1   Selective autophagic clearance of protein aggregates is mediated by the autophagy receptor,

2   TAX1BP1

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9   **Abstract**

10   Misfolded protein aggregates can disrupt cellular homeostasis and cause toxicity, a

11   hallmark of numerous neurodegenerative diseases. Protein quality control by the ubiquitin

12   proteasome system (UPS) and autophagy is vital for clearance of aggregates and maintenance of

13   cellular homeostasis[1]. Autophagy receptor proteins bridge the interaction between ubiquitinated

14   proteins and the autophagy machinery allowing selective elimination of cargo[2]. Aggrephagy is

15   critical to protein quality control, but how aggregates are recognized and targeted for degradation

16   is not well understood. Here we examine the requirements for 5 autophagy receptor proteins:

17   OPTN, NBR1, p62, NDP52, and TAX1BP1 in proteotoxic stress-induced aggregate clearance.

18   Endogenous TAX1BP1 is both recruited to and required for the clearance of stress-induced

19   aggregates while overexpression of TAX1BP1 increases aggregate clearance through autophagy.

20   Furthermore, TAX1BP1 depletion sensitizes cells to proteotoxic stress and Huntington's disease-

21   linked polyQ proteins, whereas TAX1BP1 overexpression clears cells of polyQ protein

22   aggregates by autophagy. We propose a broad role for TAX1BP1 in the clearance of cytotoxic

23   proteins, thus identifying a new mode of clearance of protein inclusions.

24   **Main**

25   Maintenance of cellular and organismal health is intricately connected to protein quality

26   control. A balance exists between protein translation, folding, and degradation that maintains the

27   stoichiometry and function of cellular protein complexes and organelles. If this balance is

28   perturbed, the accumulation of misfolded proteins can be toxic to the cell and is associated with

29   disruption of cellular function and numerous neurodegenerative diseases such as amyotrophic

30   lateral sclerosis (ALS), Huntington's disease (HD), and Alzheimer's disease (AD)[3]. A number of

31   protein quality control pathways exist within the cell to forestall dysfunction. Molecular

32   chaperone systems function to monitor and refold proteins if possible, while misfolded or

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33   damaged proteins are targeted for elimination via autophagy or the UPS[4,5]. The UPS is generally

34   responsible for routine turnover of short-lived proteins and targeted degradation of soluble or

35   solubilized misfolded proteins while macroautophagy, a catabolic process, culminates in the

36   lysosomal degradation of long-lived proteins, damaged organelles, and portions of the

37   cytoplasm[6–9]. Since the proteasome can only accommodate unfolded polypeptide chains, it is

38   generally thought that autophagy is responsible for the removal of insoluble protein

39   aggregates[1,10]. If either autophagy or the UPS is hindered, acute or chronic proteotoxic stress,

40   such as that caused by the expression of mutated proteins in neurodegenerative disease, can

41   result in the selective accumulation of these aggregation-prone proteins[11–13]. *In vivo*, inhibition of

42   autophagy results in intracellular protein aggregation contributing to neuronal cell death and

43   neurodegeneration in mice[14,15].

44   In recent years, numerous studies have highlighted the ability of autophagy to selectively

45   eliminate specific substrates, including mature ribosomes, endoplasmic reticulum, intracellular

46   pathogens, mitochondria, and protein aggregates[16–19]. In these cases, the autophagic machinery

47   employed in nonselective bulk degradation of cytosolic material is targeted to specific cargo.

48   Stimulation of autophagy is a promising therapeutic strategy in the treatment of protein

49   aggregation diseases and has been shown to enhance turnover of aggregated proteins, such as

50   TDP-43, in neuronal ALS models and huntingtin protein in Huntington disease models[20–22]. Key

51   to this approach is an understanding of how autophagy is selectively targeted to aggregates. In

52   addition to the specificity mediated by E3 ubiquitin (UB) ligases that target their cognate

53   substrates, there is selectivity in delivery to the proteasome via UB receptors[23] and in the

54   recruitment of autophagic machinery via autophagy receptors which associate with

55   polyubiquitylated proteins, thus linking substrates to the appropriate degradation machinery[16].

