated EMCS
63 participates in mitophagy, which in turn maintains healthy mitochondrial function, and
64 energy homeostasis. These findings not only advance our knowledge of organelle
65 interactions and mitophagy in general, but also bridge the gap in our understanding of how
66 EMCSs and mitophagy are regulated *in plantae*.

67

68 Results

69 TraB1 localizes to the mitochondria outer membranes and interacts with VAP27 at 70 ER-Mitochondria contact sites

71 In eukaryotic cells, the endoplasmic reticulum (ER) contacts with most
72 membranes^{2,7,21,24,25,26,27,28}. Similarly, the ER is also connected to the mitochondria through
73 ER-Mitochondria Contact Sites (EMCSs), which are essential in lipid metabolism, autophagy,
74 mitochondrial function and morphogenesis^{3,4,5,11,29}. Across eukaryotes, the ER-integral
75 membrane protein VAP27 (also known as VAP/Scs2) serves as a tethering factor between
76 the ER and various organelles^{18,19,30,31}. We therefore investigated whether VAP27 may
77 mediate contact sites between the ER and mitochondria in plants. In Arabidopsis lines co-
78 expressing VAP27-1-GFP and the mitochondrial matrix marker, Mito-mCherry, we observed
79 that some mitochondria partially co-localise with VAP27-1 on the ER surface (Figure S1a,
80 arrow); such a phenomenon was not observed in control cells expressing the ER lumen
81 marker, GFP-HDEL (Figure S1b), suggesting VAP27-1 may play an important role in mediating
82 ER-mitochondria tethering in plants.

83 To screen for the tethering proteins through which VAP27 mediates EMCSs, TAP (Tandem
84 affinity purification) tagging was performed using transgenic Arabidopsis cell cultures. After
85 affinity purification followed by a MS proteomic screen, we identified TraB1a (Figure 1a), a
86 mitochondrial protein with unknown function, as an interacting protein of VAP27-1. There
87 are two closely related TraB1 proteins encoded by the Arabidopsis genome, TraB1a and
88 TraB1b, both of which co-localize with VAP27-1-RFP at numerous donut-shaped structures
89 when co-expressed (Figure 1a-b). These structures are likely to be a hybrid membrane
90 consisting of the mitochondrial membrane and the ER membrane because both the GFP-
91 TraB1a/TraB1b and VAP27-1-YFP signal co-localise at circular structures surrounding Mito-
92 mCherry (Figure 1d). This phenomenon is likely caused by a direct interaction between TraB
93 and VAP27-1, as it is absent in cells only expressing VAP27-1-RFP (Figure 1e).

94 FRET-FLIM was used to further study these interactions. The fluorescence lifetimes of GFP-
95 TraB1a and GFP-TraB1b are reduced significantly in the presence of RFP-VAP27-1, indicating
96 physical interactions (Figure 1f-g). Furthermore these *in vivo* interaction data were further
97 confirmed using co-immunoprecipitation assays, and also BiFC. Taken together we conclude
98 that TraB1 and VAP27-1 interact (Figure 1h-i) and that this interaction may serve to mediate
99 the formation of EMCS.

cells expressing VAP27-1-RFP alone. **g.** FRET-FLIM further proved the interactions between TraB1a/VAP27-1 and TraB1b/VAP27-1. The fluorescence life-time of GFP-TraB1a (control) and GFP-TraB1b changes significantly (life-time reduces from 2.50 ± 0.01 to 2.31 ± 0.02 ns; and from 2.50 ± 0.01 to 2.21 ± 0.02 ns, respectively) in the presence of RFP-VAP27-1. **h.** Split-YFP based BiFC study showed nYFP-VAP27-1 and cYFP-TraB1 producing signals when co-expressed in *N. benthamiana*. **i.** The interaction between VAP27-1 and TraB1 is confirmed by a co-precipitation assay (GFP-Trap) using GFP-TraB1a and VAP27-1-RFP expressing plants. The majority of the GFP-TraB1a protein is trapped in the pellet fraction (left), where VAP27-1-RFP is also pulled-down in the presence of GFP-TraB1a (right). **j.** Phylogenetic tree of TraB homologues showing that Arabidopsis TraB1a and TraB1b originate from the same clade, whereas TraB2 belongs to a separate clade that is more evolutionarily similar to their mammalian homologues. **k.** In Arabidopsis, GFP-TraB1a localizes to the outer membrane of mitochondria that is labelled by the mitochondrial matrix marker, Mito-mCherry. **l.** GFP-TraB1b localizes to the ER network as well as the outer mitochondrial membrane. **m.** RFP-TraB2 mostly localizes to the cytoplasm. **n.** In citrus, only one isoform of TraB1 exists, which also localizes to the outer mitochondrial membrane in *N. benthamiana* leaf epidermal cells. $N \geq 10$ for every FRET-FLIM analysis, error bars are SEM, *** $P < 0.001$ in Student's *t* tests (Scale bar = 10 μ m).

127

128 **Phylogenetic study of TraB proteins and their function in regulating mitochondrial** 129 **activity**

130 TraB1 proteins contain a C-terminal transmembrane domain, and a TraB-homology domain
131 which currently has unknown function in eukaryotes (Figure 1a). In Arabidopsis, TraB1a and
132 TraB1b are highly conserved, whilst TraB2 is more divergent (Figure 1j). GFP-TraB1a localises
133 to ring-like structures that appear to be the outer membrane of mitochondria (OMM, Figure
134 1k), while the subcellular localisation of TraB1b, the closest homologue of TraB1 (sharing
135 73.4% peptide similarity), is found both at the ER network and the mitochondrial outer
136 membranes (Figure 1i). These results are consistent with previous observations from a
137 proteomic analysis^{32,33}, where it was found that endogenous TraB1a is enriched in
138 mitochondria in both Arabidopsis, and citrus fruits. Plant TraB2 isoforms (which are mainly
139 localized to the cytoplasm; Figure 1m) are more closely related to the mammalian and
140 bacterial TraB proteins, suggesting that this is likely to be an ancient form appearing earlier
141 in evolution (Figure 1j). The mitochondrial localisation of TraB1 is likely to be conserved in
142 many higher plants, as the citrus homologue of TraB1, CsTraB1 also localised to the OMM
143 (Figure 1n).

144 TraB1a and TraB1b is expressed in most plant tissues with the strongest expression found in
145 roots and flowers (Figure S2a-b). Under the control of their own promoters, expression of
146 both GFP-TraB1a and GFP-TraB1b is further confirmed in vegetative tissues (Figure S2c-e). As
147 they exhibit similar expression profiles and sequence homology, further studies were
148 performed mainly using TraB1a as the example.

149 **TraB1 proteins are important in mitochondrial movement, morphology and energy**
150 **metabolism.**

151 Endogenous TraB1a is found localized to the outer mitochondria membrane and stays
152 closely associated with VAP27 in root cells (Figure 2a-b). This was demonstrated by
153 immunofluorescence using an antibody that is specific to TraB1a (Figure S2f). These results
154 confirm the live cell imaging data, suggesting that the endogenous TraB1 is also likely to
155 facilitate the ER-mitochondria association. In the presence of over-expressed GFP-TraB1a or
156 VAP27-1-RFP, the percentage of stationary mitochondria (that stay immobile for more than
157 6s, Figure 2c) increased significantly, likely due to an increment of ER-mitochondria tethering
158 (Figure 2d-e). Enhanced ER-mitochondrial association at the ultrastructural level is observed
159 in cells over-expressing GFP-TraB1a (Figure 2f-h). Taken together, we have identified a novel
160 EMCS complex comprising the ER-localised VAP27 and the OMM-localised TraB1.

161 When TraB1a is over-expressed in *N. benthamiana* leaf epidermal cells, the mitochondria
162 became enlarged and swollen (Figure S3a), and the morphology of mitochondria changes
163 from a tubular/oval shape to a spherical shape (Figure S3b). Such phenotypes are commonly
164 observed when the functions of mitochondria are inhibited^{34,35,36}. At the ultrastructural level,
165 these mitochondria appear to be aggregated and more electron transparent, suggesting
166 mitochondrial function is likely impaired (Figure S3c-d). TMRM stains the mitochondria
167 according to their electron potential, which can be used as an indicator for mitochondrial
168 activity. In cells treated with antimycin (a disruptor of ATP synthesis), a significant reduction
169 of TMRM fluorescence is seen (Figure S3e). Interestingly, the TMRM signal is weaker in
170 Arabidopsis stably expressing GFP-TraB1a (Figure S3f) in contrast to control plants
171 expressing Mito-YFP (Figure S3g), indicating that the expression of GFP-TraB1a results in
172 decreased mitochondrial membrane potential and activity (Figure S3h-i).

