1   **A CRISPR screening approach for identifying novel autophagy-related factors and**

2   **cytoplasm-to-lysosome trafficking routes**

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15   **Running title:** Genetic landscapes of mammalian selective autophagy

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18   **Summary**:

19   Selective autophagy comprises cytoplasm-to-lysosome trafficking routes that transport cargos

20   using double-membrane vesicles (autophagosomes). Cargos are detected by receptor proteins,

21   which typically also bind to lipid-conjugated LC3 proteins on autophagosome membranes. We

22   dissected lysosomal delivery of four SQSTM1-like receptors by genome-wide CRISPR

23   screening looking for novel autophagy-related (ATG) factors and trafficking routes. We

24   uncovered new mammalian ATG factors including TMEM41B, an endoplasmic reticulum

25   membrane protein required for autophagosome membrane expansion and/or closure.

26   Furthermore, we found that certain receptors remain robustly targeted to the lysosome even in

27   the absence of ATG7 or other LC3 conjugation factors. Lastly, we identified a unique genetic

28   fingerprint behind receptor flux in $ATG7^{KO}$ cells, which includes factors implicated in nucleating

29   autophagosome formation and vesicle trafficking factors. Our work uncovers new ATG factors,

30   reveals a malleable network of autophagy receptor genetic interactions, and provides a valuable

31   resource (http://crispr.deniclab.com) for further mining of novel autophagy mechanisms.

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Introduction:

Macroautophagy (hereafter autophagy) is an umbrella term for a series of vesicle formation and transport mechanisms that deliver cytoplasmic material to the lysosome for degradation (Bento et al., 2016; Mizushima et al., 2011; Nakatogawa et al., 2009). During bulk autophagy, regions of cytoplasm are nonspecifically encapsulated into de novo-generated, double-membrane vesicles. These vesicles (autophagosomes) are then trafficked to lysosomes where, upon autophagosome-lysosome fusion, the inner autophagosomal membrane and its contents are degraded by lysosomal hydrolases.

There are approximately 40 autophagy-related (ATG) factors that operate at distinct stages of the autophagosome biogenesis process (Mizushima et al., 2011). Nucleation of the cup-shaped, autophagosome precursor membrane (phagophore) is thought to be mediated by the RB1CC1/ATG101/ATG13 scaffolding complex and post-Golgi vesicles containing ATG9A (Itakura and Mizushima, 2010; Kishi-Itakura et al., 2014; Koyama-Honda et al., 2013). This is followed in short succession by phagophore membrane recruitment of a phosphoinositide-3 kinase (PI3K) complex and phosphatidylinositol-3 phosphate effectors such as WIPI2 (Dooley et al., 2014). Subsequent expansion of the phagophore membrane and vesicle closure is associated with lipid conjugation of LC3-family proteins (Atg8 in yeast) via a ubiquitin-like conjugation cascade dependent on ATG7 (E1-like), ATG3 (E2-like) and ATG5/ATG12/ATG16L1 (E3-like) (Geng and Klionsky, 2008; Ichimura et al., 2000). In addition to their role in bulk autophagy, these ATG factors work together with a diverse group of proteins called autophagy receptors to enable capture of specific cytoplasmic targets, such as damaged organelles, intracellular pathogens, and protein aggregates, during autophagosome formation (Zaffagnini and Martens, 2016). In the majority of

57   known selective autophagy cases, receptor-bound targets become tethered to growing phagophores

58   via receptor-mediated interactions with lipidated LC3 (Stolz et al., 2014).

59   Defining the precise role of lipidated LC3 during either bulk or selective autophagosome

60   formation has been historically challenging for several reasons. First, the six mammalian LC3-

61   family proteins have similar sequences and perform related functions. A recent study overcame

62   this issue of LC3 redundancy as it pertains to Parkin-dependent mitophagy by generating a sixtuple

63    $LC3^{KO}$ ($LC3^{NULL}$) cell line (Nguyen et al., 2016). Envelopment of receptor-bound mitochondria

64   by the phagophore membrane persisted in $LC3^{NULL}$ cells but the resulting autophagosomes were

65   defective for fusion with the lysosome. Second, there has been a shortage of tools for visualizing

66   autophagosome formation without relying on microscopic reporters of LC3 conjugation. To get

67   around this problem, another recent work monitored bulk autophagy in cells lacking LC3

68   conjugation factors using localization of Syntaxin 17 (STX17), a SNARE protein that is recruited

69   to autophagosomes following vesicle closure (Itakura et al., 2012). Autophagosome-like structures

70   (LC3 negative, Syntaxin 17 positive) formed and fused with the lysosome but failed to undergo

71   inner autophagosomal membrane degradation, potentially because of a vesicle closure defect

72   (Tsuboyama et al., 2016).

73   There are also cytoplasm-to-lysosome pathways that appear to operate normally in the

74   absence of LC3 conjugation while still relying on other ATG factors to varying degrees. One

75   pioneering study found conditions during which bulk protein degradation was mediated by double-

76   membrane autophagosomes formed by an ATG5/7-independent mechanism (Nishida et al., 2009).

77   Yet another alternative route to the lysosome was revealed in a very recent study of how the

78   NCOA4 autophagy receptor enables iron starvation to induce degradation of ferritin (a

79   cytoplasmic, iron-binding protein aggregate) (Goodwin et al., 2017). With the notable exception

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80   of ATG9, ferritin destruction persisted in the absence of virtually all other known ATG factors,

81   including RB1CC1 (alternatively named FIP200), a scaffold protein essential for normal

82   phagophore nucleation (Goodwin et al., 2017). In sum, these discoveries are beginning to re-shape

83   the monolithic view of how ATG factors and autophagy receptors function into a more complex

84   web of overlapping pathways for robust cytoplasm-to-lysosome delivery.

85   Here, we further explored the possible existence of new ATG factors and alternative

86   routes to the lysosome by focusing on SQSTM1 and SQSTM1-like family of autophagy

87   receptors (NDP52, TAX1BP1, and NBR1) (Birgisdottir et al., 2013). Inspired by the pH-

88   sensitive, tandem-fluorescent reporter for monitoring LC3 lysosomal delivery (tfLC3) (Kimura

89   et al., 2007; Pankiv et al., 2007), we used tfReceptors as phenotypic reporters for genome-wide

90   CRISPR screening. This approach recovered virtually all known ATG factors and identified

91   several novel candidates as screen hits. Further follow-up by quantitative microscopy and

92   biochemical analysis revealed that TMEM41B is a previously uncharacterized endoplasmic

93   reticulum (ER) membrane protein with a critical role in phagophore expansion and/or vesicle

94   closure. In addition, we found that lysosomal targeting of NBR1 (and to lesser extent SQSTM1

95   and TAX1BP1) persisted in cells lacking LC3 conjugation factors. A subsequent screen

96   performed using $ATG7^{KO}$ tfNBR1 cells enabled us to compare ATG7-dependent and ATG7-

97   independent genetic modifiers across the entire genome. Notably, all forms of NBR1 lysosomal

98   delivery were absolutely dependent on ATG factors implicated in phagophore nucleation

99   (RB1CC1, ATG101, and ATG9A) but ATG7-independent delivery had a unique requirement for

100   additional factors with known roles in alternative forms of vesicle trafficking.

101   **RESULTS:**

102   **Validation of tfReceptors as phenotypic reporters of autophagy**

103   SQSTM1 and members of the SQSTM1-like family of proteins are among the most

104   widely studied autophagy receptors in mammalian cells (Zaffagnini and Martens, 2016). We

105   constructed six homologous gene cassettes encoding the tandem-fluorescent (tf) protein RFP-

106   GFP by itself (tfEmpty) or as an N-terminal fusion to either LC3 (isoform B) or SQSTM1 and

107   three SQSTM1-like family members: NBR1, TAX1BP1, and NDP52 (Fig S1A). Following

108   cassette integration at the AAVS1 locus, we measured tf flux to the lysosome as the ratio of RFP

109   fluorescence to GFP fluorescence, the latter of which is selectively quenched when exposed to

110   the low pH of the lysosomal lumen (Fig 1A) (Kimura et al., 2007; Pankiv et al., 2007). As

111   expected, inducing autophagosome formation by inhibiting mTOR with torin preferentially

112   increased lysosomal delivery of tfSQSTM1 and tfLC3 over tfEmpty in HEK293T cells (Fig 1B).

113   Notably, we confirmed that the torin-induced increase in the Red:Green ratio was due

114   specifically to a decrease in GFP fluorescence as expected for this reporter system (Fig S1B).

115   Finally, we validated that both basal and torin-induced lysosomal targeting of tfSQSTM1 was

116   dependent on two phagophore nucleation factors, *RB1CC1* and *ATG13* (Fig 1C, Fig S1C).

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118   **Genome-wide screens for tfReceptor modifiers detect known ATG factors to saturation**

119   To get an unbiased view of autophagy pathways, we expressed our tf proteins in K562

120   cells (a myelogenous leukemia line), which can be cultured in suspension to facilitate genetic

121   screening. Cells expressing either tfLC3 or tfEmpty served as receptor-agnostic controls.

122   Bafilomycin A1 (BafA1), a known inhibitor of autophagosome-lysosome fusion, blocked basal

123   targeting of tfLC3 and tfReceptors to the lysosome (Fig 2A). Conversely, lysosomal targeting of

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124   tfLC3 and tfReceptors was enhanced following treatment with torin (Fig 2A). Notably, tfEmpty

125   did not respond to these treatments, implying that nonselective engulfment of tfReceptors into

126   autophagosomes is negligible under these conditions (Fig 2A).

127   Next, we performed genome-wide, pooled CRISPR knock-out screens in K562 cells co-

128   expressing Cas9 with either tfLC3 or tfReceptors (Fig 2B). Following transduction with a library

129   of lentivirus-encoded single guide RNAs (sgRNA; 19,114 genes), we used FACS to collect the

130   top and bottom ~30% of cells ranked on the basis of their Red:Green ratios. Read counts of

131   sgRNAs derived from each cell fraction were obtained by an established Illumina sequencing

132   approach before being processed and analyzed using MAGeCK (Li et al., 2014b, 2015). The

133   resulting output for each gene includes a beta score, a proxy for its enrichment as a phenotypic

134   suppressor or enhancer (Fig 2C). An interactive interface for these data is also available online at

135   http://crispr.deniclab.com.

136   Inspection of deletions that decrease tfLC3 lysosomal targeting revealed that we

137   successfully recovered most known autophagy-related factors, thereby validating our

138   experimental setup (Fig 2C). Comparing genetic modifiers across tfLC3 and tfReceptors

139   revealed a strong phenotypic overlap comprising most well-established ATG factors, as well as

140   positive and negative regulators of mTORC1. In addition, our analysis distinguished between

141   related protein complex pairs based on their subunit ablation phenotypes. For example, in the

142   case of the homotypic fusion and vacuole protein sorting (HOPS) and class C core

143   vacuole/endosome tethering (CORVET) tethering complexes, we found evidence that only

144   HOPS was required for autophagy. Similarly, we found that the BLOC-one-related complex

145   (BORC) facilitated autophagy while the biogenesis of lysosome-related organelles complex

146   (BLOC-1) was dispensable. Lastly, our analysis identified factors whose precise functions in

147   autophagy have not been firmly defined (*e.g.* VPS37A, C18orf8), as well those that are

148   completely uncharacterized (*e.g.* TMEM41B).

149   To validate the top screen hits, we transduced our panel of K562 cell lines with individual

150   sgRNAs and measured phenotypes of single gene knockouts (Fig 3A,B, Fig S3A). This approach

151   allowed us to eliminate rare screen hits (*e.g.* sgUROD) that were spurious because they modified

152   the Red:Green ratio by changing RFP rather than GFP fluorescence (Fig S3B). The vast majority

153   of tested sgRNAs confirmed that they were true screen hits whose phenotypic strengths as single

154   gene deletions largely mirrored their relative beta score rankings (Fig 3B and 2C). Lastly, we

155   controlled for potential tf-specific artifacts by detecting accumulation of endogenous LC3 and

156   SQSTM1 in cells ablated for certain novel factors among the screen hits (Fig S3C). In sum, our

157   genetic screening strategy robustly identified known ATG factors and enabled us to pursue new

158   factors whose important roles in autophagy might have been overlooked.

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160   **TMEM41B is a novel, ER membrane protein required for phagophore maturation**

161   Despite being a complex membrane-mediated process, there is only a handful of integral

162   membrane proteins known to be directly involved in autophagosome formation. For this reason,

163   we focused on TMEM41B, a largely uncharacterized factor predicted to encode a multi-pass

164   membrane protein with a C-terminal ER retention signal (Jackson et al., 1990). A global,

165   subcellular-localization approach previously assigned TMEM41B's residence to the ER (Itzhak

166   et al., 2016). To confirm this finding and also to facilitate subsequent microscopy analysis of

167   autophagosome formation in $TMEM41B^{KO}$ cells, we turned to adherent HEK293T cells. Using a

168   split-fluorescent protein approach (Kamiyama et al., 2016), we tagged the N-terminus of

169   endogenous TMEM41B (predicted to by cytosolic) with the 11th beta strand of GFP (GFP11) and

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170   co-expressed a complementary GFP fragment (beta strands 1 through 10 [GFP1-10]). This

171   resulted in the expected reticular GFP fluorescence signal that colocalized with the ER marker

172   calnexin (Fig S4A).