56   Numerous types of selective autophagy have been examined, and much progress has been

57   made in determining the basis of cargo selectivity. The autophagy receptor proteins, OPTN,

58   NDP52, TAX1BP1 and p62 are known to be important in xenophagy[24,25], while OPTN, NDP52,

59   and to a lesser extent, TAX1BP1 are essential for PINK1/Parkin-mediated mitophagy[17,26,27]. A

60   screen in yeast identified the ATG8 and UB-binding protein, CUET, and its mammalian

61   homologue, TOLLIP, to contribute to autophagic removal of expanded polyQ isoforms of

62   huntingtin[28]. NBR1 and p62 are linked to aggrephagy in mammalian cells[29]; however, p62 and

63   NBR1 knockout (KO) mice exhibit only a mild increase in ubiquitylated aggregates suggesting

64   that additional receptors are involved[30]. To identify such autophagy receptor proteins, we

65   examined production and clearance of puromycin-induced truncated and misfolded proteins in

66   the cytosol[31], termed "DRiPs" for **d**efective **ri**bosomal **p**roducts[32] in individual and

67   combinatorial knockouts of the autophagy receptor proteins, NBR1, p62, NDP52, OPTN, and

68   TAX1BP1 (Fig. 1A, 1B, 1C, Supplementary Fig. 1A). Consistent with the literature[33,34], we

69   found a drastic reduction in puromycin-induced foci formation in p62 KO and NBR1 KO cell

70   lines, as well as in NDP52 KO cells compared to WT cells after 2h puromycin (Fig. 1C, 1D, 1E).

71   Though aggregate formation was decreased in NBR1 and NDP52 KO cell lines, any foci that did

72   form were effectively cleared after 3h washout of puromycin (Fig. 1C, 1F, 1G). In contrast,

73   individual OPTN KO and TAX1BP1 KO cell lines showed robust UB-positive foci formation,

74   equal to or greater than WT cells after 2h puromycin (1C, 1D, 1E) and a significant block in

75   aggregate clearance after puromycin washout. More than twice as many cells with foci (~54%)

76   persisted in OPTN KO cells compared to WT (~22%), while in TAX1BP1 KO cells, nearly 4

77   times as many cells retained foci (~85%) (Fig. 1C, 1F, 1G). In TAX1BP1 KO cells with foci, the

78   number of foci per cell, although equivalent to WT at 2h puromycin (Supplementary Fig. 1B,

79   1C), was substantially higher than WT cells upon puromycin washout, indicating a block in

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80   clearance (Supplementary Fig. 1D). Distinct roles therefore exist for autophagy receptors in

81   protein aggregate formation and clearance and our data reveal a new autophagy receptor

82   involved in aggregate clearance.

83   We observed complementary results upon exposure of cells to low levels of proteasome

84   inhibition which induces accumulation of misfolded protein into juxtanuclear aggresome-like

85   structures removed via autophagy[1]. Imaging revealed decreased UB-labeled foci formation in

86   p62 KO, NBR1 KO and NDP52 KO cells (Supplementary Fig. 1E). Similarly, immunoblot of

87   cellular lysates after fractionation revealed no accumulation of insoluble UB-positive protein in

88   the p62 KO and decreased levels in NBR1 KO and NDP52 KO cells relative to WT cells (Fig.

89   1H, Supplementary Fig. 1F, 1G). However, in OPTN KO and TAX1BP1 KO cells, imaging

90   revealed greater UB-foci accumulation than in WT cells (Supplementary Fig. 1E). Consistent

91   with these observations, UB-conjugated protein was increased in the insoluble fraction of

92   TAX1BP1 KO (~4X increase) and OPTN KO (~2X increase) compared to WT cells (Fig. 1H,

93   Supplementary Fig. 1F), consistent with a block in aggrephagy.

94   We compared accumulation of UB-positive punctae upon short term exposure to MG132

95   or Bafilomycin A, an autophagy inhibitor, to determine whether there is basal accumulation of

96   misfolded or aggregated protein in TAX1BP1 KO cells. We saw little effect in WT cells (Fig. 1I,

97   1J); but in TAX1BP1 KO cells, both inhibitors resulted in formation of UB-positive foci (Fig. 1I,

98   1J), though MG132 to a much greater extent (Fig. 1I, 1J). This implies that the UPS is