173 **The function of mitochondria is affected in the *trab1a/b* mutant, indicated by a** 174 **change in morphology, reduction of ATP and starch accumulation**

175 Considering the high possibility of functional redundancy, the T-DNA double mutant of
176 *trab1a/trab1b* was generated to further study the function of TraB1 (Figure S4a-b). The
177 structure and morphology of mitochondria were analysed using light and electron
178 microscopy. In the *trab1a/trab1b* T-DNA mutants, a large proportion of the mitochondria
179 show a spherical shape and become aggregated which is in contrast to that observed in the
180 wild type (Figure 2i-k). At the ultrastructural level, the structure of the mitochondria is
181 disrupted in the *trab1a/b* mutants (Figure 2l-m). Moreover, the total ATP level and ATP/ADP
182 ratio is reduced significantly in the double mutant (Figure 2n), indicating an aberrant energy
183 metabolism.

184 In plants, starch is an energy reserve and its level in plants is closely related to energy
 185 homeostasis^{37,38}. In agreement with the energy metabolism defect, we found strong starch
 186 accumulation in the *trab1a/trab1b* mutant (Figure 2o-q). In order to eliminate the influence
 187 from photosynthesis, and observe starch consumption directly related to mitochondrial
 188 activity, we further analysed the change in starch levels under dark conditions. We found a
 189 decrease in the reduction of the starch level in the *trab1a/trab1b* mutants compared to the
 190 wild type (Figure 2r), suggesting starch consumption is inhibited. Meanwhile, another
 191 independent *trab1a/b* double mutant was also created using CRISPR/Cas9 (Figure S5a) and a
 192 similar starch phenotype was observed (Figure S5c-d). In summary, these observations are
 193 consistent with the functions of TraB1 in regulating mitochondrial activity and ER-
 194 mitochondria tethering, since mitochondrial function and integrity are regulated by
 195 EMCSs^{39,40,41,42}.

Figure 2. TraB1 proteins are essential for normal mitochondrial movement, morphology and energy metabolism. **a-b.** Arabidopsis expressing Mito-mCherry and VAP27-RFP was immuno-labelled with a TraB1a antibody; the endogenous protein localized at the outer mitochondrial membrane, and in contact with VAP27-labelled ER membrane, respectively. **c.** An example of stationary mitochondria (arrow) that stay immobile during the six second time course. **d-e.** The percentage of stationary mitochondria (immobile for at least six seconds) increased significantly in the presence of GFP-TraB1a or VAP27-1. **f-h.** TEM study of wild type Arabidopsis (f) and stable Arabidopsis transformed with GFP-TraB1 (g), the distance between ER and mitochondria is significantly reduced compared to wild type plants (h). **i-k.** In the *trab1a/trab1b* T-DNA mutant Arabidopsis line stably expressing Mito-mCherry, the morphology of mitochondria is significantly altered, as indicated by the increased population of circular mitochondria (aspect ratio at 1-1.3, k). Mitochondrial aggregations (j, inset) were also frequently observed. **l-m.** In the *trab1a/trab1b* Arabidopsis, mitochondrial structures are also disrupted at the ultrastructural level (m); their internal membrane cisternae are more fragmented and the matrix becomes more electron-transparent compared with that in the wild type (l). **n.** The levels of ATP, ADP and AMP are all significantly reduced in the *trab1a/trab1b* mutant, suggesting a disruption in energy metabolism and mitochondrial function. **o-q.** The starch levels in Col-0, *trab1a/trab1b* T-DNA plants (4-5 weeks old) were analysed by Lugol staining and the *trab1a/trab1b* loss-of-function mutant accumulated high levels of starch by the end of the light cycle (p). Such

215 results were confirmed by spectrophotometry (q). r. Under dark conditions, starch consumption is
216 reduced in the *trab1a/trab1b* mutant, suggesting a reduction in mitochondrial respiration. N ≥ 15 for
217 mitochondria movement analysis; N ≥ 20 for TEM analysis; N = 9 for starch quantification; N ≥ 100 for
218 mitochondria morphology analysis; N = 8 for ATP measurement, error bars are SEM, * 0.01 < P < 0.05,
219 *** P < 0.001 in Student's t tests (Scale bar = 10 μm for light microscopy; Scale bar = 500 nm for TEM).

220 **TraB1 interacts with ATG8 through the conserved AIM motif**

221 As the molecular functions of TraB1 are yet uncharacterised, we performed further
222 sequence analysis and we found two ATG8-interacting motifs (AIM) in both proteins,
223 suggesting it may interact with ATG8 and participate in the autophagy pathway^{43,44}.
224 Therefore, stable Arabidopsis lines expressing GFP-ATG8a and RFP-TraB1a were generated.
225 We found that ATG8a signal is recruited to TraB1 labelled mitochondria under normal
226 growth conditions (Figure 3a); such co-localisation is rarely found in cells expressing CFP-
227 ATG8a and Mito-mCherry (Figure 3b).

228 Next, we mutated the putative AIM motifs of TraB1a (denoted as TraB1aΔAIMs, Figure S4c).

229 GFP-TraB1aΔAIMs localizes to the mitochondria like the full-length protein (Figure 3c).

230 However, its co-localization with ATG8 was found to be significantly reduced (Figure 3d).

231 FRET-FLIM was used to study the interaction of TraB1aΔAIMs with ATG8 (Figure 3e-g). We

232 observed that the fluorescence lifetimes of GFP-TraB1a are reduced significantly in the

233 presence of RFP-ATG8e, indicating physical interactions, whereas the GFP lifetimes exhibit

234 little reduction when GFP-TraB1aΔAIMs is expressed with RFP-ATG8e using the same

235 conditions (Figure 3g), indicating that GFP-TraB1a ΔAIMs does not interact with RFP-ATG8e.

236 Similarly, co-localization and interaction between TraB1b and ATG8 was confirmed *in vivo*

237 (Figure S6a-c). Using one-on-one yeast-two-hybrid assays, the yeast strains only grew on

238 selective medium when co-transformed with non-mutated TraB1 and ATG8, but were not

239 able to grow when co-transformed with TraB1aΔAIMs and ATG8 (Figure 3h). These data

240 indicate that the AIM motif is essential for the interaction between TraB1a and ATG8e.

241 It is known that DNP promotes mitochondrial depolarisation and mitophagy^{45,46,47}. After DNP

242 treatment, time lapse confocal microscopy revealed the dynamic recruitment of ATG8

243 labelled autophagosome membranes to the swollen mitochondria in the presence of TraB1a

244 (Figure 2i) which is consistent with a role for TraB1 in its interaction with ATG8.

Figure 3. TraB1 interacts with ATG8 through the ATG8 interacting motif (AIM). **a.** In Arabidopsis stably transformed with RFP-TraB1a and GFP-ATG8e under non-stressed conditions, GFP-ATG8e positive structures are recruited to the mitochondria that are labelled with RFP-TraB1a in hypocotyl epidermal cells. **b.** Such ATG8e-mitochondrial co-localization is rarely seen in cells expressing a mitochondrial maker, Mito-mCherry. **c-d.** *N. benthamiana* leaf epidermal cells expressing GFP-TraB1 Δ AIMs (TraB1a without the ATG8 interacting motif) with a mitochondrial marker (c) and an autophagosome marker (d). GFP-TraB1 Δ AIMs still localizes to the mitochondrial outer membrane but did not co-localise with ATG8. **e-g.** FRET-FLIM further proved that the interactions between TraB1a and ATG8 is reliant on the AIM motifs. The fluorescence life-time of GFP-TraB1a (control) changes significantly (life-time reduces from 2.49 ± 0.01 to 2.30 ± 0.02 ns) in the presence of RFP-ATG8e, while the fluorescence life-time of GFP-TraB1 Δ AIMs (control) only dropped by 0.04 ns in the presence of RFP-ATG8e; such a difference is too little to indicate an interaction. **h.** Using a one-on-one yeast-2-hybrid assay, ATG8e was confirmed to interact with full length TraB1a but not with the TraB1 Δ AIMs mutant. **i.** Following DNP-induced mitochondrial depolarization in Arabidopsis root cells expressing GFP-ATG8e and RFP-TraB1a, the ATG8e labelled autophagosome membrane was recruited to a swollen mitochondrion that is labelled by TraB1a during a course of 45 seconds. $N \geq 10$ for every FRET-FLIM analysis, error bars are SEM, * $0.01 < P < 0.05$, *** $P < 0.001$ in Student's t tests (Scale bar = $10 \mu\text{m}$).