173   Next, we made a stable $TMEM41B^{KO}$ cell line (Fig S4B) and established that this neither

174   elicited an ER stress response nor diminished its induction by tunicamycin, an inhibitor of

175   protein glycosylation in the ER (Fig S4C). Several lines of evidence argue that autophagy is

176   severely blocked in the absence of TMEM41B. First, $TMEM41B^{KO}$ cells accumulated LC3 in its

177   lipidated form (LC3-II), a phenotype that was rescued by transgenic expression of TMEM41B

178   (Fig S4D). In addition, we found no evidence for further LC3 accumulation in $TMEM41^{KO}$ cells

179   following addition of BafA1 (Fig 4A). Second, we also detected accumulation of autophagy

180   receptors (e.g. SQSTM1, etc) in $TMEM41B^{KO}$ cells (Fig 4B). Third, autophagy flux was

181   abolished in $TMEM41B^{KO}$ cells as measured using tfLC3 and tfSQSTM1, which phenocopied the

182    $ATG7^{KO}$ control (Fig S4E, S4F).

183   To define which step in autophagy requires TMEM41B, we first monitored a signal

184   induction pathway for autophagosome initiation. mTOR kinase activity normally represses

185   autophagy initiation by inhibiting the ULK1 kinase (Alers et al., 2012). Active ULK1

186   phosphorylates ATG13, a component of the RB1CC1 scaffolding complex required for

187   phagophore nucleation (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). We found

188   that torin treatment stimulated ATG13 phosphorylation at Ser318, a known ULK1 site,

189   comparably in wildtype and $TMEM41B^{KO}$ cells (Fig 4C). For comparison, this signaling assay

190   robustly detected loss of ULK1 activity in cells lacking RB1CC1, a known ULK1 kinase-

191   coactivator (Fig 4C). These data argue that TMEM41B is required for a step downstream of

192   phagophore nucleation.

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194   To monitor phagophore elongation in $TMEM41B^{KO}$ cells, we visualized several markers

195   of this process by cell microscopy. Semi-automated and custom-written scripts for image

196   processing and segmentation allowed us to make quantitative phenotypic measurements. We

197   found that $TMEM41B^{KO}$ cells displayed ~3-fold more LC3+/ SQSTM1+ punctae than wildtype

198   cells under basal conditions (Fig 5A, 5B). To further test if these structures correspond to

199   intermediates in autophagosome formation, we analyzed localization of the early autophagy

200   marker WIPI2 in $TMEM41B^{KO}$ cells under basal and torin-induced conditions. WIPI2 transiently

201   associates with nascent phagophores before it dissociates prior to vesicle closure (Dooley et al.,

202   2014; Polson et al., 2010). We found that torin treatment did not further increase the level of

203   LC3+ punctae in $TMEM41B^{KO}$ cells and that the majority of LC3+ structures were positive for

204   the early autophagosome marker WIPI2 (Fig 5C, 5D). For comparison, torin induced a strong

205   increase in LC3+ punctae formation in wildtype cells and these LC3+ structures had relatively

206   little overlap with WIPI2 (Fig 5D). Notably, in $TMEM41B^{KO}$ cells both LC3+ and WIPI2+

207   structures were dependent on the upstream autophagy factor RB1CC1 for formation (Fig 5E, 5F,

208   Fig S5A).

209   As another way of visualizing terminal steps in autophagosome formation, we turned to

210   STX17, a SNARE protein that is recruited to mature autophagosomes following vesicle closure

211   of elongated phagophores (Itakura et al., 2012). A recent study showed that STX17+ structures

212   accumulate in the absence of ATG7 and we confirmed this finding (Fig S5B, S5C) (Tsuboyama

213   et al., 2016). By contrast, we found no appreciable amounts of STX17 on LC3+ structures that

214   accumulate in $TMEM41B^{KO}$ cells (Fig S5B, S5C). As a final test of whether LC3+ structures in

215    $TMEM41B^{KO}$ cells represent closed autophagosomes defective for fusion with the lysosome, we

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216   carried out an LC3 protease protection assay (Fig 6A-C). To maximally accumulate any sealed

217   autophagosomes, we inhibited autophagosome-lysosome fusion with BafA1 for 18 hours before

218   gently lysing cells by mechanical disruption. Trypsin treatment of control wildtype cell extracts

219   revealed that lipidated LC3 (LC3-II) was protease-resistant in the absence but not presence of

220   Triton X-100, a non-ionic detergent that solubilizes membranes. By contrast, LC3-II was

221   protease-sensitive in $TMEM41B^{KO}$ and control $RB1CC1^{KO}$ cells (Fig 6B, 6C). Taken together,

222   our microscopy and biochemical evidence support the view that TMEM41B is required for a late

223   step in phagophore maturation into fully-sealed autophagosomes.

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225   **Receptor-specific engagements with ATG7-independent autophagy**

226   Returning to other results from our genetic screens, we noted that NBR1 stood out

227   because of its unusually weak dependence on ATG7 and other LC3 lipidation factors (Fig 7A). A

228   recent study showed that loss of RB1CC1 induces an alternative lysosome trafficking route for

229   NCOA4 and TAX1BP1 (Goodwin et al., 2017). To examine if NBR1 can also access this route,

230   we analyzed $RB1CC1^{KO}$ tfNBR1 cells but found that no evidence supporting this possibility,

231   either under basal conditions or following torin treatment (Fig 7B, Fig S7A-C). By contrast, in

232    $ATG7^{KO}$ cells lysosomal delivery of tfNBR1 was strongly stimulated by torin and inhibited by

233   BafA1 (Fig S7C). To estimate the extent of NBR1's engagement with ATG7-independent

234   autophagy in K562 cells, we treated ATG9A deletions as true autophagy nulls and calculated

235   that 65% of tfNBR1 autophagy flux was maintained in an ATG7-independent context (Fig 7C).

236   Similarly, 30% of TAX1BP1 and 15% of SQSTM1 was delivered to the lysosome in the absence

237   of ATG7, while delivery was negligible for NDP52 and LC3.

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Forward genetic screen for modifiers of ATG7-independent autophagy

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240   To directly probe the genetics of ATG7-independent autophagy by an unbiased approach,

241   we performed a forward genetic screen in K562 $ATG7^{KO}$ cells co-expressing Cas9 and tfNBR1

242   (Fig 7D). From our list of screen hits and subsequent hit validation by individual sgRNA

243   experiments, we found that lysosomal delivery of NBR1 in the absence of LC3 lipidation is

244   absolutely dependent on several canonical ATG factors, including ATG9A, RB1CC1 and

245   ATG101, as well as components of the late endocytic pathway including RAB7A and HOPS

246   (Fig 7E). Next, we compared modifiers of tfNBR1 in wildtype and $ATG7^{KO}$ backgrounds to look

247   for interesting genetic interactions. This analysis revealed that loss of ATG7 induced complete

248   buffering to several ATG factors, including ATG3 and ATG10. Furthermore, we detected

249   synthetic genetic interactions between ATG7 and several factors with poorly-defined roles in

250   autophagy (*e.g.* EI24), as well as TMEM41B and the autophagy receptor TAX1BP1. Lastly, we

251   found evidence that NBR1 autophagy flux in the absence of LC3 lipidation is dependent on

252   certain vesicle trafficking (*e.g.* RAB1A and TRAPP complex components).

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256 **Discussion:**

257   The power of forward genetics in yeast is the foundation on which the field of autophagy

258   research firmly stands (Harding et al., 1995; Thumm et al., 1994; Tsukada and Ohsumi, 1993).

259   Yeast genetic screens were instrumental in identifying the first autophagy receptor and the ~30

260   core ATG factors required for autophagosome biogenesis in yeast (Scott et al., 2001). Despite deep

261   conservation between yeast and mammalian ATG factors, there are many mammalian-specific

262   forms of autophagy that remain poorly understood. The recent emergence of new CRISPR/Cas9-

263   based technologies has begun facilitating efforts to define novel mammalian autophagy factors

264   and pathways by forward genetic screening.

265   Here, we combined the power of CRISPR screening with that of tandem fluorescent

266   reporters for monitoring cytoplasm-to-lysosome targeting of several known autophagic factors. To

267   facilitate discovery, these data are also presented online in an interactive format at

268   http://crispr.deniclab.com. Among our screen hits, we found virtually all known ATG factors, plus

269   autophagy modulators that only became discovered while this work was in preparation (Jia et al.,

270   2017; Vaites et al., 2017), as well as fully uncharacterized proteins. Within this latter category, we

271   identified TMEM41B as an ER membrane protein important for a late step in phagophore

272   elongation and/or vesicle closure. Going forward, it will be important to test potential connections

273   between TMEM41B and other ER-associated autophagy factors (*e.g.* VMP1). Another promising

274   extension of our work will be to apply CRISPR screening to additional tf-based reporters of

275   cytoplasm-to-lysosome targeting, including autophagy targets themselves.

276   Following primary screening, our focus was drawn to one receptor, NBR1, because of its

277   unusually persistent autophagy flux in the absence of many ATG factors, including those essential

278   for LC3 lipidation. This initial finding was surprising to us because of the well-established

279   paradigm that NBR1 and most other autophagy receptors tether their targets to lipidated LC3 via

280   their LC3-interacting regions (LIRs) (Birgisdottir et al., 2013). To get a better handle on this

281   unexpected result, we carried out a secondary screen in $ATG7^{KO}$ tfNBR1 cells. Our specific choice

282   for this LC3-lipidation null background was driven by two considerations. Historically, ATG7-

283   independent autophagosomes were among the first forms of alternative autophagy to be reported

284   (Nishida et al., 2009). Practically, $ATG7^{KO}$ cells are widely used in autophagy research as an

285   autophagy-null control.

286   We found that genetic ablation of ATG9A, RB1CC1, and ATG101 consistently gave rise

287   to the most severe loss-of-function phenotypes for both ATG7-dependent and ATG7-

288   independent autophagy. In the absence of ATG7, tfNBR1 autophagy flux became increasingly

289   dependent on certain ATG factors (*e.g.* ATG2B, WDR45) and less on others, including certain

290   late endosomal factors (*e.g.* EPG5 and VPS37A). This type of buffering genetic interaction

291   places LC3, EPG5, and VPS37A in the same genetic pathway. Consistently, both LC3-family

292   proteins and EPG5 have recently been defined as vesicle/target-membrane specificity

293   determinants in autophagosome-lysosome fusion (Nguyen et al., 2016; Wang et al., 2016). Thus,

294   our working model is that autophagosomes formed during ATG7-independent autophagy are

295   trafficked to lysosomes by an alternative trafficking mechanism mediated by factors that we

296   found were selectively required for ATG7-independent but not ATG7-dependent autophagy (*e.g.*

297   RAB1A, GDI2, TRAPPC2L) . A mutually non-exclusive hypothesis emerges from our

298   observation that NBR1 trafficking becomes co-dependent on TAX1BP1 in the absence of ATG7.

299   This autophagy receptor is known to interact with myosin IV and was previously postulated to

300   control vesicle trafficking by the actin/myosin network (Tumbarello et al., 2015). Intriguingly, a

301   recent study showed how activation of TBK1 kinase enables TAX1BP1 to direct lysosomal

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302   targeting of ferritin by a novel ESCRT-mediated process (Goodwin et al., 2017). Our work

303   implicates TAX1BP1 in another noncanonical cytoplasm-to-lysosome pathway and should help

304   raise the interest level in this understudied autophagy receptor.

305   Mechanistically, it remains unclear how NBR1, as well as TAX1BP1 and SQSTM1 to

306   lesser extents, engages the autophagosome biogenesis process in the absence of LC3 lipidation. In

307   yeast, several receptors are known to locally induce autophagosome formation by binding to a C-

308   terminal region of Atg11 (Kamber et al., 2015), which has weak homology to a C-terminal

309   sequence of RB1CC1 (Li et al., 2014a; Yorimitsu and Klionsky, 2005). Analogous mechanisms

310   could explain our observation that NBR1's lysosomal delivery remains selective in the absence of

311   ATG7 albeit in a manner still dependent on RB1CC1 and ATG101. Consistently, there is evidence

312   that mammalian autophagy receptors (both mitophagy receptors (Lazarou et al., 2015) and TRIMs

313   (Kimura   et   al.,   2016))   can   recruit   an   RB1CC1-containing   complex

314   (RB1CC1/ATG101/ATG13/ULK1) to locally induce autophagosome formation.