99   compensating for an increased basal proteotoxic load in TAX1BP1 KO cells, most likely due to a

100   block in TAX1BP1-mediated selective aggrephagy. Interestingly, proteotoxic stress induced a

101   dramatic increase in TAX1BP1 protein levels (Fig. 2A, 2B). Additionally, a large proportion of

102   TAX1BP1 accumulates with insoluble ubiquitinated protein after MG132 treatment (Fig. 2C).

103   TAX1BP1, NDP52, and CALCOCO1 are paralogous proteins of the CALCOCO gene

104   family, all containing a SKICH domain, a putative canonical LC3-interacting region (LIR) motif

105   and coiled-coil regions while differing in conservation of the C-terminal zinc finger domains in

106   various species[25]. Therefore, we suggest that the specificity of TAX1BP1 and NDP52 may have

107   diverged over time, leading to a specific function for TAX1BP1 in aggrephagy. TAX1BP1 is the

108   most evolutionarily conserved member of the CALCOCO gene family and present widely in

109   vertebrates, unlike NDP52 which is sporadically lost or truncated[25]. Examination of NDP52 and

110   TAX1BP1 expression levels in a panel of human tissue lysates revealed that TAX1BP1 is highly

111   expressed in brain, while NDP52 was undetectable and likely does not function in neuronal

112   tissues (Fig. 2D). We then examined primary rat cortical neurons exposed to proteotoxic stress.

113   As in HeLa cells, UB-conjugated protein accumulated in the insoluble fraction upon MG132

114   exposure (Fig. 2E). As in human tissue, TAX1BP1 was expressed robustly in the rat cortical

115   neuron lysate and furthermore, accumulated to a greater extent in the insoluble fraction with

116   increased exposure to proteotoxic stress compared to other autophagy receptor proteins except

117   p62 (Fig. 2F). Consistent with our observations in HeLa cells, TAX1BP1 staining was diffuse in

118   untreated primary rat cortical neurons and in neurons derived from human induced pluripotent

119   stem cells (iPSCs)[35], but colocalized robustly with UB upon MG132 treatment (Supplemental

120   Fig. 2A, 2B). To our knowledge, this is the first study to identify a role for TAX1BP1 in

121   aggrephagy and suggests that TAX1BP1 plays a specific role in neuronal aggrephagy.

122   To further validate a specific role for TAX1BP1 in aggrephagy, we reintroduced GFP-

123   TAX1BP1 at near endogenous levels in the TAX1BP1 KO HeLa cell line (Supplementary Fig.

124   3A). In untreated cells, GFP-TAX1BP1 was diffuse or present in small punctae (Fig. 3A) and

125   colocalized with UB-positive foci upon 2h puromycin treatment (Fig. 3A). After puromycin

126 washout, UB foci were cleared to a similar extent in the TAX1BP1-rescued TAX1BP1 KO cells

127 as in WT cells and GFP-TAX1BP1 returned to a diffuse or punctate appearance (Fig. 3A, 3B).

128   To further confirm the effect of TAX1BP1 on aggregate clearance, we titered expression

129 of N- or C-terminally tagged TAX1BP1 constructs in the TAX1BP1 KO background to create

130 stable high- or low-expressing rescue lines (Supplementary Fig. 3A, 3B). Aggregate formation

131 and clearance was similar to WT in the low-expressing TAX1BP1 rescue lines (Fig. 3C, 3D,

132 Supplementary Fig. 3B). However, upon 2h puromycin treatment, UB-positive foci were present

133 in only ~20% of the high TAX1BP1-expressing rescue cell lines compared to ~65% of WT and

134 ~90% of TAX1BP1 KO cells. (Fig. 3C). Furthermore, upon puromycin washout, foci were

135 cleared to a greater extent in TAX1BP1 high-expressing rescue lines in which only ~5-10% of

136 cells retained aggregates compared to 30% in WT cells, and >70% in the parental TAX1BP1 KO

137 (Fig. 3D), suggesting an active role for TAX1BP1 in promoting aggregate clearance.