The turnover of TraB1 is dependent on the autophagy pathway

Concanamycin A (ConcA) is a V-ATPase inhibitor that inhibits lytic vacuole activities and induces autophagosome accumulation (Figure 4a-b)⁴⁸ and we have used it to determine the

autophagy flux of GFP-TraB1a under different conditions. In lines co-expressing RFP-TraB1 and GFP-ATG8a, large numbers of the TraB1 labelled puncta co-localize with ATG8 (Figure 4c-d) after Conc A treatment, suggesting that TraB1 is targeted to the vacuole with the autophagosomes. The number of vacuole-accumulated autophagosomes increases significantly in the presence of GFP-TraB1 (Figure 4e). Similarly, in cells expressing GFP-TraB1a and Mito-mCherry, both proteins co-localize in the vacuole after Conc A treatment (Figure 4f-i), and the number of vacuole-accumulated mitochondria increased significantly (Figure 4j) in contrast to the control cells only expressing Mito-mCherry (Figure 4f-g). These data suggest a possible function for TraB1 in promoting autophagy/mitophagy. Furthermore, in transgenic Arabidopsis expressing both GFP-TraB1b and Mito-mCherry, strong co-localisation and vacuole accumulation of these proteins was found after Conc A treatment (Figure S6d-e).

The internalization of GFP-TraB1a to the vacuole is dependent on autophagy, as no vacuole accumulation of TraB1a is found after Conc A treatment of the same GFP-TraB1a transgenic line crossed into either the *atg5* and *atg7* autophagy deficient mutants (Figure 4k-l). In parallel, we generated Arabidopsis transgenic lines expressing the ATG8 binding-defective GFP-TraB1a Δ AIMs mutant construct, in which little GFP-TraB1a Δ AIMs vacuole accumulation is observed following Conc A treatment (Figure 5m-n). Therefore, these results suggest that the vacuole internalization of TraB1 is dependent on both the interaction with ATG8 and a functional autophagy pathway.

To further confirm the autophagic degradation of TraB1, protein levels of GFP-TraB1 in autophagy deficient backgrounds was monitored using western blotting⁴⁸. The levels of full-length protein and their degradation products (free GFP cleaved from the fusion protein) were analysed. As expected, the level of full-length GFP-TraB1a protein was found to be higher in *atg5*, *atg7* mutants under normal growth conditions, whilst the level of free GFP resulting from protein degradation was much lower compared to the Col-0, indicating impairment of TraB1 degradation in the autophagy mutants (Figure 5o-p). When treated with Conc A, the protein levels of GFP-TraB1 accumulated to a higher level in the wild type, indicating that blocking autophagic degradation prevents TraB1 degradation. Meanwhile, ConcA treatment of both *atg5* and *atg7* mutants did not result in a pronounced accumulation of GFP-TraB1a levels (Figure 5o-p). Transgenic plants expressing GFP-TraB1a Δ AIMs were also analysed and we found that the intensity ratio of free GFP/full-length protein is reduced significantly in GFP-TraB1 Δ AIMs plants (Figure 4q-r), indicating the

300 impaired degradation of TraB1 when its interaction with ATG8 is inhibited.

301

302 **Figure 4. TraB1a over-expression promotes autophagy flux and the degradation of mitochondria**
303 **through the autophagy machinery.** a-d. Transgenic Arabidopsis plants expressing GFP-ATG8a or GFP-
304 ATG8a+RFP-TraB1a grown in MS growth media without stress. After Conc A treatment, GFP-ATG8a
305 labelled autophagosome structures were identified inside the vacuole (b); autophagosomes that
306 contain mitochondrial signal (labelled by RFP-TraB1a) are also abundant in cells expressing GFP-
307 ATG8a and RFP-TraB1a (d). In contrast, little vacuole signal was seen in cells treated with DMSO as the
308 control (a, c). e. The number of autophagosomes in the vacuole after conc A treatment was analysed
309 statistically; more autophagosomes were found in cells over-expressing TraB1a. f-i. Transgenic
310 Arabidopsis plants expressing Mito-mCherry or Mito-mCherry + GFP-TraB1a grown in MS growth
311 media without stress. After Conc A treatment, numerous mitochondria (either labelled with Mito-
312 mCherry or co-labelled with Mito-mCherry + GFP-TraB1a) accumulated in the vacuole (g,i). In contrast,
313 little vacuole signal was seen in cells treated with DMSO as the control (f, h). j. The number of
314 vacuole-accumulated mitochondria increased in cells over-expressing TraB1a after Conc A treatment.
315 k-l. GFP-TraB1a localization in wild type and an autophagy deficient mutant (*atg5*) under non-stressed
316 conditions upon Conc A treatment. No punctate structures are found to accumulate inside the
317 vacuole of cells in the *atg5* mutant, indicating that the transport of TraB1-labelled mitochondria into
318 the vacuole relies on the core autophagy machinery. m-n. Stable Arabidopsis expressing GFP-
319 TraB1ΔAIMs were treated with Conc A (m), the vacuole-accumulation of GFP positive signal is

significantly reduced in comparison with Arabidopsis expressing non-mutated GFP-TraB1a (n). **o-p.** Western blot analysis of GFP-TraB1 (GFP-TB1) levels in *Col-0*, *atg5* and *atg7* Arabidopsis seedlings with and without Conc A treatment (o), the result shows a reduced level of degradation of GFP-TraB1 in both of the autophagy defective mutants, as indicated by the reduction in free GFP levels and ratio of free GFP/Full-length protein (p). **q-r.** Western blot analysis of the protein levels of GFP-TraB1 and GFP-TraB1ΔAIMs in Arabidopsis seedlings under non-stressed condition (q), the result shows that the degradation of GFP-TraB1ΔAIMs is reduced, as indicated by the reduction in free GFP level and ratio of free GFP/Full-length protein (r). N ≥ 15 for image quantification; N = 2 for every western blot analysis, error bars are SEM, *** P < 0.001 in Student's t tests (Scale bar = 10 μm; Scale bar = 15 mm for root analysis).

Interestingly, the impairment of autophagic degradation of GFP-TraB1 also resulted in developmental defects. Both *atg5* and *atg7* plants expressing GFP-TraB1 are smaller than *Col-0* lines expressing GFP-TraB1 (Figure S7). Similarly, Arabidopsis plants expressing GFP-TraB1ΔAIMs are also dwarfed (Figure S8), indicating that the autophagy-dependent protein turnover of TraB1 is essential for normal plant development, and over-accumulation may lead to mitochondrial dysfunction (Fig.S3a-d). Taken together, in addition to the function as an ER-Mitochondria tethering factor by its interaction with VAP27-1, TraB1 proteins also interact with ATG8 and are degraded via the autophagy pathway. However, whether TraB1 is part of the machinery for mitophagy, or simply acts as a cargo for autophagic degradation is the next question to be determined.

Stress induced mitophagy is affected in the *trab1a/b* mutant, suggesting that TraB1 is likely to be a mitophagy receptor

It is known in yeast and mammalian systems that the ER-Mitochondrial interfaces are essential for autophagosome biogenesis and mitophagy^{17,49,50}, so it is also reasonable to propose that TraB1-VAP27 mediated ER-Mitochondrial tethering and TraB1-ATG8 regulated mitophagy are two closely related processes. Transgenic Arabidopsis expressing GFP-TraB1 and VAP27-RFP were treated with Conc A. The majority of the TraB1 labelled mitochondria are found in close contact with VAP27 labelled ER membrane after internalization to the vacuole, suggesting that ER-Mitochondria stay in contact during mitophagy (Figure 5a-b). With the help of immunogold labelling, anti-VAP27 gold particles were found to be enriched at the ER-Mitochondrial interface of mitophagosome-like structures (Figure 5c).

To investigate whether TraB1 and VAP27 are important for regulating mitophagy, we examined mitophagy activity in their mutant plants. Mito-mCherry was transformed into wild type Arabidopsis and the *trab1a/b* double mutant, and the level of mitophagy flux (indicated by the number of internalized mitochondria) was measured after ConcA

356 treatment. Under normal growth conditions, the number of vacuole-internalized
357 mitochondria was found to be similar in both Col-0 and *trab1a/b* but a significant reduction
358 was observed in the *trab1a/b* mutant when these plants were subjected to DNP-induced
359 mitochondrial depolarization (Figure 5d). This result indicates that TraB is needed for stress-
360 induced mitophagy above the normal basal levels. In addition, two independent *vap27-*
361 *1/3/4/6* quadruple mutants were generated (Figure S9a) using CRISPR/Cas9 with the
362 intention of disrupting the VAP27-TraB1 interaction at EMCSs, as these ER localized VAP27s
363 exhibit partial co-localization with TraB1a (Figure S9b-d). Both mutants exhibit defects in
364 stress-induced mitophagy similar to the *trab1a/b* mutant (Figure 5d-e).