315   LC3 positive and negative double-membrane vesicles have previously been shown to co-

316   exist in certain cell types (Nishida et al., 2009). To what extent do receptors then engage alternative

317   autophagy pathways in cells permissive for LC3 lipidation? Answering this important question

318   will necessitate development of receptor-based reporters that differentiate flux through related

319   pathways comprising many overlapping components. At one extreme, it is possible that LIR-

320   containing receptors normally engage only LC3-marked autophagosomes. This would then imply

321   that ablation of ATG7 induces compensatory mechanisms, which bypass the need for receptor-

322   LC3 interactions. It also remains unclear what distinguishes receptors that engage ATG7-

323   independent autophagy from those that don't (*e.g.* NBR1 vs NDP52). Since NBR1 is known to

324   interact with a diverse set of autophagy targets (Deosaran et al., 2013; Kenific et al., 2016; Kirkin

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325   et al., 2009; Lamark et al., 2009), one intriguing possibility is that its engagement with this

326   alternative autophagy pathway is target dependent.

327   Our systematic, genome-wide analysis of NBR1 autophagy flux casts a new light on the

328   roles of ATG factors within the autophagy cannon. We found that some are absolutely essential

329   (*e.g.* RB1CC1 and ATG9A) whereas others (*e.g.* WIPI2 and ATG14) only become critical in the

330   absence of ATG7. At a molecular level, RB1CC1 and ATG9A are among the first ATG factors to

331   be recruited to ER-associated sites at which LC3-marked autophagosomes eventually form

332   (Koyama-Honda et al., 2013). RB1CC1 doesn't have a strong yeast homolog by sequence analysis,

333   but is very likely structurally and functionally equivalent to Atg17 and Atg11 in yeast (Li et al.,

334   2014a). Recent studies have proposed that Atg17 is sufficient to remodel Atg9 vesicles into a cup-

335   shaped structure (Bahrami et al., 2017; Ragusa et al., 2012). Our work makes it further imperative

336   to establish if RB1CC1 scaffolding of ATG9A vesicles is a conserved phagophore nucleation

337   mechanism. Even more speculatively, our data suggests that autophagosomes that are produced by

338   a common phagophore nucleation process can then engage a variable set of mechanisms involved

339   in downstream autophagy steps. Further mapping of pairwise genetic interactions between ATG

340   factors is a promising approach for teasing apart these complexities inherent in the mammalian

341   ATG network.

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346   **Figure legends:**

347   **Figure 1. tfReceptors perform comparably to tfLC3 as reporters of autophagy.**

348   **(A)** Schematic depicting how tfReceptors measure autophagy flux. tfReceptors bind to targets

349   and interface with remaining autophagy machinery. GFP fluorescence is selectively quenched in

350   low pH environments, leading to an increased Red:Green ratio upon exposure of tfReceptor to

351   the lumen of the lysosome.

352   **(B)** HEK293T cells expressing indicated tf expression cassettes from the AAVS1 locus were

353   analyzed by flow cytometry under basal conditions and after 18h treatment with 250nM torin.

354   Plots show median Red:Green ratios with inner quartiles (boxed regions) and top and bottom

355   decade indicated (whiskers). n > 10,000 cells for each sample.

356   **(C)** Wildtype and indicated HEK293T knockout cells expressing tfSQSTM1 from the AAVS1

357   locus were treated and analyzed as in part B. n > 10,000 cells for each sample.

358   See also, Figure S1.

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360   **Figure 2. Genome-wide, pooled CRISPR screening for modifiers of tfReporters reveals**

361   **known and novel autophagy-related genes.**

362   **(A)** K562 cells expressing the indicated tf proteins from the AAVS1 locus were analyzed by

363   flow cytometry under basal conditions and after 18h treatment with 250nM torin or 100nM

364   BafilomycinA1 (BafA1). Plots show median Red:Green ratios with inner quartiles (boxed

365   regions) and top and bottom decade indicated (whiskers). n > 10,000 cells for each sample.

366   **(B)** Schematic depicting a pooled CRISPR screening strategy for defining genetic modifiers of

367   tfReporters (tfLC3 and tfReceptors). K562 cells expressing Cas9 (zeocin) were transduced with

368   the Brunello lentiviral sgRNA library (puromycin). The top (stimulated tfReporter delivery to the

369   lysosome) and bottom (inhibited delivery) 30% of the Red:Green ratio cell distribution cells

370   were collected. sgRNA sequences from collected cells were obtained by an Illumina sequencing

371   approach and analyzed by MAGeCK.

372   **(C)** A heat map of beta scores for each gene and each tfReporter are shown. Beta scores for each

373   gene-reporter pair were averaged across replicates. The maximum beta score for each tfReporter

374   was used to normalize its heatmap values. For convenience, genes are clustered loosely based on

375   their known function. Score extremes were color-coded from Red (autophagy suppression) to

376   Blue (autophagy enhancement) with average scores uncolored (white).

377

18

378   **Figure 3. Ablation of novel screen hits robustly yields autophagy phenotypes in single gene**

379   **knockout experiments.**

380   **(A)** K562 cells co-expressing Cas9 and tfSQSTM1 were transduced with individual sgRNAs

381   against the indicated genes or with negative sgRNA controls. Cells were pre-treated with torin to

382   maximize the dynamic range of tfSQSTM1 signals and analyzed by flow cytometry (n > 5000

383   cells) to determine median Red:Green ratios. Shown are violin plots generated in R using

384   ggplot2. The median of ratios for affected cell subpopulations in each sample are indicated by a

385   black line. Bimodal populations were deconvolved in an unbiased manner using the BifurGate

386   function of FlowJo. Red dotted line across all samples corresponds to the median ratio for mock

387   treated cells (Control 1). See Supplemental Figure S3A for similar data from the remaining

388   tfReporters.

389   **(B)** Heat map indicating the phenotypic strengths of indicated gene knockouts (as autophagy

390   suppressors) derived from the primary data in part A and Figure S3A. The median Red:Green

391   ratio of each population was used to calculate the fold-repression according to the following

392   formula: $(\text{ratio}_{\text{sgGene}}-\text{ratio}_{\text{sgATG9A}})/(\text{ratio}_{\text{sgControl}}-\text{ratio}_{\text{sgATG9A}})$, with the assumption that sgATG9

393   yields a true autophagy-null phenotype. Deeper shades of red indicate stronger suppressor

394   phenotypes. Black boxes indicate unscored cases.

395   See also, Figure S3.

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398   **Figure 4. Autophagy flux is disrupted in *TMEM41B^{KO}* cells despite normal induction of**

399   **autophagy by torin.**

400   **(A)** Wildtype and indicated HEK293T knockout cells were treated with 250nM Bafilomycin A1

401   (BafA1) for the times indicated. The corresponding cell extracts were normalized by total protein

402   and resolved by SDS-PAGE analyzed by immunoblotting (IB) for LC3. I and II indicate the

403   unmodified and lipidated forms of LC3.

404   **(B)** Extracts derived from wildtype and *TMEM41B^{KO}* HEK293T cells were resolved by SDS-

405   PAGE followed by immunoblotting (IB) with indicated antibodies. All samples were normalized

406   by total protein using a BCA assay prior to loading. I and II indicate the unmodified and

407   lipidated forms of LC3.

408   **(C)** Wildtype and indicated HEK293T knockout cells cell were treated with 250nM torin for 3

409   hours or left untreated. The corresponding cell extracts were resolved by SDS-PAGE and

410   analyzed immunoblotting (IB) with antibodies against indicated proteins or specific

411   phosphorylation sites. All samples were normalized by total protein using a BCA assay prior to

412   loading and even loading was verified by monitoring tubulin levels. I and II indicate the

413   unmodified and lipidated forms of LC3.

414   See also, Figure S4.

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20

417   **Figure 5. *TMEM41B^{KO}* cells accumulate unresolved intermediates in autophagosome**

418   **biogenesis.**

419   **(A)** Representative confocal micrographs (as maximum intensity projections) of wildtype and

420   *TMEM41B^{KO}* HEK293T cells. Selected regions (white box) of micrographs are shown as insets

421   of single and merged channels from immunofluorescence against indicated proteins. LC3,

422   magenta; SQSTM1, green; merged, white; Hoechst, blue. Scale bars: large panels, 5µm; small

423   panels, 1µm.

424   **(B)** Plots showing means of LC3$^+$/SQSTM1$^+$ punctae in wildtype and *TMEM41B^{KO}* HEK293T

425   cells imaged in part A (see Materials and Methods for details of quantitation) with inner quartiles

426   (boxed regions), 1.5 interquartile ranges (whiskers), and outliers (dots) indicated. Sample size (n)

427   for each sample is indicated.

428   **(C)** Wildtype and indicated HEK293T knockout cells were treated with 250nM torin for the

429   indicated times prior to analysis by confocal microscopy. Shown are representative confocal

430   micrographs (as maximum intensity projections). Selected regions (white boxes) of micrographs

431   are shown as insets of single and merged channels from immunofluorescence against indicated

432   proteins. LC3, magenta; WIPI2, green; merged, white; Hoechst, blue.

433   **(D)** Plots showing means of indicated punctae in wildtype and HEK293T knockout cells imaged

434   in part C with inner quartiles (boxed regions), 1.5 interquartile ranges (whiskers), and outliers

435   (dots) indicated. Sample size (n) for each sample is indicated.

436   **(E)** Representative confocal micrographs (as maximum intensity projections) of wildtype and

437   indicated single and double knockouts (DKO) of HEK293T cells analyzed by confocal

438   microscopy. Selected regions (white box) of micrographs are shown as insets of single and

21

439   merged channels from immunofluorescence against indicated proteins. LC3, magenta; WIPI2,

440   green; merged, white; Hoechst, blue. Scale bars: large panels, 5μm; small panels, 1μm.

441   **(F)** Plots showing means of indicated punctae in cells imaged in part **E** with inner quartiles

442   (boxed regions), 1.5 interquartile ranges (whiskers), and outliers (dots) indicated. Sample size (n)

443   for each sample is indicated.

444   See also, Figure S5.

445

446

447   **Figure 6. Autophagosome biogenesis in *TMEM41B^{KO}* cells is arrested prior to full vesicle**

448   **closure.**

449   **(A)** Schematic of protease protection assay for detecting closed autophagosomes. Lipidated LC3

450   (LC3-II) is indicated on the autophagosomal membrane. Dashed lines indicate proteolyzed LC3.

451   **(B)** Wildtype and indicated HEK293T knockouts were treated for 18h with BafilomycinA1 prior

452   to gentile, mechanical lysis. The corresponding cell extracts were treated as indicated prior to

453   being resolved by SDS-PAGE and analyzed by immunoblotting (IB) with indicated antibodies. I

454   and II indicate unmodified and lipidated forms of LC3.

455   **(C)** Quantitation of protease-protection data from experiments in part B. Bar graphs show the

456   mean +/- SD of each sample from three independent experiments. p values were determined

457   using a student's t-test. **, $p < 0.01$.

458

23

459   **Figure 7. ATG7-dependent and ATG7-independent autophagy: a comparison of genetic**

460   **landscapes.**

461   **(A)** Gene correlation plot (19,117 genes) of average beta scores for tfNBR1 and tfSQSTM1.

462   Representative autophagy-related genes are indicated in black. Dashed lines indicate top 1% of

463   beta scores.

464   **(B)** K562 cells co-expressing Cas9 with indicated tfReporter were transduced with individual

465   sgRNAs against shown genes or with sgRNA controls. Cells were pre-treated with torin to

466   maximize the dynamic range of tfSQSTM1 signals and analyzed by flow cytometry (n>5000

467   cells) to determine their Red:Green ratios. Shown are violin plots generated in R using ggplot2.

468   The median of ratios for affected cell subpopulations in each sample are indicated by a black

469   line. Bimodal populations were deconvolved in an unbiased manner using the BifurGate function

470   of FlowJo. Red dotted line across all samples corresponds to the median ratio for mock treated

471   cells (Control 1).

472   **(C)** Dot plots of data from triplicate experiments showing the indicated tfReporter fractions

473   delivered to the lysosome by ATG7-independent mechanisms. The median Red:Green ratio for

474   each reporter was calculated for sgControl 1 and sgATG9A to set the upper and lower bounds,

475   respectively. The median Red:Green ratio measured in sgATG7 cells was then used to calculate

476   the fraction of tfReporter delivered to the lysosome according to the following formula:

477    $(\text{ratio}_{\text{sgATG7}}-\text{ratio}_{\text{sgATG9A}})/(\text{ratio}_{\text{sgControl 1}}-\text{ratio}_{\text{sgATG9A}})$. P-values were determined using a student's

478   t-test. ***, $p <0.001$; **, $p <0.01$; *ns*, not significant.