138   Because we did not observe robust foci formation in the high-expression TAX1BP1-

139 rescue lines (Fig. 3C, Supplementary Fig. 3B), we treated cells with Bafilomycin A during

140 puromycin treatment and washout to block autophagy. In WT cells, Bafilomycin A resulted in

141 increased aggregate retention after washout (Fig. 3E, 3F, larger field of view shown in

142 Supplementary Fig 3C). However, in TAX1BP1 KO cells, Bafilomycin A barely exacerbated the

143 defect in aggregate clearance, suggesting that the autophagy pathway is nonfunctional in the

144 presence of proteotoxic stress (Fig. 3E, 3F). Bafilomycin A was sufficient to restore UB-positive

145 foci in TAX1BP1 rescue cell lines (Fig. 3E, 3F, Supplementary Fig. 3C), resulting in UB-

146 positive foci which colocalized with FLAG-TAX1BP1 and tended to loosely accumulate in the

147 perinuclear region, similar to that observed upon addition of Bafilomycin A to WT cells (Fig. 3E,

148 Supplementary Fig. 3D). Our data demonstrate that TAX1BP1-medated aggregate clearance is

149   dependent upon expression level and capable of accelerating degradation of insoluble protein

150   aggregates by promoting selective flux through the autophagy pathway.

151   TAX1BP1 contains several protein-interacting domains, including an N-terminal SKICH

152   domain, at least 3 less studied coiled-coil regions, a canonical LIR motif and putative

153   noncanonical CLIR motif, as well as two C-terminal zinc finger (ZF) UB-binding

154   domains[24,25,27,28]. We created a variety of truncation and point mutants to test the requirements

155   for these domains in TAX1BP1-mediated aggrephagy (Fig. 4A). Each mutant was stably

156   expressed in the TAX1BP1 KO background and assessed for puromycin-induced aggregate

157   formation and clearance. Aggregate formation occurred with minor variation similarly in WT

158   and all mutant cell lines as well as a GFP-only control (Fig. 4Bi,ii, 4C, larger fields in

159   Supplementary Fig. 4A). However, substantial differences were observed amongst the mutants in

160   aggregate clearance. N-terminal SKICH deletion (ΔSKICH) as well as both the LIR (W49A) and

161   CLIR (V143S) point mutants localized to UB-positive foci and rescued clearance to the same

162   extent as WT TAX1BP1 (Fig. 4Biii, iv, v, 4D, Supplementary Fig. 4A). The structure of the

163   TAX1BP1 SKICH domain shows that the canonical LIR (W49) is mostly buried within the

164   hydrophobic interior of the folded SKICH domain and therefore is likely not a functional LIR

165   domain[36]. A truncation mutant lacking both C-terminal ZF domains did not localize to

166   puromycin-induced foci and was unable to rescue aggregate clearance (Fig. 4Bvi, 4D). To

167   determine whether one or both ZF domains was essential, we created point mutants targeting

168   both ZF domains (ZF1/ZF2: Q743A/E747K/ Q770A/E774K) as well as each domain

169   individually (ZF1:Q743A/E747K, ZF2:Q770A/E774K). The double mutant, ZF1/ZF2, as well as

170   the single ZF2 mutant, failed to localize to UB foci after puromycin treatment and both were

171   unable to rescue aggregate clearance (Fig. 4Bviii, ix, 4D). In contrast, point mutation of ZF1 had

172   little effect on TAX1BP1 localization and rescued aggregate clearance similarly to WT

173   TAX1BP1 (Fig. 4Bvii, 4D). This is consistent with a prior report that only the ZF2 of TAX1BP1

174   is capable of binding UB[25]. An N- and C-terminally truncated mutant (ΔSKICH/ΔZF) did not

175   localize to aggregates but instead formed aberrant clusters in the cytosol and was inferior to the

176   ZF mutants in its ability to rescue clearance (Fig. 4Bx, 4D, Supplementary Fig. 4A).

177   Very similar results were observed in triple knockout (TKO: TAX1BP1 KO, OPTN KO,

178   NDP52 KO) cells (Supplementary Fig. 4B, 4C), ruling out potential redundancy that may mask

179   requirements for TAX1BP1 domains through functionally related autophagy receptors. Low,

180   near-endogenous expression of TAX1BP1 rescued aggregate clearance to that of WT cells while

181   higher expression increased clearance compared to WT cells (Fig. 4E, 4F, Supplementary Fig.