365 We hypothesise that heightened levels of impaired mitochondria (TMRM negative) resulting
366 from DNP-induced depolarisation (Figure S10a) requires mitophagy to maintain
367 mitochondrial homeostasis, in which TraB1-VAP27 plays an important role. Significant
368 accumulation of depolarized mitochondria is found in the loss-of-function mutants of
369 *trab1a/b* and *vap27-1/3/4/6* (Figure S10b-d). In conclusion our data demonstrate that TraB1:
370 1. is OMM-localized; 2. interacts with ATG8; 3. is transported to the vacuole using the
371 autophagy machinery; 4. over-expression enhances mitophagy and furthermore, 5. in TraB1
372 knock out mutants, DNP induced mitophagy is partially impaired. These data favour the
373 hypothesis that TraB1 is a novel mitophagy receptor, as well as performing a function in ER-
374 Mitochondria tethering.

375 Mitochondrial damage releases reactive oxygen species and other toxic compounds^{16,51},
376 which induces cell stress. Therefore, unsuccessful removal of damaged/depolarised
377 mitochondria may have negative impacts on plant growth^{52,53}. To test this hypothesis, the
378 *Arabidopsis trab1a/b* and *vap27-1/3/4/6* mutants were subjected to acute DNP treatments
379 and their growth was analysed 2 days after recovery on DNP-free media. We observed that
380 *trab1a/b* and *vap27-1/3/4/6* mutant plants exhibit relatively weak recovery compared to
381 Col-0 plants after 2 days (Figure 5f, Figure S11), suggesting that both mutants are defective
382 in the maintenance of mitochondrial homeostasis, which we attribute to defects in
383 mitophagy. This observation is consistent with our previous observations that impairment of
384 TraB-promoted mitophagy (through overexpression of GFP-TraB1ΔAIMs) results in growth
385 defects (Figure S8), and demonstrates the importance of endogenous TraB in plant
386 mitophagy. As the positive control, the autophagy defective mutants (*atg5* and *atg7*) were
387 treated with DNP and studied in the same way; reduced recovery was also prominent
388 (Figure 5f-g).

Figure 5. Mitochondrial stress induced mitophagy is inhibited in the *trab1a/trab1b* mutants.

a. Transgenic Arabidopsis expressing GFP-TraB1 and VAP27-1-RFP were treated with Conc A. The majority of TraB1-labelled mitochondria are found in contact with VAP27-labelled ER membrane inside the vacuole, suggesting that these two structures are internalised together. **b.** High magnification images of **a.** **c.** Anti-VAP27 immunogold labelling of transgenic Arabidopsis Hypocotyl cells expressing VAP27 under non-stressed condition. **d-e.** Arabidopsis plants (Col-0; *trab1a/trab1b*; *vap27-1,3,4,6*) stably expressing Mito-mCherry were treated with DNP and Conc A to study the activity of mitophagy. Under non-stressed conditions, the mitochondrial turnover rate is similar in all plants analyzed. However, the number of vacuole-accumulated mitochondria is significantly reduced in both the *trab1a/trab1b* and the *vap27-1,3,4,6* (two independent lines, A1 and B2) mutants treated with DNP, suggesting that an interruption of ER-Mitochondria tethering affects mitochondrial turnover following mitochondrial stress/mitochondrial damage. **f-g.** The recovery of Arabidopsis seedlings after 4 hours of DNP treatment was studied. The root growth of the *trab1a/b* mutant plants is significantly reduced compared to that in the wild type, suggesting that the DNP induced mitochondrial dysfunction has more impact on plant development when the function of TraB is impaired. N ≥ 20 for confocal quantification; N ≥ 90 for root analysis; error bars are SEM, *** P < 0.001 in Student's t tests (Scale bar = 10 μm for confocal; Scale bar = 200 nm for TEM).

Discussion

410 It is known that ER-Mitochondria interactions are essential for mitochondrial division and
411 signal/substrate exchange between the two compartments. In animal cells, the protein
412 composition of EMCs has been extensively studied in the last decade, and proteins such as
413 MFN, Miro and dynamin related proteins are known to have important functions in
414 regulating mitochondrial function and morphogenesis^{11,36,42,49,54,55}. In this study, we have
415 identified the evolutionarily conserved TraB1 protein family as regulators of two putative
416 interrelated pathways. Firstly, they interact with ER-localized VAP27 to regulate ER-
417 Mitochondrial interactions, and secondly, they interact with ATG8 to regulate mitochondrial
418 degradation working as putative mitophagy receptors. Our data indicates that TraB1 has
419 important roles in mitochondrial homeostasis through regulation of mitophagy, which is
420 essential for normal plant growth under stress conditions.

421 It is known in yeast and mammalian systems that the ER-Mitochondrial interfaces are
422 essential for autophagosomes biogenesis^{3,17,40,42,49,50,56}, so it is also reasonable to propose
423 that TraB1-VAP27 mediated ER-Mitochondria tethering and TraB1-ATG8 regulated
424 mitophagy are two closely related processes. In animal cells, the homologues of VAP27
425 (VAPA/B) interact with multiple autophagy proteins, including ULK1/ATG1 and WIPI2/ATG18,
426 to modulate autophagosome biogenesis²⁶. In plants, VAP27 also functions in regulating
427 actin-dependent autophagosome biogenesis through recruitment of AtEH1/Pan1²¹.
428 Therefore, it is likely that the core autophagy machinery can also be recruited to the VAP27-
429 mediated membrane interfaces, the VAP27-TraB1-mediated EMCs, and function in
430 selective autophagy processes (Figure 6).

431 In the *trab1a/b* mutant, mitochondrial structure and function are impaired, causing an
432 overall reduction in ATP production and starch consumption (Figure 3n-r). These phenotypes
433 are indicative of defective mitochondrial function and are likely linked to the function of
434 TraB1 in mitophagy and ER-Mitochondria tethering, both of which are important in
435 maintaining mitochondrial homeostasis and a healthy population of mitochondria through
436 turnover of damaged mitochondria. In animal cells, it has been observed that damaged
437 sections of mitochondria become dissected from the entire mitochondrial network, and are
438 degraded through mitophagy. Unsuccessful removal of damaged mitochondria causes
439 overall mitochondria dysfunction and cell stress/death^{10,13,47,52,53,57,58}. Consistent with this,
440 TraB1-loss of function results in the accumulation of damaged mitochondria under stress
441 conditions caused by DNP treatment (Figure 5d, Figure S11).

Figure 6. Proposed model for TraB1-VAP27 mediated Mitochondria-ER tethering and mitophagy. a. Under normal conditions, mitochondria are tethered to the ER through the TraB1-VAP27 complex at EMCSs; this link is essential for mitochondrial function (e.g. movement, morphology and energy metabolism). **b.** TraB1 also interacts with ATG8, a function that is essential for the mitochondrial stress-response. When the number of damaged mitochondria increases, TraB1 recruits ATG8 at the EMCS to regulate the formation of mitophagosomes. VAP27 may also recruit other unidentified autophagy proteins, such as ULK1/ATG1 and WIPI2/ATG18 to facilitate autophagosome maturation, as has been reported in mammalian cells.

In conclusion, we have characterised a novel EMCS complex mediated by the interaction of VAP27 with the OMM-integral protein, TraB1, which has important roles in mitochondrial function through regulating ER-Mitochondrial tethering and mitophagy (Figure 6a, b). Firstly, TraB1 interacts with ER-localized VAP27 to regulate ER-Mitochondrial interactions. Secondly, TraB1 interacts with ATG8 to regulate mitochondrial degradation working as putative mitophagy receptors, which is essential for normal plant growth in stressed conditions. We hypothesise that TraB1-mediated EMCSs are likely to regulate mitophagy, which in turn maintains healthy mitochondrial homeostasis and function. Whilst the mechanisms of EMCSs are already well known in animals and yeast, TraB1 is the first identified protein to have a dual function in ER-mitochondria tethering and mitophagy in eukaryotes, and these data aid in bridging the gap in our understanding of how EMCSs and mitophagy are regulated in *plantae*.

Materials and methods

Molecular cloning

The cDNA of TraB1a, TraB1b and TraB2 were cloned into pMDC43 or pK7WGR2 via gateway

cloning (BP Clonase™ II, Invitrogen 11789020; LR Clonase™ II, Invitrogen 11791020), to facilitate their expression as an N-terminal GFP or RFP fusion proteins. ATG8e was cloned using Phanta Max Super-Fidelity DNA Polymerase (Vazyme, P505) into pK7WGR2 (N-terminal RFP fusion protein) and pK7WGC2 (N-terminal CFP fusion protein) via gateway cloning. To make the AIM motif mutation of TraB1a, two predicted AIM motifs (Figure S4c) were mutated (F21A, I24A and F279A, L282A) using their site-mutagenesis primers and a Mut Express II Fast Mutagenesis Kit-V2 (Vazyme, C214).