479   **(D)** Gene correlation plot (19,117 genes) of average beta scores for tfNBR1 in wildtype and

480   *ATG7^{KO}* cells. Notable autophagy-related genes are highlighted in black. Also highlighted (but

24

481   without their gene names) are components of the TRAPP (blue) complex that scored as hits.

482   Dashed lines, top 1% of beta scores.

483   **(E)** Wildtype and $ATG7^{KO}$ K562 cells co-expressing Cas9 and tfNBR1 were transduced with

484   individual sgRNAs for indicated genes. The median Red:Green ratio of each population was

485   used to calculate the fold-repression according to the following formula: $(\text{ratio}_{sgGene}-$

486    $\text{ratio}_{sgATG9A})/(\text{ratio}_{sgControl}-\text{ratio}_{sgATG9A})$, with the assumption that sgATG9 yields a true

487   autophagy-null phenotype. Deeper shades of red indicate stronger suppressor phenotypes. Genes

488   were clustered on the basis of their patterns of genetic interactions.

489

490   **Figure S1 – Figure 1, supplement 1. Generation and validation of tfReceptor autophagy**

491   **reporters.**

492   **(A)** Diagram of gene cassette used for expressing all tf proteins in this study. Salient cassette

493   features are color coded. Shown below are ATG factors that were expressed as N-terminal tf

494   fusions with their length, common names, and RefSeq accession number listed left to right. SA,

495   splice acceptor; P2A, self-cleaving peptide; Puro^R/BSD^R, puromycin or blasticidin resistance

496   cassette; BGH pA, bovine growth hormone polyadenylation signal; CAG, CAG promoter

497   sequence; AAVS1, AAVS homology arms. All inserts were positioned at the KpnI restriction

498   site.

499   **(B)** HEK293T cells expressing the indicated tf proteins from the AAVS locus were grown under

500   basal conditions or treated with torin. Shown are flow cytometry traces of GFP and RFP

501   fluorescence (arbitrary units), both as individual signals and as a ratio (Red:Green).

502   **(C)** Extracts derived from cells with indicated genotypes were normalized by total protein levels

503   using a BCA assay and resolved by SDS-PAGE followed by immunoblotting (IB) with indicated

504   antibodies.

505

506   **Figure S3 – Figure 3, supplement 1. Confirmation of sgRNA effects on autophagy**

507   **reporters.**

508   **(A)** K562 cells co-expressing Cas9 and indicated tfReporters were transduced with individual

509   sgRNAs against the shown genes or with negative sgRNA controls. Cells were treated and

510   analyzed as in Figure 3A. These data are represented as part of the heatmap in Figure 3B. n >

511   5000 cell each.

512   **(B)** K562 cells co-expressing Cas9 and tfLC3 were transduced with sgRNAs against the

513   indicated genes or with a negative sgRNA control. Shown are flow cytometry traces of GFP and

514   RFP fluorescence (in arbitrary units), both as individual signals and as a ratio (Red:Green). Cells

515   were treated and analyzed as in Figure 3A.

516   **(C)** K562 cells expressing Cas9 were transduced with individual sgRNA against the indicated

517   genes or negative sgRNA controls. The corresponding cell extracts were resolved by SDS-PAGE

518   followed by immunoblotting (IB) with indicated antibodies. I and II indicate the unmodified and

519   lipidated forms of LC3, respectively. Numbers indicate the indicated protein ratios and were set

520   to 1 for the Control 1 in each boxed data set.

521

522

523

524 **Figure S4 – Figure 4, supplement 1. TMEM41B deletion inhibits autophagy.**

525 **(A)** Wildtype HEK293T cells (top) or expressing endogenous TMEM41B with an N-terminal

526 GFP11 tag (bottom) were transduced with a lentivirus expressing GFP1-10 and analyzed by

527 confocal microscopy. Shown are confocal slice micrographs of GFP fluorescence and calnexin

528 immunofluorescence, both as individual signals and merged.

529 **(B)** Sequence of mutant alleles in *TMEM41B^{KO}* HEK293T cells. Wildtype allele with indicated

530 splice site is shown for comparison. Color coding was used to distinguish intron and exon

531 sequences.

532 **(C)** Extracts derived from wildtype and *TMEM41B^{KO}* HEK293T cells were normalized for total

533 protein by BCA and resolved by SDS-PAGE followed by immunoblotting (IB) with indicated

534 antibodies.

535 **(D)** Extracts derived from wildtype and *TMEM41B^{KO}* HEK293T cells expressing indicated tf

536 fusions of TMEM41B were normalized for total protein by BCA resolved by SDS-PAGE

537 followed by immunoblotting (IB) with indicated antibodies. I and II indicated unmodified and

538 lipidated forms of LC3.

539 **(E and F)** Wildtype and indicated HEK293T knockout cells expressing indicated tfReporters

540 were analyzed by flow cytometry under basal conditions and after 18h treatment with 250nM

541 torin or 100nM BafilomycinA1 (BafA1). Plots show mean Red:Green ratios with inner quartiles

542 (boxed regions) and top and bottom decade indicated (whiskers). n > 5000 cells each sample.

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546   **Figure S5 – Figure 5, supplement 1. TMEM41B deletion arrests autophagy on-pathway**

547   **prior to autophagosome closure.**

548   **(A)** Extracts derived from wildtype and indicated HEK293T single and double knockout (DKO;

549   *TMEM41B^{KO}/RB1CC1^{KO}*) cells were resolved by SDS-PAGE and analyzed by immunoblotting

550   (IB) for indicated proteins. I and II indicate unmodified and lipidated forms of LC3. Also shown

551   are PCR results from a T7 endonuclease assay used to confirm TMEM41B gene knockouts.

552   **(B)** Wildtype and indicated HEK293T knockout cells expressing GFP-STX17TM were treated

553   with 250nM torin for one hour or left untreated (mock) prior to confocal microscopy. Shown are

554   representative confocal micrographs (as maximum intensity projections). Selected regions (white

555   boxes) of micrographs are shown as insets of single and merged channels from intrinsic GFP

556   fluorescence or immunofluorescence against indicated proteins. LC3, magenta; STX17, green;

557   merged, white; Hoechst, blue. Scale bars: large panels, 5µm; small panels, 1µm.

558   **(C)** Plots showing means of indicated punctae in wildtype and HEK293T knockout cells imaged

559   in part B with inner quartiles (boxed regions), 1.5 interquartile ranges (whiskers), and outliers

560   (dots) indicated. Sample size (n) for each sample is indicated.

561

29

562 **Figure S7 – Figure 7, supplement 1. Comparison of *ATG7^{KO}* and *RB1CC1^{KO}* isolates.**

563 **(A)** K562 cells (far left lane) expressing tfNBR1 were transduced with sgRB1CC1 and sgATG7.

564 Following clonal expansion of transductants, six candidates were selected for analysis by SDS-

565 PAGE and immunoblotting (IB) of their cell extracts with indicated antibodies, as appropriate.

566 Wildtype K562 cells and the parent tfNBR1 integrant cell line were also analyzed for

567 comparison. Red boxes indicate isolates used in subsequent studies.

568 **(B)** K562 tfNBR1 transductants described in part A and six WT clones were analyzed by flow

569 cytometry (n>5000 cells). Shown are plots of median for individual isolates (dots) and the mean

570 for the isolates combined (dashed lines). p-values (*ns*, not significant) shown were determined

571 using a student's t-test. Isolates used in subsequent studies are indicated in red.

572 **(C)** K562 knockout isolates described in parts A and B and their wildtype parent were analyzed

573 by flow cytometry under basal conditions and after 18h treatment with 250nM torin or 100nM

574 BafilomycinA1 (BafA1). Plots show mean Red:Green ratios with inner quartiles (boxed regions)

575 and top and bottom decade indicated (whiskers). n > 5000 cells each sample.

576

577

578   **Figure 2–source data 1. Read counts from all screens.**

579   Normalized read counts from each sequencing experiment. See 'read_me' tab for sample

580   identity. Up, top 30% of cells by Red:Green ratio; Down, bottom 30% of cells by Red:Green

581   ratio.

582

583   **Figure 2–source data 2. Beta scores from all screens.**

584   Individual beta scores from each experiment are listed by gene. Beta values from all replicates

585   were averaged and are shown. Averaged values are color coded from red (suppressor) to white

586   (neutral) to blue (enhancer). To view the underlying beta values for each replicate of a given

587   reporter, expand the desired column by clicking the '+' at the top the column.

588

589 **Table S1. sgRNA oligos**

590 List of 20-nucleotide oligos (plus overhangs) used to clone individual sgRNAs.

591

592   **Table S2. Other oligos**

593   List of oligos used in T7 endonuclease assays to confirm TMEM41B deletion and oligos used for

594   endogenous labeling of TMEM41B with the 11th beta strand of GFP.

595

596   **Table S3. List of primers for cloning tfReporters**

597   List of primers used to clone LC3B and receptors into the KpnI site of tfEmpty to generate each

598   tfReporter.

599

600   **Table S4. Illumina primers for Brunello library**

601   List of primers used for Illumina sequencing of the Brunello library. Staggered oligos were

602   pooled prior to PCR amplification. In contrast, a unique barcode primer was used for each

603   sample. Green, Illumina P5 or P7 sequence; blue, sequencing primer annealing region; underline,

604   stagger; red, region complementary to vector backbone (for PCR); purple, unique barcode (6-

605   mer);

606

607

608

609

610   **Methods**:

611   **Antibodies:** The following antibodies were used in this study. For immunoblotting (IB), all

612   primary antibodies were used 1:1000 except where otherwise noted; secondary antibodies were

613   used 1:3000 (HRP) or 1:10000 (fluorescent). For immunofluorescence (IF), antibody dilutions

614   are noted following each antibody; secondary antibodies were used 1:500. Primary antibodies:

615   mouse anti-SQSTM1 ([1:200 – IF] ab56416, Abcam), rabbit anti-TAX1BP1 (5105, CST), rabbit

616   anti-NDP52 (9036, CST), rabbit anti-LC3B ([1:2000 – IB] NB100-2220, Novus), rabbit anti-

617   LC3A/B ([1:100 – IF] 12741S, CST), rat anti-tubulin ([1:500 – IB] sc-53030, Santa Cruz), anti-

618   p70-S6K (9202S,CST), anti- p70-S6K(pT389) (9205S, CST), rabbit anti-ATG13 (ABC344,

619   EMDmillipore), rabbit anti-ATG13(pS318) (NBP2-19127, Novus), anti-WIPI2 ([1:150 – IF]

620   ab105459, Abcam), rabbit anti-ATG7 (8558, CST), anti-RB1CC1 (12436S, CST), mouse anti-

621   GFP (11814460001, Sigma), mouse anti-SERCA2 (ab2861, Abcam), rabbit anti-Calnexin (2679,

622   CST); secondary antibodies (IB): goat anti-mouse IgG HRP (170-6516, Bio-rad), goat anti-rabbit

623   IgG HRP (170-6515, Bio-rad), goat anti-rat IgG Alexa Fluor 488 (A11006, Invitrogen), goat

624   anti-mouse IgG Cy5 (A10524, Invitrogen), goat anti-rabbit IgG Alexa Fluor 546 (A11010,

625   Invitrogen); secondary antibodies (IF): goat anti-rabbit IgG Alexa Fluor 568 (A11036,

626   Invitrogen), goat anti-mouse IgG Alexa Fluor Plus 488 (A32723, Invitrogen).

627

628   **Chemicals and reagents:** The following chemicals and reagents were used in this study: torin1

629   (sc-396760, Santa Cruz), BafilomycinA1 (tlrl-baf1, Invivogen), poly-l-lysine (P4707-50ML,

630   Sigma), Lipofectamine 3000 (L3000008, Life Technologies), nucleofector kit T (VACA-1002,

631   Lonza), polybrene (H9268-5G, Sigma), normocin (ant-nr-1, Invivogen), puromycin (ant-pr-1,

632   Invivogen), blasticidin (ant-bl-1, Invivogen), zeocin (ant-zn-1, Invivogen), normal goat serum

37

633   (ab7481, Abcam), Phusion High-Fidelity DNA polymerase (M0530L, NEB), T5 exonuclease

634   (M0363S, NEB), Taq DNA ligase (M0208L, NEB).

635

636   **Vectors:** pHR-SFFV-GFP1-10 (Addgene plasmid # 80409) and pCDNA CMV mCherry-GFP-

637   11 were gifts from Bo Huang. pMRXIP GFP-Stx17TM was a gift from Noboru Mizushima

638   (Addgene plasmid # 45910). Human Brunello CRISPR knockout pooled library was a gift from

639   David Root and John Doench (Addgene #73178). lentiCRISPRv2 puro was a gift from Brett

640   Stringer (Addgene plasmid # 98290). lentiGuide-puro was a gift from Feng Zhang (Addgene

641   plasmid # 52963). ptfLC3 was a gift from Tamotsu Yoshimori (Addgene plasmid # 21074).