182   4C). In contrast, neither the ΔSKICH, ΔZF nor ΔSKICH/ΔZF mutants were able to fully rescue

183   aggregate clearance demonstrating the need for these protein-interaction and UB-binding

184   domains (Fig. 4F, Supplementary Fig. 4C). Thus, full length TAX1BP1 was able to rescue

185   aggregate clearance in the absence of NDP52 and OPTN, indicating that TAX1BP1 promotes

186   aggrephagy (Fig. 4F).

187   We also examined whether TAX1BP1 overexpression in WT cells beyond the level of

188   endogenous TAX1BP1 expression could promote aggregate clearance, thus addressing the

189   therapeutic potential of TAX1BP1. TAX1BP1 protein expressed in WT cells behaved similarly

190   to the endogenous protein, remaining diffuse in untreated cells and colocalizing with UB-stained

191   foci upon puromycin treatment (Fig. 4G, Supplementary Fig. 4D). Aggregate formation upon 2h

192   puromycin treatment was decreased in both low (~20% decrease) and high (~30% decrease) -

193   overexpressing TAX1BP1 cells (Fig. 4H). Clearance of aggregates in WT cells with low

194   overexpression of TAX1BP1 was similar to that in WT cells expressing only endogenous

195   TAX1BP1 (Fig. 4I, Supplementary Fig. 4D), whereas, higher levels of TAX1BP1 expression

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196   increased clearance upon puromycin washout – only ~10% of cells retained UB-positive foci in

197   contrast to more than 30% of WT cells (Fig. 4G, 4I).

198   Overexpression of WT TDP-43 and polyQ huntingtin fragments is cytotoxic in *in*

199   *vitro* and *in vivo* model system[20,37–44]. Stimulation of autophagy increases clearance of WT

200   and mutant TDP-43, huntingtin fragments and attenuates cytotoxicity[20,43,45–48]. Therefore, WT,

201   TAX1BP1 KO, and high-expressing TAX1BP1-rescue cells were exposed to a variety of

202   stresses, including low dose proteasome inhibition and expression of aggregation-prone proteins,

203   such as the ALS-associated, EGFP-TDP-43, and varied length model substrates carrying

204   expanded glutamine tracts (polyQ) expressed from exon 1 of the huntingtin-encoding gene

205   (Supplementary Fig. 5A, 5B). Cells were either infected or transfected on day 1 and assessed

206   daily for six days. Loss of TAX1BP1 decreased viability compared to WT cells upon exposure to

207   each form of proteotoxic stress (Fig. 5A, Supplementary Fig. 5C). Expression of TAX1BP1 in

208   the knockout line was able to partially restore cell viability (Fig. 5A, Supplementary Fig. 5C).

209   To determine whether TAX1BP1 can aid in clearance of Htt-polyQ proteins, we infected

210   WT, TAX1BP1 KO, and TAX1BP1-rescue cells with virus to express HttQ23-EGFP, HttQ74-

211   EGFP and HttQ103-EGFP and assessed aggregate levels 4 days post-infection (Supplementary

212   Fig. 5A). Due to the low number of glutamine repeats, HttQ23-EGFP does not form inclusions

213   and therefore is a useful control when comparing to the aggregate-forming HttQ74-EGFP and

214   HttQ103-EGFP proteins. HttQ23-EGFP expression was robust and as expected the protein

215   remained largely diffuse and cytosolic in both WT and TAX1BP1 KO cells (Fig. 5B). In

216   contrast, TAX1BP1-deficient cells exhibited increased focal aggregates of both Htt74Q-EGFP

217   and Htt103Q-EGFP compared to WT cells (Fig. 5B, 5C, 5D). Rescue of the TAX1BP1 KO line

218   with expression of FLAG-TAX1BP1 restored clearance of HttQ74-EGFP and Htt103Q-EGFP to

219   that of WT cells, indicating that TAX1BP1 is highly effective in directing clearance of

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220 huntingtin polyQ proteins (Fig. 5B, 5C, 5D). Endogenous TAX1BP1 also colocalized with

221 HttQ103-EGFP aggregates in WT cells (Fig. 5E) appearing to enclose aggregates in many

222 instances (Fig. 5F, Supplementary Figure 5D).