For the expression of TraB1a and TraB1b under their own promoters, *pTraB1a*:GFP-TraB1a and *pTraB1b*:GFP-TraB1b native expression constructs were generated. Approximately 1500bp of promoter region directly upstream of the TraB1a and TraB1b start codons was PCR amplified with 5' PstI and 3' BamHI restriction sites using their gene specific primers. The *pCAMV 35s* promoters of pMDC43-TraB1a and pMDC43-TraB1b were excised by PstI/BamHI double restriction digest, and PstI/BamHI-digested *pTraB1a* and *pTraB1b* fragments were inserted respectively using T7 DNA Ligase (NEB, M0318S). For generation of recombinant TraB1a-6xHIS protein, full length TraB1a cDNA was PCR amplified with 5' NdeI and 3' NotI restriction sites using gene-specific primers, and subcloned into the NdeI/NotI restriction sites of pET24 using T7 DNA ligase (NEB, M0318S) to facilitate its expression as a C-terminal 6xHIS fusion protein in *E. coli*.

For yeast-two-hybrid constructs, full-length CDS of ATG8e was sub-cloned into pGADT7-GW plasmid using Gateway. TraB1a and TraB1aΔAIMs coding sequences without the transmembrane domains were cloned into pGBKT7 plasmid by Gateway LR reaction. All primers used in this study were listed in Supplementary Table S1.

Transgenic Arabidopsis lines

Mito-CFP, Mito-YFP, Mito-mCherry, GFP-HDEL organelle markers have been described previously⁵⁹. To generate the GFP-ATG8a and RFP-TraB1a Arabidopsis lines, the GFP-ATG8a Arabidopsis line^{21,60} was dipped with RFP-TraB1a via Agrobacterium-mediated transformation. Arabidopsis lines co-expressing GFP-TraB1a + Mito-mCherry, GFP-TraB1a + VAP27-1-RFP and GFP-TraB1b + Mito-mCherry were generated by crossing using stable transgenic plants of expressing single constructs. The GFP-TraB1a/*atg5*, GFP-TraB1/*atg7* were generated by crossing of stable transgenic GFP-TraB1a plants and the *atg5* and *atg7* mutants. Other Arabidopsis mutants generated in this study and are described in detail below. Please refer to Supplementary Table S2 and S3 for a complete list of all plasmid and transgenic Arabidopsis lines in this study.

Arabidopsis Mutant analysis

The *trab1a-1* (GABI KAT_369F08) T-DNA mutant line was obtained from GABI Kat, and the *trab1b-1* (SALK_059433) T-DNA mutant line was obtained from NASC, and the *trab1a-2*, *trab1b-2*, and *vap27-1,3,4,6* CRISPR mutants were generated in this study. The *atg5* (SAIL_129_B07) and *atg7* (SAIL_11_H07) mutants have been described as previously⁶¹.

For T-DNA mutant lines, homozygosity was confirmed using the genotyping primers listed in Supplementary Table S1. Total RNA was extracted from seedlings (Qiagen) and cDNA was synthesised using Superscript III (Invitrogen). RT-PCR was used to confirm the absence of full-length transcripts in the T-DNA mutants using the primers listed in Supplementary Table S1. RT-PCR amplification of the housekeeping gene, *EF1α*, was performed as a control, and equal amounts of Col-0 and mutant cDNA was used.

For CRISPR/Cas9-mediated generation of *trab1a* and *trab1b* mutants, two gRNA spacer sequences on the + strand were chosen as Cas9 targets: for TraB1a; target 1 (5'-GCCGCCGCTAAACACAGAAT-3') and target 2 (5'-GAAGACAGCTTGAAACAGTA-3') were chosen. For TraB1b; target 1 (5'-GGAGAAGTTGGAGCTGCCTG-3') and target 2 (5'-ATTGCAGATAAAGGAACTGA-3') were chosen. For *vap27-1/vap27-3* double mutants, two gRNA spacer sequences on the + strand of each gene were chosen as Cas9 targets. For VAP27-1, target 1 (5'-AGGTCTACTTGCGAAGTTCT-3') and target 2 (5'-ATACTGGAGTTGTTCTCCCG-3') were chosen; for VAP27-3, target 1 (5'-CGATAATTATGTCGCCTTCA-3' and target 2 (5'-ATCCAAAGAAGTACTGCGTT-3') were chosen. Target specificities were evaluated with CAS-OFFINDER, using an algorithm for potential off-target sites of Cas9 RNA-guided endonucleases (Bae et al., 2014). The two respective TraB1a and TraB1b gRNA expression modules, were constructed via PCR using pCBC-DT1T2 as a template according to Xing et al.⁶² and Golden Gate assembled into the vector pHEE401E allowing egg cell-specific EC1.2en:EC1.1 promoter-controlled expression of 3× FLAG-NLS-zCas9-NLS⁶³. The four VAP27-1/VAP27-3 gRNA expression modules were constructed via PCR using pCBC-DT1T2, pCBC-DT2T3 and pCBC-DT3T4 as a template according to Xing et al.⁶² and assembled via HiFi Gibson Assembly into pHEE401E. For *vap27-1,3,4,6* CRISPR mutants, the two VAP27-4/VAP27-6 gRNA expression module was transformed into *vap27-1/vap27-3* CRISPR mutants generated above. The two gRNA expression module was constructed via Restriction-ligation cloning into pKSE401⁶³, and the target (5'-TGGGACTCTTGCATCATTAG-3') of VAP27-4, target (5'-GGACGAACACAGTATTTGCG-3') of VAP27-6 were chosen respectively. Transformants were genotyped and sequenced using the primers listed in Supplementary Table S1.

535 **Generation of antibodies and immunostaining.**

536 Polyclonal antibodies were raised to full-length TraB1a-6xHIS. TraB1a-6xHIS was expressed
537 in *E. coli* (Rosetta2; Novagen), and purified using Nickel-Agarose beads (Qiagen). Polyclonal
538 antibodies were raised in mice as previously described⁶⁴.

539 For immunostaining, 5–7-day old seedlings were fixed in 4 % PFA in PEM buffer (50 mM
540 PIPES pH 6.9, 5 mM EGTA, 1 mM MgSO₄) for 90 minutes. Fixed roots were washed (5x 10
541 min) with PEM buffer and cell walls were digested using 0.5 % cellulase, 0.05 % pectolyase,
542 2 % driselase in PEM buffer + 0.4 M mannitol for 15 minutes. Roots were washed (3x 10
543 minutes in PEM) and were gently squashed on a poly-L-lysine-coated coverslip. Roots were
544 washed (3x 10 minutes in PEM) and permeabilised in PEM + 0.1% triton X100 for 15 minutes.
545 Roots were washed (3x 10 minutes in PEM) and incubated in blocking buffer 2% BSA in PEM)
546 for 1 hour. Samples were incubated in primary antibody at room temperature for one hour
547 and then at 4°C overnight. Samples were then washed in Phosphate Buffered Saline solution
548 (PBS; 6x 30 minutes) and were stained with secondary antibody at room temperature for
549 one hour and then at 4°C overnight. Samples were washed in PBS (6x 30minutes) and were
550 mounted in Vectashield and imaged using Airy scanning super-resolution confocal
551 microscopy.

552 For co-staining of TraB1a with VAP27-1 and mitochondria, Arabidopsis stable lines
553 expressing native VAP27-1-RFP or Mito-mCherry were stained with anti-TraB1a mouse
554 primary antibody at a dilution of 1:500 and anti-RFP rat primary antibody (Chromotek),
555 followed by secondary antibody incubation with FITC conjugated against mouse, and TRITC
556 conjugated against rat (Jackson ImmunoResearch).