642   pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid #

643   42230). AAVS1-CAG-hrGFP was a gift from Su-Chun Zhang (Addgene plasmid # 52344).

644   psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260). pCMV-VSV-G was a gift

645   from Bob Weinberg (Addgene plasmid # 8454). pUMVC was a gift from Bob Weinberg

646   (Addgene plasmid # 8449). pFUGW-EFSp-Cas9-P2A-Zeo (pAWp30) was a gift from Timothy

647   Lu (Addgene plasmid # 73857).

648

649   **Isothermal assembly:** PCR fragments were generated using 2X phusion master mix (M0531S,

650   NEB) and insert-specific primers that appended a 30bp overlap with target DNA. Vector

651   backbones were linearized by restriction enzyme and dephosphorylated by calf intestinal

652   phosphatase (M0290S, NEB). Prior to assembly, all DNA fragments were gel purified (D4002,

653   Zymo Research). 50ng of linearized vector DNA were combined with isomolar amounts of

654   purified insert(s). 5ul of the resulting DNA mix was added to isothermal assembly master mix

655   and incubated at 50 C for 20 minutes (Gibson et al., 2010). Assembled product was transformed

38

656   into NEB Stable competent cells (C3040H, NEB) and plated on LB+agar plates (plus appropriate

657   antibiotics) to isolate single isolates. Single isolates were grown in LB broth + antibiotics and

658   plasmid DNA was purified using a Qiagen miniprep kit (27106, Qiagen). Sequences were

659   verified by Sanger sequencing (Eton Bioscience Inc).

660

661   **sgRNA oligonucleotide (oligo) ligation protocol:** sgRNA oligos were ordered from Eton

662   Biosciences. Oligo sequences are listed in Table S1. To generate the necessary overhangs, all

663   oligos were in the form: Forward: 5' CACCGNNNNNNNNNNNNNNNNNNNN – 3'; Reverse:

664   5'- AAACNNNNNNNNNNNNNNNNNNNNC – 3'. Oligos were diluted to 10uM in distilled

665   water. 50pmol each of forward and reverse oligo were combined in a 25ul reaction and

666   phosphorylated with T4 polynucleotide kinase (M0201S, NEB) in 1X T4 DNA ligase buffer

667   (B0202S, NEB) for 30 minutes at 37 degrees Celsius. Phosphorylated oligos were boiled for 5

668   min at 95 degrees Celsius and slow cooled (0.1 degrees Celsius/sec) to facilitate annealing.

669   Annealed oligos were diluted 1:100 and 2μl of insert were ligated into 20ng digested vector

670   (pLentiuGuide-puro, BsmBI site; pLentiCRIPSR v2, BbsI site; pX330-derivatives, BbsI site)

671   using T4 DNA ligase (M0202S, NEB). Ligation was allowed to proceed for 15 minutes at room

672   temperature. Ligated products were transformed into NEB Stable cells.

673

674   **T7 endonuclease (Surveyor) assay:** Genomic DNA (gDNA) was extracted using QuickExtract

675   buffer (QE0905T, Epicentre) according to the manufacturer's instructions. gDNA was

676   subsequently normalized to 200ng/μl. 600ng gDNA was used per 100 μl PCR to amplify the

677   targeted region of interest. Primers are listed in Table S2. The resulting PCR amplicon was

678   purified using a Zymoclean Gel DNA Recovery Kit (D4002, Zymo Research) and normalized to

679   20ng/µl in 19ul 1X NEBuffer 2 (B7002S, NEB). Amplicon was boiled and cooled (-1 degrees

680   Celsius/sec) to allow for hybridization. 1µl T7 endonuclease I (M0302, NEB) was added and

681   incubated for 15 min at 37 degrees Celsius. Reaction was quenched by adding 1.5µl 0.25M

682   EDTA and analyzed in a 2% UltraPure Agarose gel (16500500, Invitrogen).

683

684   **Generation of AAVS1-targeting tfReporters**: The hrGFP fragment was excised from AAVS1-

685   CAG-hrGFP using SalI/EcoRV and replaced with RFP-GFP subcloned from ptfLC3 to generate

686   pCS418 tfEmpty (puro). LC3B (NP_074058.2), SQSTM1 (NP_003891.1), NDP52

687   (NP_005890.2), NBR1 (NP_006015.4) or TAX1BP1 (NP_005822.1) were PCR amplified and

688   subcloned into the KpnI site of pCS418. Primers are listed in Table S3. To generate blasticidin

689   resistant versions of each cassette, the puromycin resistance gene was excised from pCS418 with

690   an XhoI/SpeI digest and replaced with an analogous geneblock fragment (oVD5075, Integrated

691   DNA technologies) encoding a blasticidin resistance gene (BSD).

692

693   **Tissue Culture:** All cells were grown in a standard water-jacketed incubator with 5% CO_2.

694   K562 cells were grown in IMDM media (30-2005, ATCC) with 10% FBS (30-2020, ATCC) and

695   1x penicillin/strep. Cells were maintained below 1 million cells per milliliter. HEK293T cells

696   were grown in DMEM media (30-2002, ATCC) with 10% FBS and 1x pen/strep. Normocin

697   (1:500) was used as a common additive. All cells were passaged less than 25 times. For

698   passaging, cells were trypsinized with Trypsin-EDTA (25300-054, Life Technologies).

699   Puromycin (2µg/ml), blasticidin (5µg/ml) and zeocin (50µg/ml) were added when necessary for

700   selection.

701

40

702   **Cell Line Authentification:** Genomic DNA was isolated from HEK293T and K562 cells using

703   the GenElute™ Mammalian Genomic DNA Miniprep Kits (Sigma-Aldrich). STR profiling and

704   allele identification were performed by the Molecular Diagnostics Laboratory of Dana-Farber

705   Cancer Institute. Briefly, isolated genomic DNA was analyzed with the GenePrint® 10 short

706   tandem repeat (STR) profiling kit (Promega) and Amelogenin for gender identification.

707   GeneMapper v4 Fragment Analysis software (Applied Biosystems) and GenePrint10® allele

708   panel (Promega) custom bin files were used to identify the alleles at eight STR loci (TH01,

709   TPOX, vWA, CSF1PO, D16S539, D7S820, D13S317 and D5S818). The ATCC STR Profile

710   Database was used to verify that the identified alleles matched those of the expected cell type.

711

712   **Transient transfection and nucleofection**: Prior to transfection, HEK293T cells were seeded in

713   OptiMEM Reduced Serum media (51985-034, ThermoFisher). At 90% confluency, cells were

714   transfected overnight using Lipofectamine 3000 reagent according to the manufacturer's

715   recommendations. The following morning media was exchanged and cells were passaged for

716   another 24 hours prior to drug treatment. K562 cells were nucleofected using a Nucleofector™

717   2b Device (Lonza) using nucleofector kit T (VACA-1002, Lonza) and protocol T-016.

718   Transfected/nucleofected DNA was prepared using a ZymoPURE™ Plasmid Midiprep Kit

719   (D4200, Zymo Research).

720   **Generation of gene knock-out cell lines using CRISPR-Cas9 gene editing:** For the generation

721   of stable knock-outs, HEK293T and K562 cells were transfected or nucleofected, respectively, as

722   described above. Oligo sequences for sgRNAs were generated by CHOPCHOP or extracted from

723   the Brunello library and cloned into the indicated vectors as outlined above under 'sgRNA

724   oligonucleotide (oligo) ligation protocol' (Doench et al., 2016; Labun et al., 2016). Oligos are

41

725   listed under Table S2. Cells were diluted by limiting dilution or cell sorting into 96 well plates

726   and clonally expanded. Expanded cells were confirmed for knock-out by western blot or PCR/T7

727   endonuclease testing.

728   **Generation of GFP1-10 cell line:** HEK293T cells were transduced with a lentiviral GFP1-10

729   expression cassette. Clonal cell lines were established by limiting dilution and validated for

730   GFP1-10 insertion by transient transfection with mCherry-GFP-11. Four successful clones were

731   saved and one was used for all further experiments. To tag TMEM41B, cells were transfected

732   with pCS651 (coexpressing Cas9-T2A-BFP, sgTMEM41B) and oVD6217 (an oligo containing

733   5'homology arm-GFP11-linker-3' homology arm). Successful integrants were identified and

734   sorted by FACS.

735

736   **AAVS1 integration:** Cell lines were co-transfected with pX330-U6-Chimeric_BB-CBh-

737   hSpCas9-AAVS1- gRNA and AAVS1-tfReporter template vectors. 48 hours post-transfection

738   cells were incubated with appropriate selection media and passaged for 14 days. Red+/Green+

739   cells were sorted and propagated.

740

741   **Lentiviral generation:** Lentivirus was generated in HEK293T cells using Lipofectamine 3000.

742   Cells were grown overnight in Opti-MEM media (5% FBS, no antibiotics) (31985062,

743   ThermoFisher) to 90% confluency. Cells were than transfected with pVSV-G, pSPAX2 and

744   packaging constructs at a 1:3:4 ratio. Transfection proceeded for 6-8 hour before media was

745   refreshed. Virus was collected and pooled at 24 and 48 hours post-transfection. Virus was

746   pelleted at 1000g 2X 10 min, aliquoted and frozen in single-use aliquots. For retrovirus

747   production, all methods were the same except pUMVC was exchanged for pSPAX2.

748

749   **Viral transduction:** Cells were incubated in appropriate media containing 8µg/ml polybrene and

750   lacking penicillin/streptomycin. Cells were transduced overnight. Media was exchanged for

751   media lacking polybrene for 24 hours prior to antibiotic selection.

752

753   **Protease protection assay:** Our protocol for mammalian protease protection assay was based on

754   Zhao et al. (Zhao et al., 2013). Cells were seeded so they would be 70% confluent at 5pm. Media

755   was then exchanged into media containing 100nM Bafilomycin A1. Cells were incubated for 15

756   hours. After incubation, cells were trypsinized and pelleted. Cells were washed 1X with cold

757   PBS and resuspended in pre-chilled lysis buffer (20mM Hepes KOH pH7.4, 0.22M mannitol,

758   0.07M sucrose). Cells were lysed by extrusion through a 30 gauge needle 10-15 times. Samples

759   were pelleted 2X at 500g for 10 min at 4 degrees Celsius to pellet debris. When indicated,

760   samples were incubated with 1X trypsin (T1426-100MG, Sigma; 100X stock: 1mg/ml) and/or

761   0.5% Triton X-100 for 35 minutes at 30C. Reactions were quenched in 1X hot Laemmli sample

762   buffer and boiled at 65 degrees for 10 minutes.

763

764   **Immunoprecipitation:** Cells were collected and resuspended in IP buffer (50mM HEPES pH

765   7.4, 150mM NaCl, 2mM EDTA, 1% Triton X-100). Cells were incubated on ice for 30 minutes

766   and pelleted twice at 5000g for 5 minutes at 4C. Supernatant was applied to pre-washed GFP-

767   Trap or RFP-Trap magnetic agarose (Chromotek) and incubated for 1 hour at 4C. Beads were

768   washed 4x 5 min with two tube changes. Protein was eluded by boiling at 70C in 1X SDS buffer.

769

43

770 **Gel electrophoresis and western blotting:** Cells were trypsinized at 75% confluency and

771 quenched in an equal amount of media. Cells were lysed for 15 minutes on ice in lysis buffer

772 (50mM HEPES pH 7.4, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 2X cOmplete protease

773 inhibitor tablet [Roche]). For phosphorylation analysis, lysis buffer was supplemented with

774 phosphatase inhibitors (10X: 100mM NaF, 10mM Na_3VO_4, 100mM $NaPP_i$). Lysates were

775 cleared 2X at 1000g for 5 minutes. Post-spin supernatants were used as input. Protein levels in

776 supernatants were normalized using a BCA protein assay (Pierce #23227). Normalized samples

777 were boiled in 1X (final concentration) Laemmli Loading Buffer (3X stock: 189 mM Tris pH6.8,

778 30% glycerol, 6% SDS, 10% beta-mercaptoethanol, bromophenol blue). Gel electrophoresis was

779 performed at 195V for 70 minutes in Novex 4-20% Tris-Glycine gels. Total protein analysis was

780 performed using SYPRO Ruby (Thermo Fisher) according the manufacturer's recommendations

781 (short protocol). For western blotting, samples were transferred for 60 minutes to 0.2µm PVDF

782 membranes (Millipore Sigma #ISEQ00010) using a Semi-dry transfer cell (Bio-Rad).