557 Live cell Imaging and Image analysis

558 *Nicotiana benthamiana* plants were grown in a growth room or greenhouse with long-day
559 conditions. Transient expression was performed by leaf infiltration according to Sparkes et
560 al⁶⁵. The transformed *N. benthamiana* tissues were imaged two days after infiltration using a
561 laser scanning confocal microscope (Leica SP8). For each experiment, at least three
562 independent infiltrations were performed. Images were taken in multi-track mode with line
563 switching when multifuorescence was used. For GFP/RFP or FITC/TRITC combination,
564 samples were excited at 488 and 552 nm and detected at 510–550 and 590–650 nm,
565 respectively. For CFP/GFP/RFP or GFP/YFP/mCherry combination, CFP or GFP was excited at
566 458 nm and detected at 470–510 nm; GFP or YFP was excited at 514 nm and detected at
567 550–580 nm; RFP or mCherry was excited at 552 nm and detected at 590–650 nm. Images
568 were taken using a 63x oil immersion objective (NA = 1.4), with scan speed of 400 Hz at a
569 resolution of 1024 × 1024 pixels. To capture the dynamics of TraB, VAP27, mitochondria and
570 autophagosomes in Arabidopsis, cells from the root elongation zone and cotyledon were
571 acquired at a fixed rate of 0.876 s per time point for 1 min. Airy scanning super-resolution
572 confocal imaging was performed using the Zeiss LSM 880 confocal laser scanning microscope
573 in fast AiryScan mode, and using a Zeiss C PL APO x63 oil-immersion objective lens (NA = 1.4).
574 Airy scanning confocal image raw data was processed using the Airyscan Processing tool on
575 Zeiss Zen Black software. FRET-FLIM was performed as previously described¹⁸. For BiFC
576 analysis, full-length CDS of TraB1a and VAP27-1 were sub-cloned into pCL112-nYFP and
577 pCL113-cYFP plasmids, respectively⁶⁶. Transiently transformed *N. benthamiana* were imaged
578 two days after infiltration using a Leica SP8 confocal microscope. Images were excited at 514
579 nm and detected at 550–580 nm for YFP. For TMRM staining, seedlings were grown on MS
580 medium for 5 days, and transferred into MS liquid medium with 500 nM TMRM for 10 min.
581 Samples were excited at 552 nm and detected at 590–640 nm. Mitochondrial aspect ratio
582 and fluorescence intensity was measured using Image J.

583 Phylogenetic analysis

584 To identify TraB family homologues, the predicted proteins of each genome were searched
585 using BLASTP with *Arabidopsis thaliana* TraBs as input sequences. Used databases were NCBI
586 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), Phytozome
587 (<https://phytozome.jgi.doe.gov/pz/portal.html>), and Orange Genome Annotation Project
588 (<http://citrus.hzau.edu.cn/cgi-bin/orange/search>). Multiple alignments were constructed
589 with the TraB domain amino acid sequence through SMART database ([http://smart.embl-](http://smart.embl-heidelberg.de)
590 [heidelberg.de](http://smart.embl-heidelberg.de)). The TraB family tree was generated from multiple alignments by applying
591 the Maximum Likelihood method based on the JTT matrix-based model⁶⁷ to a bootstrapped
592 dataset with 1000 replicates. Initial tree(s) for the heuristic search were obtained
593 automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise
594 distances estimated using a JTT model, and then selecting the topology with superior log
595 likelihood value. The tree is drawn to scale, with branch lengths measured in the number of
596 substitutions per site. The analysis involved 44 amino acid sequences. All positions
597 containing gaps and missing data were eliminated. There was a total of 74 positions in the
598 final dataset. Evolutionary analyses were conducted in MEGA7⁶⁸ v1.0.5877. See
599 Supplementary Sequence data for a complete list of all protein sequences.

600 Arabidopsis phenotype studies

601 For general growth phenotype studies, seedlings were grown on half MS medium for 7 days,

602 and transferred to soil in a growth chamber (22 °C, 70% relative humidity, 14 h light/10 h
603 dark cycle). For DNP treatment and recovery, Arabidopsis seedlings were grown on MS
604 medium for 5 days, and transferred onto medium supplemented with either DMSO or 50 µM
605 DNP for 4 h, then the seedlings were transferred onto MS medium to recover for 2 days.
606 Images were captured using a digital camera (Canon, EOS 80D), and the root length was
607 measured using Image J.

608 **Yeast-two-Hybrid**

609 Testing and control combinations of plasmids were co-transformed into yeast AH109 strain
610 using the Frozen-EZ Yeast Transformation II Kit (ZYMO, T2001) and cultured on a SD/-Leu-Trp
611 selective plate at 30 °C for 2–4 days. Three independent colonies of each combination of
612 plasmids were picked and dotted on a SD/-Leu-His-Trp selective plate with 5 mM 3-AT.
613 Combinations of pGBKT7-53 + pGADT7-T and pGBKT7-lam + pGADT7-T were used as positive
614 and negative controls, respectively.

615 **Proteomics screen, co-immunoprecipitation and western blotting**

616 The TAP-tag purification and mass spectrometry was performed by a proteomic service
617 performed in Ghent University, as described previously⁶⁹. The immunoprecipitation assays
618 were performed using GFP-Trap_A (Chromotek, gtma-20) as described previously⁶. For
619 detection, the membrane was incubated in 2xTBST buffer with 5% milk prior to primary
620 antibody incubation (1:500 for anti-VAP27; 1:2000 for anti-GFP, Biorbyt orb323045) at room
621 temperature for 3 h. After three washes in TBST buffer, the membrane was probed with HRP
622 conjugated mouse secondary antibody (Yeasen, 33201ES60) at 1:5000 and developed using
623 a Super ECL reagent (Yeasen, 36208ES60).

624 **Electron Microscopy**

625 All samples were prefixed in 2.5 % glutaraldehyde (v/v in 0.1 M phosphate buffer, pH 7.2) for
626 2 h, and then rinsed 3 times with 0.1 M phosphate buffer (pH 7.2). They were post-fixed in 1 %
627 OsO₄ for 2 h, followed by three 15 min rinses with phosphate buffer. Afterwards, the
628 samples were dehydrated through an acetone series (30 %, 50 %, 70 %, 90 %, 100 %, 100 %, 100 %)
629 (v/v in dd H₂O) at room temperature, samples were incubated for 20 min at each
630 concentration. Then the samples were infiltrated in a graded scale of 3:1, 1:1, 1:3 (v/v)
631 acetone/SPI-PON 812 resin and, as the last step, in 100 % (v/v) SPI-PON 812 resin (SPI
632 Supplies, West Chester), for 12 h per step. Samples were embedded in SPI-PON 812 resin
633 and polymerized at 60 °C for 48 h. Ultrathin sections (80 nm) were prepared using an EM
634 UC7 Ultracut ultramicrotome (Leica, UC7). Sections were observed and photographed using
635 a transmission electron microscope (Hitachi H-7650) at an accelerating voltage of 80.0 kV.
636 The samples for immunogold labelling were fixed and probed with a VAP27 antibody as
637 describe previously¹⁹.

638 **Drug treatment and autophagy assays**

639 For Conc A treatment, Arabidopsis seedlings were treated with either DMSO or 1 µM Conc A
640 for 8–12 h prior to imaging. For the Conc A & DNP treatment, Arabidopsis seedlings were
641 first grown on MS medium for 5 days, and transferred into liquid MS medium supplemented
642 with Conc A for 12 h, then DNP was added into the same medium to a concentration of 50
643 µM, the seedlings were incubated for an additional 2 h prior to microscopy.

644 For autophagy-flux tests, seedlings were treated with 1 µM Conc A before western blotting,

the dilution ratio of primary antibody was 1:2000 for anti-GFP (Biorbyt orb323045) and HRP conjugated mouse secondary antibody (Yeasten, 33201ES60) was 1:5000.

Real-time qPCR

RNA from various samples (e.g. roots, stems, leaves, flowers) were extracted according to the instructions of the Universal Plant RNA Isolation Kit (NOBELAB, RNE35). The concentration of RNA was measured by NANODROP 2000 ultraviolet. 1 µg RNA was used to synthesize the first-strand cDNA by HiScript II Q RT SuperMix (Vazyme, R223). Finally, the gene expression of *AtTraB1a* and *AtTraB1b* were detected in a qPCR system (Roche, Lightcycler 480). ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711) was used to perform the reaction. The relative expression level of each gene was quantified with the comparative threshold cycle method, using actin as the internal reference. Reactions for each of the three biological replicates were performed in duplicate. The primer sequences used in RT-qPCR are shown in Supplementary Table S1.

Starch Assay

Rosettes of four-five weeks old plants were collected and immediately transferred to 95% ethanol, and boiled until completely decoloured (about 10–15 mins). Samples were stained in 5% Lugol's solution (5% [w/v] I₂ and 10% [w/v] KI) for 10 min, and then destained in water until a clear background was obtained before imaging⁷⁰. For starch content measurement, lugol-stained samples were washed using deionised water until decoloured, air dried at room temperature and ground into powder. The powdered tissue was washed three times with 2 ml of diethyl ether and subsequently washed twice with 80% ethanol, and then twice with ddH₂O. The washed powder was boiled in 5 mL ddH₂O for 15 min. 980 µL of the supernatant was mixed with 20 µL of staining solution [5% (w/v) I₂ and 2% (w/v) KI]. This reaction was subjected to colorimetric determination at 660 nm. The standard curve was performed with standard starch (Sigma, 33615).

ATP measurement

ATP, ADP, and AMP content was measured using HPLC. Sample preparations and adenylate standards for the measurement were performed as previously reported⁷¹. The analysis of adenosines was performed by HPLC on a C₁₈ column (Acclaim® PolarAdvantage II, 4.6 × 150 mm, 3 µm, 120 Å) connected to an HPLC system (1525 binary HPLC pump coupled with 2998 photodiode array detector and 2707 autosampler, Waters). The HPLC analysis was carried out as described previously⁷². The gradient for separation of adenosine derivatives was optimized as follows: 0 min, 100% A; 2 min, 95% A and 5% B; 6 min, 80% A and 20% B; 7 min, 75% A and 25% B; 8–10 min, 100% A (running buffer A: 0.06 M K₂HPO₄, 0.04 M KH₂PO₄, pH 7.0; running buffer B: 100% Acetonitrile). The flowrate is set to 1.2 ml/min.

Statistical analysis

All statistical graphs were performed using the GraphPad Prism software (ver. 7.00). The results were compared using the Student's t tests and data were expressed as the mean ± SEM (Std. Error of Mean) with each symbol. P value below 0.05 was considered significant.

Accession numbers

The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are TraB1a (AT1G05270), TraB1b (AT2G32340), TraB2 (AT5G52030), VAP27-1 (AT3G60600),

VAP27-3 (AT2G45140), VAP27-4 (AT5G47180), VAP27-6 (AT4G00170), ATG8a (AT4G21980), ATG8e (AT2G45170), ATG5 (AT5G17290), and ATG7 (AT5G45900).

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Author Contribution

P.W. and P.J.H. conceived and supervised the project, C.L. and P.D. performed most of the experiments, C.L., P.D., P.J.H. and P.W. wrote the manuscript. T.Z. and J.K. generated the CRISPR mutant. Y.F. performed the phylogenetic studies. X.L. and Y.C. helped on ATP analysis and HPLC studies. G.D.J. performed the TAP-tag MS screen. T.Z., and Y.F. helped with confocal imaging, material preparation and data interpretation.

Data availability

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files, or from the corresponding author upon reasonable request.

Competing Interests

The authors declare no competing interests.

References

1. Hawes, C., Kiviniemi, P. & Kriechbaumer, V. The endoplasmic reticulum: a dynamic and well-connected organelle. *Journal of Integrative Plant Biology* 57(1), 50–62 (2015).
2. Wu, H., Pedro, C. & Gia, K. V. Here, there, and everywhere: The importance of ER membrane contact sites. *Science* 361, 6401 (2018).
3. Hamasaki, M. et al. Autophagosomes form at ER-mitochondria contact sites. *Nature* 495(7441), 389–393(2013).
4. Kornmann, B. et al. An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science* 325(5939), 477–481 (2009).

- 723 5. Jaipargas, E. A., Barton, K. A., Mathur, N. & Mathur, J. Mitochondrial pleomorphy in
724 plant cells is driven by contiguous ER dynamics. *Frontiers in plant science* 6, 783 (2015).
- 725 6. Zhang, H. & Hu, J. Shaping the endoplasmic reticulum into a social network. *Trends in*
726 *cell biology* 26(12), 934–943 (2016).
- 727 7. Ng, A. Y. E., Ng, A. Q. E. & Zhang, D. ER-PM contacts restrict exocytic sites for polarized
728 morphogenesis. *Current Biology* 28(1), 146–153 (2018).
- 729 8. Bayer, E. M., Sparkes, I., Vanneste, S. & Rosado, A. From shaping organelles to signalling
730 platforms: the emerging functions of plant ER–PM contact sites. *Current Opinion in Plant*
731 *Biology* 40, 89–96 (2017).
- 732 9. Friedman, J. R. et al. ER tubules mark sites of mitochondrial division. *Science* 334(6054),
733 358–362 (2011).
- 734 10. López-Crisosto, C. et al. ER-to-mitochondria miscommunication and metabolic diseases.
735 *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1852(10), 2096–2105
736 (2015).
- 737 11. White, R. R. et al. Miro2 tethers the ER to mitochondria to promote mitochondrial fusion
738 in tobacco leaf epidermal cells. *Communications Biology* 3(1), 1–8 (2020).
- 739 12. Jaipargas, E. A., Barton, K. A., Mathur, N. & Mathur, J. Mitochondrial pleomorphy in
740 plant cells is driven by contiguous ER dynamics. *Frontiers in plant science* 6, 783 (2015).
- 741 13. Novak, I. et al. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO*
742 *reports* 11(1), 45–51 (2010).
- 743 14. Hanna, R. A. et al. Microtubule-associated protein 1 light chain 3 (LC3) interacts with
744 Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via
745 autophagy. *Journal of Biological Chemistry* 287(23), 19094–9104 (2012).
- 746 15. Van Wijk, K. J. Protein maturation and proteolysis in plant plastids, mitochondria, and
747 peroxisomes. *Annual review of plant biology* 66, 75–111 (2015).
- 748 16. Huang, S., Van Aken, O., Schwarzländer, M., Belt, K. & Millar, A. H. The Roles of
749 Mitochondrial Reactive Oxygen Species in Cellular Signaling and Stress Response in
750 Plants. *Plant Physiology* 171(3), 1551–1559 (2016).
- 751 17. Böckler, S. & Westermann, B. Mitochondrial ER contacts are crucial for mitophagy in
752 yeast. *Developmental cell* 28(4), 450–458 (2014).
- 753 18. Wang, P. et al. The plant cytoskeleton, NET3C, and VAP27 mediate the link between the
754 plasma membrane and endoplasmic reticulum. *Current Biology* 24(12), 1397–1405
755 (2014).
- 756 19. Wang, P., Richardson, C., Hawkins, T. J., Sparkes, I., Hawes, C. & Hussey, P. J. Plant VAP
757 27 proteins: domain characterization, intracellular localization and role in plant
758 development. *New Phytologist* 210(4), 1311–1326 (2016).
- 759 20. Stefano, G. et al. Plant endocytosis requires the ER membrane-anchored proteins
760 VAP27-1 and VAP27-3. *Cell reports* 23(8), 2299–2307 (2018).

- 761 21. Wang, P. et al. Plant AtEH/Pan1 proteins drive autophagosome formation at ER-PM
762 contact sites with actin and endocytic machinery. *Nature communications* 10(1), 1–16
763 (2019).
- 764 22. Saravanan, R. S. et al. The targeting of the oxysterol-binding protein ORP3a to the
765 endoplasmic reticulum relies on the plant VAP33 homolog PVA12. *Plant Journal* 58(5),
766 817–830 (2009).
- 767 23. Greer, M. S. et al. SEIPIN isoforms interact with the membrane-tethering protein VAP27-
768 1 for lipid droplet formation. *Plant Cell* 32(9), 2932–2950 (2020).
- 769 24. Loewen, C. J., Young, B. P., Tavassoli, S. & Levine, T. P. Inheritance of cortical ER in yeast
770 is required for normal septin organization. *The Journal of cell biology* 179(3), 467–483
771 (2007).
- 772 25. Jing, J. et al. Proteomic mapping of ER–PM junctions identifies STIMATE as a regulator of
773 Ca^{2+} influx. *Nature cell biology* 17(10), 1339–1347 (2015).
- 774 26. Zhao, Y. G. et al. The ER contact proteins VAPA/B interact with multiple autophagy
775 proteins to modulate autophagosome biogenesis. *Current Biology*, 28(8), 1234–1245
776 (2018).
- 777 27. Zang, J. et al. A novel plant actin-microtubule bridging complex regulates cytoskeletal
778 and ER structure at ER-PM contact sites. *Current Biology*, 31(6), 1251–1260 (2021).
- 779 28. Brault, M. L. et al. Multiple C2 domains and transmembrane region proteins (MCTP s)
780 tether membranes at plasmodesmata. *EMBO reports*, 20(8), e47182 (2019).
- 781 29. Xu, L. et al. Miga-mediated endoplasmic reticulum–mitochondria contact sites regulate
782 neuronal homeostasis. *Elife* 9, e56584 (2020).
- 783 30. Stefan, C. J. et al. Osh proteins regulate phosphoinositide metabolism at ER-plasma
784 membrane contact sites. *Cell* 144(3), 389–401 (2011).
- 785 31. Dong, R. et al. Endosome-ER contacts control actin nucleation and retromer function
786 through VAP-dependent regulation of PI4P. *Cell* 166(2), 408–423 (2016).
- 787 32. Li, X. et al. Isolation and comparative proteomic analysis of mitochondria from the pulp
788 of ripening citrus fruit. *Horticulture research* 8(1), 1–16 (2021).
- 789 33. Fuchs, P. et al. Single organelle function and organization as estimated from Arabidopsis
790 mitochondrial proteomics. *The Plant Journal* 101(2), 420–441 (2020).
- 791 34. El Zawily, A. M. et al. FRIENDLY regulates mitochondrial distribution, fusion, and quality
792 control in Arabidopsis. *Plant physiology* 166(2), 808–828 (2014).
- 793 35. Zhou, Y. et al. Topology-dependent, bifurcated mitochondrial quality control under
794 starvation. *Autophagy* 16(3), 562–574 (2020).
- 795 36. Wang, F. et al. Phosphorylation and ubiquitination of dynamin-related proteins
796 (AtDRP3A/3B) synergically regulate mitochondrial proliferation during mitosis. *The Plant*
797 *Journal* 72(1), 43–56 (2012).