783 Membranes were blocked for 20 minutes in TBS-T with 5% milk. Primary antibodies were

784 incubated overnight at 4 degrees Celsius. Blots were then rinse 3X 5 minutes. Secondary

785 antibodies were incubated for 1 hour at room temperature. Blots were rinsed 4X 10 minutes in

786 TBS-T and imaged using fluorescence (Typhoon Trio Imager) or chemiluminescence

787 (SuperSignal™ West Femto Maximum Sensitivity Substrate, ThermoFisher Scientific, #34095).

788 If necessary, stripping of membranes was performed using Restore Western Blot Stripping

789 Buffer (ThermoFisher Scientific #21059) for 10 minutes.

790

791 **Immunofluorescence/Immunocytochemistry:** Coverslips (Fisher Scientific, 12-548A) were

792 placed in 6-well tissue culture plates (VWR, 62406-161) and coated with poly-L-lysine (Sigma,

44

793   P4707) per the manufacturer's recommendations. Cells were seeded onto coverslips overnight so

794   that they would be 15% confluent at the time of fixation. When reported, cells were treated with

795   250nM torin for 1 to 3 hours prior to fixation. Fresh 16% PFA (Electron Microscopy Sciences,

796   #15710) was diluted to 4% in 1X Dulbecco's phosphate buffered saline with calcium chloride

797   and magnesium chloride (10X: Life Technologies, #14080-055). Coverslips were removed with

798   forceps and placed into 4% PFA for 15 minutes. PFA was aspirated and washed twice with PBS

799   (Sigma, D8537). For LC3 immunofluroescence, cells were transferred to wells containing pre-

800   chilled (-20C) methanol for 5 minutes. Slides were returned to PBS and washed 2X for 5

801   minutes. Slides were blocked at RT for 1 hour in blocking buffer (0.3% Triton X-100, 5%

802   Normal Goat Serum (NGS) in PBS) and washed once in PBS. Primary antibody was diluted in

803   5% NGS at the dilutions described elsewhere in these methods. 75ul of antibody mixture was

804   spotted on parafilm in a humidified chamber and inverted coverslips were incubated with

805   antibody overnight at 4C. After incubation, coverslips were washed 3X 10 min in PBS.

806   Secondary antibodies were diluted 1:500 in 5% NGS and inverted coverslips were incubated

807   with antibody mixture for 45 minutes. Cells were stained with a 1:10,000 dilution of Hoechst

808   33342 (ThermoFisher Scientific, H3570) for 5 minutes. Coverslips were washed 4X 10 minutes

809   in PBS and mounted on coverslips (Corning, 294875X25) using Prolong Diamond

810   (ThermoFisher Scientific, P36965).

811

812   **Confocal microscopy:** Fluorescent images were obtained using a confocal microscope with

813   Airyscan detectors (LSM880 with Airyscan, Zeiss), a 63x PlanAPO oil-immersion objective lens

814   (Zeiss), and processed with Zeiss Blue software (Zeiss).

815

816   **Image analysis:** Fluorescence microscopy images were processed using a newly developed

817   Python analysis pipeline built around the pyto_segmenter analysis package (Weir et al., 2017).

818   First, regions of images containing cells were identified. To do so we first fit a Gaussian

819   distribution to the fluorescence intensity distribution for a smoothed green channel (488 nm

820   excitation) z-stack from an empty field. Using this Gaussian fit we predicted the probability that

821   each pixel in the smoothed green channel z-stack for each field corresponded to background (non-

822   cell) or foreground (cell). We assigned each pixel with a p(background) $< 10^{-5}$ to the cells, thus

823   creating a "cell mask". After removing small specks (<100,000 pixels volume) to eliminate debris,

824   we removed out-of-focus planes from the cell mask using a Support Vector Machine (SVM)

825   classifier as described previously (Hsu and Chen, 2008). Next, nuclei were segmented from the

826   blue (DAPI) channel by slice-by-slice relative thresholding followed by watershed segmentation

827   using the pyto_segmenter package. Cells were segmented using watershed segmentation from

828   nuclei seeds. Cell edges were eroded to eliminate blurred edge excess included during the p-value

829   transformation. Cells contacting the edge of the field were removed from analysis. Next, punctae

830   were segmented in the green and red (561 nm) channels using the pyto_segmenter package with

831   empirically determined Canny edge detection thresholds. The number of total punctae and punctae

832   overlapping with objects in the other fluorescence channel were counted and tabulated data were

833   saved in .csv format. Plotting was performed using R and the ggplot2 package. See the image

834   analysis package for details. Scripts for image analysis, scripts for data plotting and tabulated

835   values from microscopy data can be found at https://github.com/deniclab/csth-

836   imaging/tree/pub_version.

837

838   **Sample size estimate and experimental replication details:** For microscopy experiments, 40

839   images were collected and the number of cells in each sample was counted using segmentation

840   scripts. Cell counts are indicated above each quantitation. Samples were masked prior to data

841   collection and analyzed using automated scripts to eliminate bias during quantification.

842   Replicates represent biological replicates in which strains were subjected to identical

843   preparations on different days.

844   For sequencing experiments, the number of replicates (2-4) are indicted in Figure 2–source data

845   1. Each replicate was a biological replicate where strains were transfected and taken through the

846   entire experiment on separate days.

847   For flow cytometry experiments, N is indicated for each experiment in the figure legend. >5000

848   cells were used for each experiment.

849

850   **Library propagation:**

851   Brunello library (2 vector system) was purchased from addgene (item #73178). 50ng of library

852   was electroporated into 25µl Endura electrocompetent cells (Lucigen, 60242-2). Cells from 8

853   electroporations were pooled and rescued in 8 ml of rescue media for 1 hour at 37 degrees

854   Celsius. 8 ml of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM

855   $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to cells and 200µl of the final solution

856   was spread onto 10 cm LB plates containing 50 µg/ml carbenicillin (80 plates total). Through a

857   dilution series, 500 million colonies were estimated, representing 7000x coverage of the library.

858   Cells were manually scraped off plates and a GenElute Megaprep kit (Sigma, NA0600-1KT) was

859   used to purify plasmid DNA.

860

861 **Library lentiviral generation:**

862 Lentivirus was generated by lipofection (Lipofectamine 3000) of HEK293T cells with 5 µg

863 psPAX2 (Addgene Plasmid #12260), 1.33 µg pCMV-VSV-G (Addgene plasmid Plasmid

864 #8454), and 4 µg library vector per 10 cm plate. Transfection was performed according to

865 manufacturer's specifications. Briefly, low-passage HEK293T cells were grown in Opti-MEM +

866 5% FBS medium to 95% confluency by time of transfection. Cells were transfected for 6 hours,

867 then media was replaced with fresh Opti-MEM + 5% FBS. 24 hours post-transfection,

868 supernatant was collected and replaced. 48 hours post-transfection, supernatant was again

869 collected, pooled with the 24 hour supernatant and clarified 2X 1000g for 10 min. Viral RNA

870 was purified using a Machery Nagel viral RNA purification kit. Viral RNA was quantified using

871 the Lenti-X™ qRT-PCR Titration Kit (Takara). A value of 849 copies/IFU, derived from a

872 control virus expressing BFP, was used to calculate viral titer.

873

874 **Transduction and cell growth:**

875 For CRISPR screening experiments, K562 cells were passaged to maintain cell density between

876 500,000 and 2 million cells/ml. Cells were propagated in IMDM + 10% FBS + pen/strep +

877 appropriate antibiotics (Blasticidin 5 µg/ml, zeocin 50 µg/ml) until 200 million cells were

878 obtained (approximately 8-10 days). All this stage, all remaining experiments were performed in

879 175 cm^2 flasks. For infection, 200 million cells were pelleted and resuspended in IMDM + 10%

880 FBS + 8 µg/ml polybrene. Date of infection was day 0. An MOI of 0.4 was used to minimize

881 multiple infection events per cell. Cells were infected overnight, pelleted and exchanged into

882 fresh media. After 24 hours, cells were split and 2 µg/ml puromycin was added. Cells were

883 continually passaged in puromycin. At day 10, cells were removed from puromycin and at day

48

884   12 cells were sorted for red:green fluorescence. 100M unsorted cells were pelleted and processed

885   as input. The top and bottom 30% of cells (based on Red:Green ratio) were taken. 100 million

886   cells were sorted for each experimental condition. Cell sorting was performed using a FACSAria

887   or BioRad S3 sorter. Cells were pelleted and stored at -80 degrees Celsius until processing.

888

889   **CRISPR screen processing:**

890   Genomic DNA was purified from collected cells using the NucleoSpin Blood XL kit (740950.1,

891   Machery Nagel) according to the manufacturer's instructions. Illumina sequencing libraries were

892   created by PCR amplifying the genomically-integrated sgRNA sequences. All genomic DNA

893   was used for each PCR. A pool of eight staggered-length forward primers was used in each PCR

894   reaction to avoid monotemplating during Illumina sequencing. Reverse primers contained unique

895   barcodes designed to allow for sequencing and differentiation of multiple libraries on a single

896   chip during Illumina sequencing. Each 50 µL PCR reaction contained 0.4 µM of each forward

897   and reverse primer mix (Integrated DNA Technologies), 1x Phusion HF Reaction Buffer (NEB),

898   0.2 mM dNTPs (NEB), 40 U/mL Phusion HF DNA Polymerase (NEB), 5 µg of genomic DNA,

899   and 3% v/v DMSO. The following PCR cycling conditions were used: 1x 98°C for 30 s; 25x

900   (98°C for 30 s, 56°C for 30 s, 72°C for 30 s); 1x 72 °C for 10 min. The resulting products were

901   pooled to obtain the sgRNA libraries. The pooled PCR products were size selected by adding

902   0.95x magnetic bead slurry as outlined by DeAngelis et al.(DeAngelis et al., 1995). The High

903   Sensitivity D1000 ScreenTape system (Agilent Technologies) was used to confirm the absence

904   of primer dimers after purification. Sample libraries were quantified by qPCR using the

905   NEBNext Library Quant Kit for Illumina (NEB). Four to five libraries were pooled to a total

906   concentration of 10 nM for simultaneous Illumina sequencing on a single chip. The libraries

49

907 were sequenced using either the HiSeq 2000 or 2500 system (Illumina). The HiSeq 2000 system

908 was run on High Output Run Mode with the TruSeq SBS V3 kit (Illumina). The HiSeq 2500

909 system was run on either the Rapid Run Mode with the HiSeq Rapid SBS V2 kit (Illumina) or

910 the High Output Run Mode with the HiSeq SBS V4 kit (Illumina). Sequencing was performed

911 per recommendations of the manufacturer with custom sequencing and indexing primers

912 (Integrated DNA Technologies). For primer sequences, see Table S4.

913

914 **NGS data analysis**:

915 The 5' end of NGS reads were trimmed to 5'-CACCG-3' using Cutadapt. The `count` function of

916 MAGeCK (version 0.5.3) was used to extract read counts for each sgRNA. The `mle` function was

917 used to compare read counts from cells displaying increased and decreased Red:Green ratios.

918 The output included both beta scores and false discovery rates. Beta scores for each sgRNA for

919 each tfReporter were averaged across 2-4 experiments. Across all experiments, average read

920 counts were 200-400 per sgRNA. To generate heatmaps for each reporter (e.g. Fig 2), the beta

921 scores for each gene were normalized by the beta score for ATG9A.

922

923 **Flow cytometry:**

924 All samples were pelleted, washed 1X in cold PBS, and filtered through strainer cap tubes

925 (VWR, 21008-948) prior to analysis. Flow cytometry data were collected on an LSRII flow

926 cytometer. Data were analyzed in FlowJo (FlowJo, LLC) and R. The biomodality of populations

927 was determined in an unbiased manner using the BifurGate tool in FlowJo. At least 5000 cells

928 were collected for all samples.