- 798 37. Avin-Wittenberg, T. et al. Global analysis of the role of autophagy in cellular metabolism
799 and energy homeostasis in Arabidopsis seedlings under carbon starvation. *The Plant Cell*
800 27(2), 306–322 (2015).
- 801 38. Barros, J. A., Siqueira, J. A., Cavalcanti, J. H., Araújo, W. L. & Avin-Wittenberg, T.
802 Multifaceted roles of plant autophagy in lipid and energy metabolism. *Trends in plant*
803 *science* 25(11), 1141–1153 (2020).
- 804 39. Naon, D. et al. Critical reappraisal confirms that Mitofusin 2 is an endoplasmic
805 reticulum–mitochondria tether. *Proceedings of the National Academy of Sciences*
806 113(40), 11249–11254 (2016).
- 807 40. Puri, R., Cheng, X. T., Lin, M. Y., Huang, N. & Sheng, Z. H. Mul1 restrains Parkin-mediated
808 mitophagy in mature neurons by maintaining ER-mitochondrial contacts. *Nature*
809 *communications* 10(1), 1–19 (2019).
- 810 41. Rasul, F. et al. Emr1 regulates the number of foci of the endoplasmic reticulum-
811 mitochondria encounter structure complex. *Nature communications* 12(1), 1–14 (2021).
- 812 42. Prudent, J. et al. MAPL SUMOylation of Drp1 stabilizes an ER/mitochondrial platform
813 required for cell death. *Molecular cell* 59(6), 941–955 (2015).
- 814 43. Noda, N. N., Ohsumi, Y. & Inagaki, F. Atg8-family interacting motif crucial for selective
815 autophagy. *FEBS letters* 584(7), 1379–1385 (2010).
- 816 44. Jacomin, A. C., Samavedam, S., Promponas, V. & Nezis, I. P. iLIR database: A web
817 resource for LIR motif-containing proteins in eukaryotes. *Autophagy* 12(10), 1945–1953
818 (2016).
- 819 45. Georgakopoulos, N. D., Wells, G. & Campanella, M. The pharmacological regulation of
820 cellular mitophagy. *Nature chemical biology* 13(2), 136–146 (2017).
- 821 46. Gao, F., Zhang, Y., Hou, X., Tao, Z., Ren, H. & Wang, G. Dependence of PINK1
822 accumulation on mitochondrial redox system. *Aging Cell* 19(9), e13211 (2020).
- 823 47. Ma, J. et al. Friendly mediates membrane depolarization-induced mitophagy in
824 Arabidopsis. *Current Biology* 31(9), 1931–1944 (2021).
- 825 48. Klionsky, D. J. et al. Guidelines for the use and interpretation of assays for monitoring
826 autophagy. *Autophagy* 17(1), 1–382 (2021).
- 827 49. McLelland, G. L. et al. Mfn2 ubiquitination by PINK1/parkin gates the p97-dependent
828 release of ER from mitochondria to drive mitophagy. *Elif* 7, e32866 (2018).
- 829 50. Gelmetti, V. et al. PINK1 and BECN1 relocate at mitochondria-associated membranes
830 during mitophagy and promote ER-mitochondria tethering and autophagosome
831 formation. *Autophagy* 13(4), 654–669 (2017).
- 832 51. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in NLRP3
833 inflammasome activation. *Nature* 469(7329), 221–225 (2011).
- 834 52. Li, F., Chung, T. & Vierstra, R. D. AUTOPHAGY-RELATED11 plays a critical role in general
835 autophagy-and senescence-induced mitophagy in Arabidopsis. *The Plant Cell* 26(2), 788–

- 836 807 (2014).
- 837 53. Broda, M., Millar, A. H. & Van Aken, O. Mitophagy: a mechanism for plant growth and
838 survival. *Trends in plant science* 23(5), 434–450 (2018).
- 839 54. Gao, H., Sage, T. L. & Osteryoung, K. W. FZL, an FZO-like protein in plants, is a
840 determinant of thylakoid and chloroplast morphology. *Proceedings of the National*
841 *Academy of Sciences* 103(17), 6759–6764 (2006).
- 842 55. Yamaoka, S. & Leaver, C. J. EMB2473/MIRO1, an Arabidopsis Miro GTPase, is required
843 for embryogenesis and influences mitochondrial morphology in pollen. *The Plant Cell*
844 20(3), 589–601 (2008).
- 845 56. Barazzuol, L., Giamogante, F., Brini, M. & Cali, T. (2020). PINK1/parkin mediated
846 mitophagy, Ca^{2+} signalling, and ER–mitochondria contacts in Parkinson’s disease.
847 *International journal of molecular sciences* 21(5), 1772.
- 848 57. Aung, K. & Hu, J. The Arabidopsis tail-anchored protein PEROXISOMAL AND
849 MITOCHONDRIAL DIVISION FACTOR1 is involved in the morphogenesis and proliferation
850 of peroxisomes and mitochondria. *The Plant Cell* 23(12), 4446–4461 (2011).
- 851 58. Li, L. et al. CaM/BAG5/Hsc70 signaling complex dynamically regulates leaf senescence.
852 *Scientific reports* 6(1), 1–12 (2016).
- 853 59. Nelson, B. K., Cai, X. & Nebenführ, A. A multicolored set of in vivo organelle markers for
854 co-localization studies in Arabidopsis and other plants. *The Plant Journal* 51(6), 1126–
855 1136 (2007).
- 856 60. Huang, X. et al. Genetic analyses of the Arabidopsis ATG1 kinase complex reveal both
857 kinase-dependent and independent autophagic routes during fixed-carbon starvation.
858 *The Plant Cell* 31(12), 2973–2995 (2019).
- 859 61. Guan, B. et al. Effect of waterlogging-induced autophagy on programmed cell death in
860 Arabidopsis roots. *Frontiers in plant science* 10, 468 (2019).
- 861 62. Xing, H. L. et al. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC plant*
862 *biology* 14(1), 1–12 (2014).
- 863 63. Wang, Z. P. et al. Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates
864 homozygous mutants for multiple target genes in Arabidopsis in a single generation.
865 *Genome biology* 16(1), 1–12 (2015).
- 866 64. Smertenko, A. P., Kaloriti, D., Chang, H. Y., Fiserova, J., Opatrny, Z. & Hussey, P. J. The C-
867 terminal variable region specifies the dynamic properties of Arabidopsis microtubule-
868 associated protein MAP65 isoforms. *The Plant Cell* 20(12), 3346–3358 (2008).
- 869 65. Sparkes, I. A., Runions, J., Kearns, A. & Hawes, C. Rapid, transient expression of
870 fluorescent fusion proteins in tobacco plants and generation of stably transformed
871 plants. *Nature protocols* 1(4), 2019–2025 (2006).
- 872 66. Boevink, P., McLellan, H., Bukharova, T., Engelhardt, S & Birch, P. In vivo protein-protein
873 interaction studies with BiFC: conditions, cautions, and caveats. *Methods in Molecular*
874 *Biology* 1127, 81–90 (2014).

- 875 67. Jones, D. T., Taylor, W. R. & Thornton, J. M. The rapid generation of mutation data
876 matrices from protein sequences. *Bioinformatics* 8(3), 275–282 (1992).
- 877 68. Kumar, S., Stecher, G. & Tamura, K. MEGA7: molecular evolutionary genetics analysis
878 version 7.0 for bigger datasets. *Molecular biology and evolution* 33(7), 1870–1874
879 (2016).
- 880 69. Gadeyne, A. et al. The TPLATE adaptor complex drives clathrin-mediated endocytosis in
881 plants. *Cell* 156(4), 691–704 (2014).
- 882 70. Wang, Y. et al. Autophagy contributes to leaf starch degradation. *The Plant Cell* 25(4),
883 1383–1399 (2013).
- 884 71. Zhang, Y., Krahner, I., Bolze, A., Gibon, Y. & Fernie, A. R. Adenine nucleotide and
885 nicotinamide adenine dinucleotide measurements in plants. *Current Protocols in Plant*
886 *Biology* 5(3), e20115 (2020).
- 887 72. Chen, J., Zhang, H., Pang, Y., Cheng, Y., Deng, X. & Xu, J. Comparative study of flavonoid
888 production in lycopene-accumulated and blonde-flesh sweet oranges (*Citrus sinensis*)
889 during fruit development. *Food Chemistry* 184, 238–246 (2015).

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