929

930

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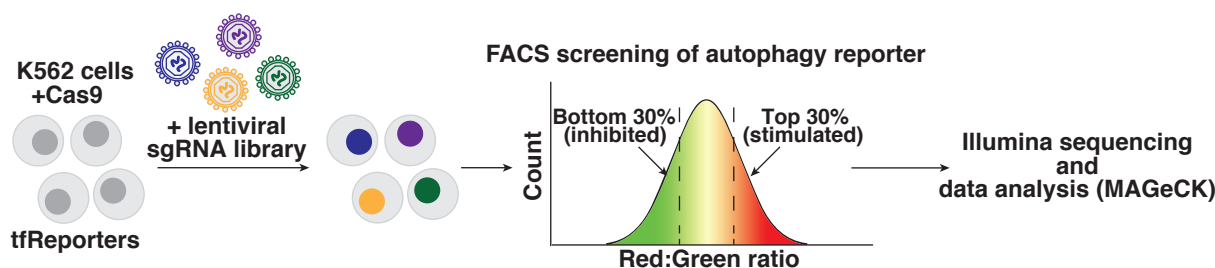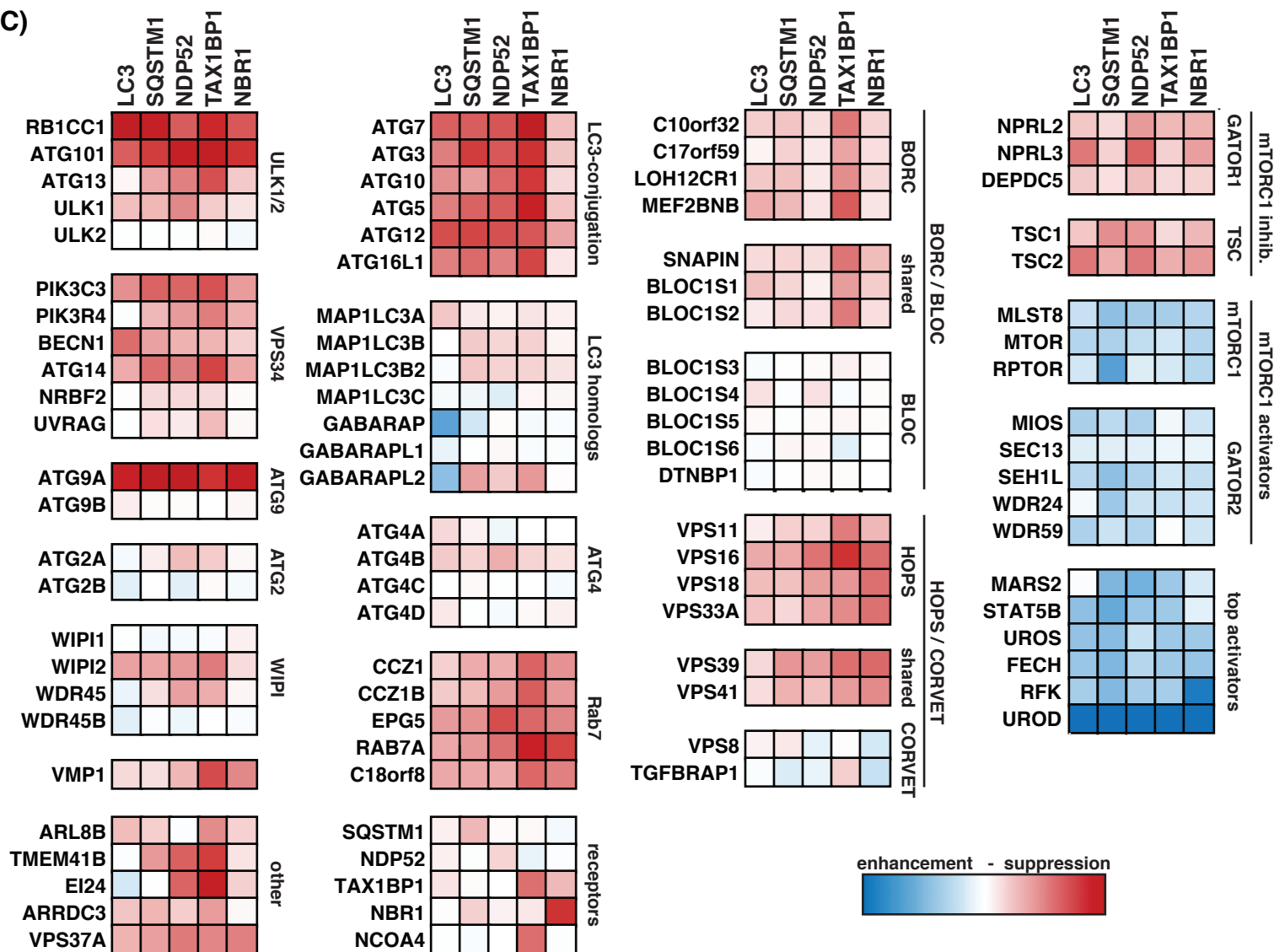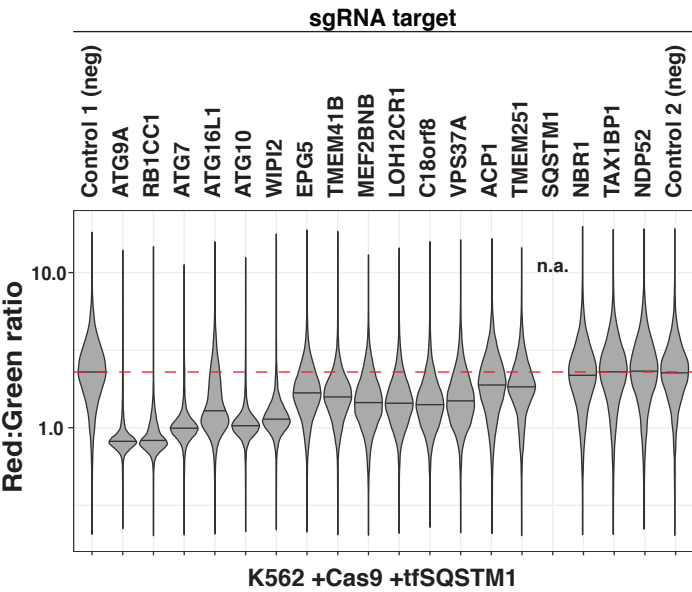1080   degeneration. J. Cell Biol. *200*, 731–741.

1081

Figure 1

Figure 2

Figure 3

A)

B)

Figure 4

A)

HEK293T + BafA1

time (h) | 0 | 1 | 3 | 7

WT — I / II

TMEM41B[KO] — I / II

RB1CC1[KO] — I / II

IB: LC3

B)

HEK293T

WT | *TMEM41B*[KO]

- SQSTM1
- TAX1BP1
- NDP52
- NBR1
- LC3 (I / II)
- tubulin

IB

C)

HEK293T

WT | *RB1CC1*[KO] | *TMEM41B*[KO]

torin | + | + | +

p70-S6K — p-T389 (mTOR site)

total

ATG13 — p-S318 (ULK1 site)

total

LC3 — I / II

tubulin

IB

Figure 5

A)

WT   *TMEM41B^{KO}*

merge / LC3 / SQSTM1

B)

total cells: 90   52

LC3+/SQSTM1+ punctae per cell

WT   *TMEM41B^{KO}*

C)

WT   *TMEM41B^{KO}*   *RB1CC1^{KO}*   *ATG7^{KO}*

0 hr torin / 1 hr torin / 3 hr torin

merge / LC3 / WIPI2

D)

WT   *TMEM41B^{KO}*   *RB1CC1^{KO}*   *ATG7^{KO}*

total cells: 87 77 87   61 36 80   81 82 83   82 81 88

Number of punctae per cell

WIPI2 / LC3 / overlap

Torin treatment time (h)

E)

WT / *TMEM41B^{KO}* / *TMEM41B^{KO}/ RB1CC1^{KO}* (DKO)

merge / LC3 / WIPI2

F)

total cells: 42 35 41

Number of punctae per cell

WIPI2 / LC3 / overlap

WT   *TMEM41B^{KO}*   DKO

Figure 6

A)

B)

C)

Figure 7

A) tfSQSTM1 vs tfNBR1 in K562 cells

B) K562 (+Cas9)

C)

D) tfNBR1 in WT vs *ATG7^KO* cells

E)

Figure S1, related to Figure 1

A)

no insert / empty	
146 aa — LC3B	NP_074058.2
440 aa — SQSTM1/p62	NP_003891.1
966 aa — NBR1	NP_005890.2
789 aa — TAX1BP1	NP_006015.4
446 aa — NDP52/CALCOCO2	NP_005822.1

B)

HEK293T

tfEmpty · tfLC3 · tfSQSTM1

GFP

RFP

Fluorescence (a.u)

ratio

RFP/GFP ratio

■ basal
■ torin

C)

HEK293T

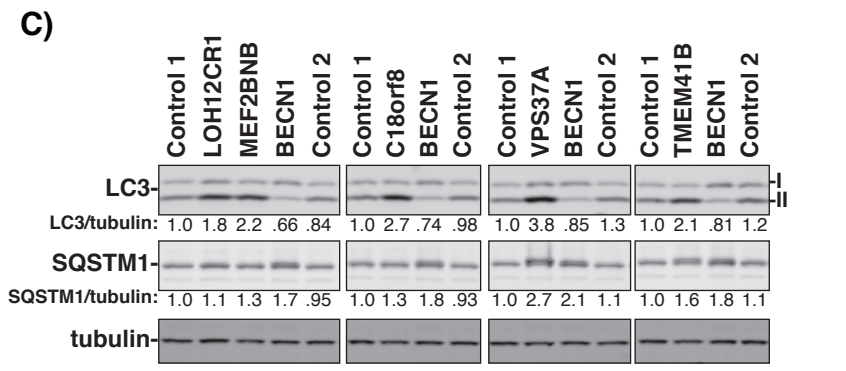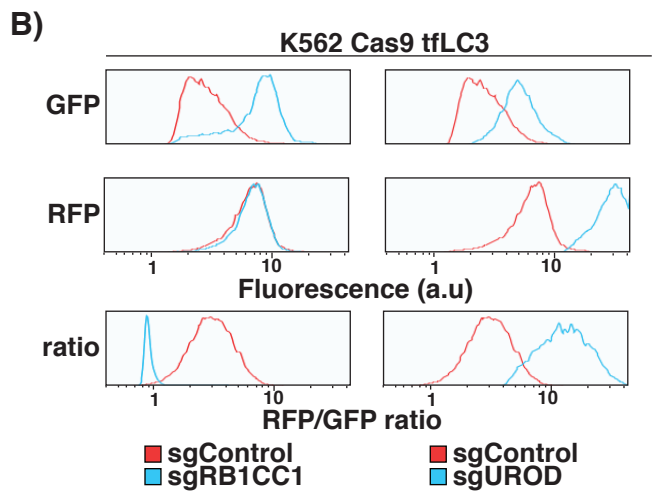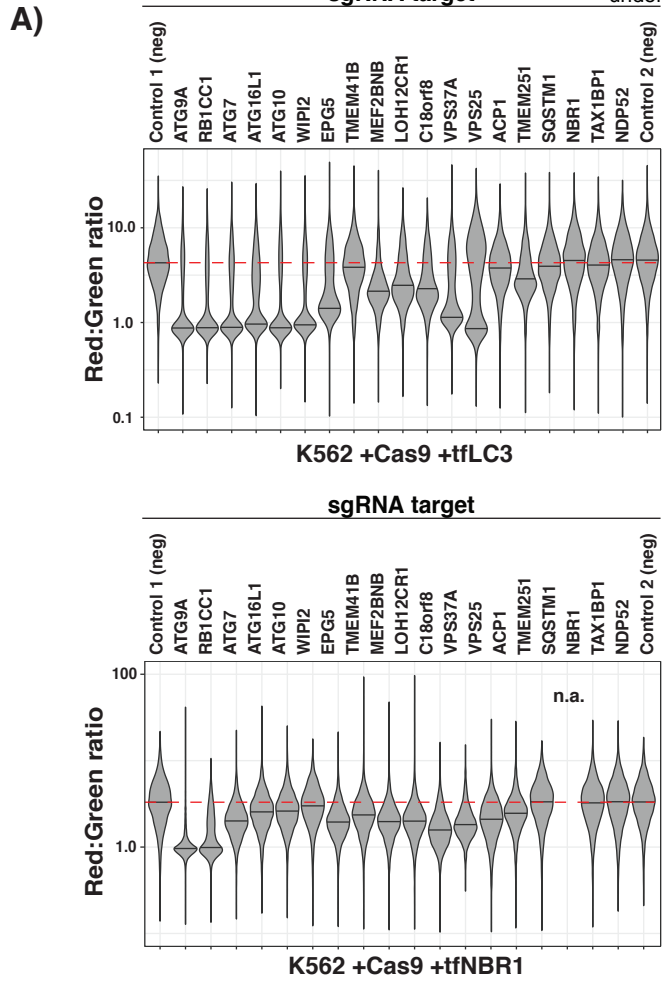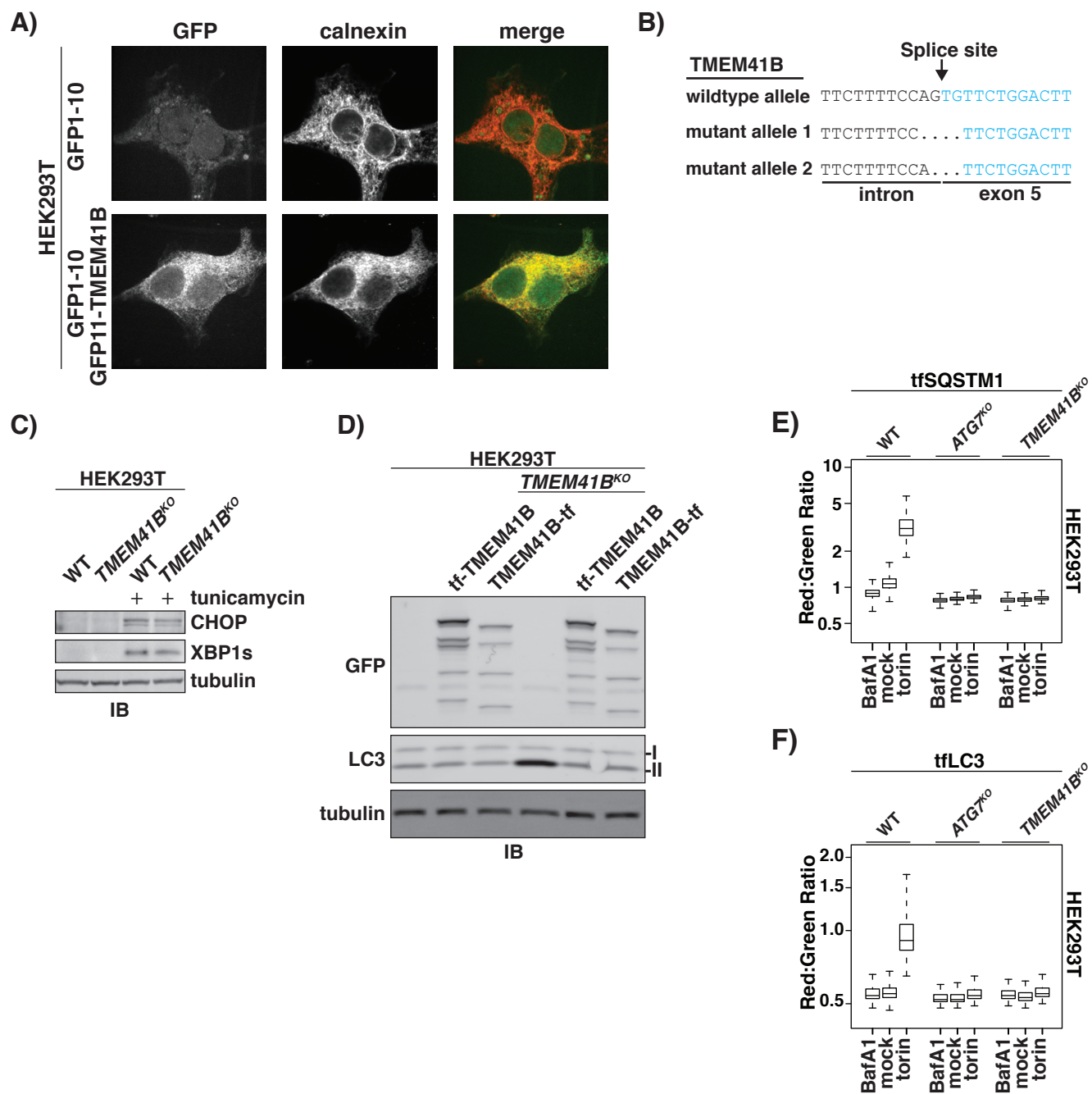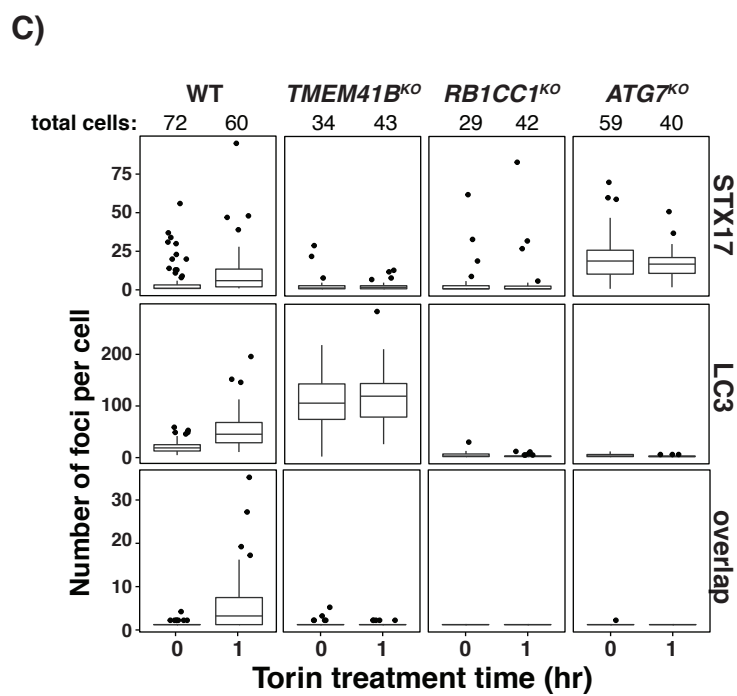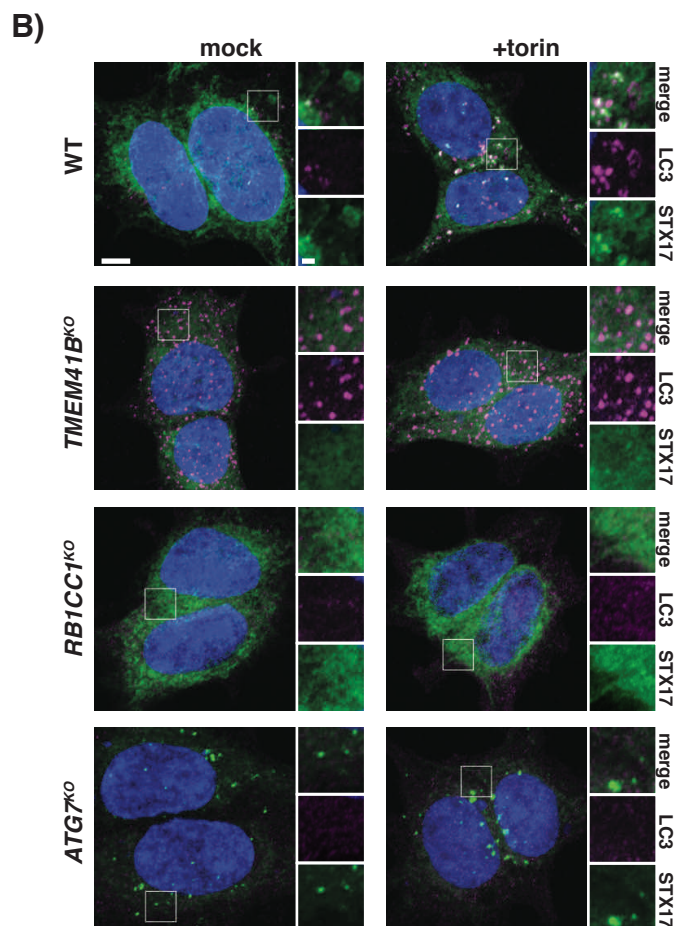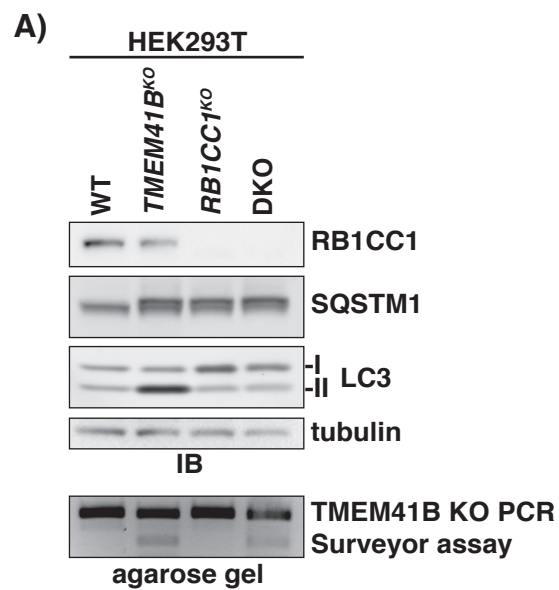WT · RB1CC1^{KO} · ATG13^{KO}

RB1CC1

ATG13

SQSTM1

IB

Figure S3, related to Figure 3
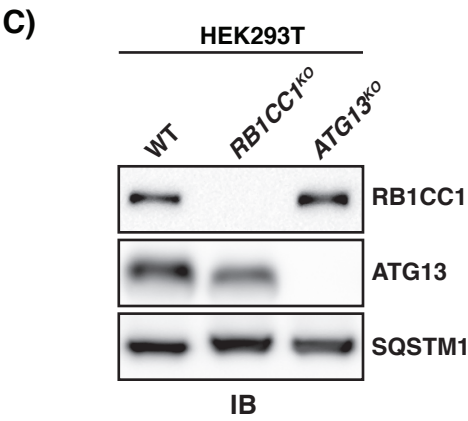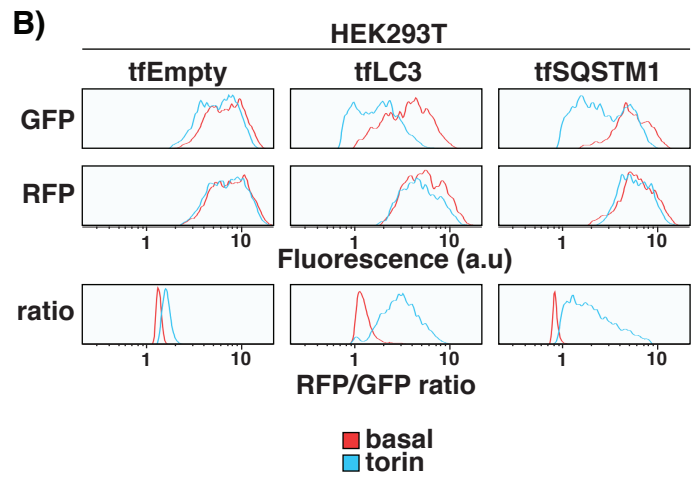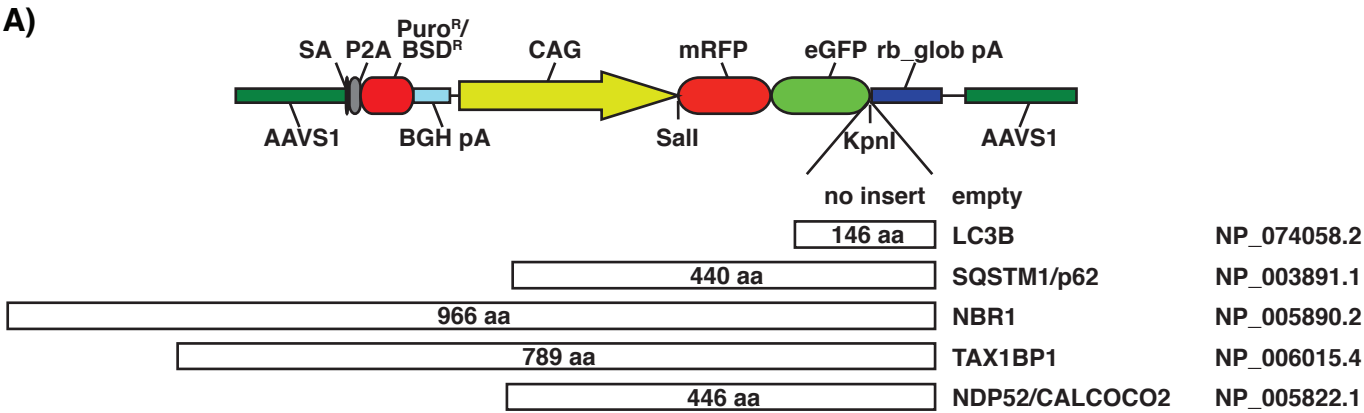
Figure S4, related to Figure 4

A)

	GFP	calnexin	merge

HEK293T

GFP1-10

GFP1-10 GFP11-TMEM41B

B)

TMEM41B

Splice site

wildtype allele   TTCTTTTCCAGTGTTCTGGACTT

mutant allele 1   TTCTTTTCC....TTCTGGACTT

mutant allele 2   TTCTTTTCCA...TTCTGGACTT

intron   exon 5

C)

HEK293T

WT *TMEM41B^{KO}* WT *TMEM41B^{KO}*

+   +   tunicamycin

CHOP

XBP1s

tubulin

IB

D)

HEK293T

TMEM41B^{KO}

tf-TMEM41B TMEM41B-tf tf-TMEM41B TMEM41B-tf

GFP

LC3 -I -II

tubulin

IB

E)

tfSQSTM1

WT   *ATG7^{KO}*   *TMEM41B^{KO}*

HEK293T

Red:Green Ratio

10
5

2

1

0.5

BafA1 mock torin   BafA1 mock torin   BafA1 mock torin

F)

tfLC3

WT   *ATG7^{KO}*   *TMEM41B^{KO}*

HEK293T

Red:Green Ratio

2.0
1.5

1.0

0.5

BafA1 mock torin   BafA1 mock torin   BafA1 mock torin

Figure S5, related to Figure 5

A)

B)

C)

Figure S7, related to Figure 7