223 Because TAX1BP1 overexpression can promote clearance of aggregates beyond that in

224 WT cells and because we found TAX1BP1 highly and specifically-expressed in brain (Figure

225 2D, 2F), we examined whether TAX1BP1 could provide a protective effect in iPSC-derived

226 neurons exposed to huntingtin proteins. iPSC-derived neurons with or without stable expression

227 of TAX1BP1 were infected with virus expressing either the non-aggregating control construct,

228 HttQ23-EGFP, (Figure 5G) or the aggregate-forming HttQ103-EGFP (Figure 5H) and imaged

229 daily for 15 days to assess viability via nuclei count (NLS-BFP). The rates of cell death, assessed

230 by comparing slopes obtained from lines fitted to cell number over time, did not differ between

231 WT and TAX1BP1-overexpressing neurons infected with the non-aggregate-forming HttQ23-

232 EGFP (Figure 5G). However, TAX1BP1 overexpression significantly improved survival in

233 neurons exposed to the aggregate-forming HttQ103-GFP protein (Figure 5H), consistent with our

234 observations in HeLa cells.

235 Here we report a broad role for TAX1BP1 in protein homeostasis. Our results

236 demonstrate that loss of TAX1BP1 leads to decreased ability to target insoluble protein for

237 degradation. Overexpression of TAX1BP1 further promotes aggregate clearance and rescues cell

238 viability upon exposure to varied proteotoxic insults, including translation stress, proteasome

239 inhibition, and exposure to aggregate-prone proteins such as TDP-43 and huntingtin-expanded

240 polyQ model substrates (Supplementary Figure 5E).

241 Aggrephagy has potential to mitigate neurodegenerative proteinopathies. Focus on the

242 proteins that provide specificity in targeting aggregates, such as TAX1BP1, may be valuable, as

243 these are likely deciding factors in directing the autophagy response. Using single or

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244   combinatorial knockouts of OPTN, NDP52, TAX1BP1, NBR1, and p62, we observed distinct

245   roles for these varied autophagy receptor proteins in aggrephagy. Though they may function *en*

246   *masse* to maintain protein homeostasis, the individual proteins exhibit functional and spatial

247   distinctions at the subcellular and tissue level. For example, autophagy receptor proteins are

248   recruited independently to distinct microdomains surrounding bacteria and mitochondria where

249   they perform nonredundant roles during xenophagy and mitophagy, respectively[26,49].

250   Furthermore, TAX1BP1 is robustly expressed in human brain lysate as well as primary rat

251   cortical neurons, distinguishing it from NDP52. TAX1BP1 is also associated with the insoluble

252   protein fraction in primary rat cortical neurons exposed to proteotoxic stress. Amongst the other

253   autophagy receptor proteins, only p62 showed similar association with the insoluble fraction.

254   TAX1BP1 and its paralog, NDP52, share similar domain structures; however, recent studies

255   suggest structural differences in the organization of the SKICH domains as well as distinct

256   ATG8 binding affinities[25,50]. TAX1BP1 is also more broadly conserved than NDP52 among

257   mammals[25], suggesting that TAX1BP1 fills nonredundant essential roles. Future studies

258   examining TAX1BP1 expression levels during aging and in animal models of neurodegenerative

259   disease are warranted.

260   TAX1BP1 recruitment to protein aggregates requires the C-terminal ubiquitin-binding

261   domain and its function in promoting aggrephagy further necessitates the N-terminal SKICH

262   domain. However, none of our TAX1BP1 mutants were completely dead in terms of rescue

263   effect, suggesting that other cellular functions of TAX1BP1 may be involved. One such role is to

264   act as a Myosin VI cargo adaptor protein for mediating autophagosome maturation, which could

265   contribute to clearance dependent upon other upstream factors, thus explaining partial rescue[25].

266   Additionally, TAX1BP1 is best studied for its role in negatively regulating nuclear factor-kb

267   (NF-κB) and interferon regulatory factor (IRF) 3 via an interaction with the deubiquitinase, A20,

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268   thus restricting pro-inflammatory signaling and immune response[51,52]. Though links between

269   TAX1BP1's role as an autophagy adaptor and the immune response are yet to be understood, it

270   is well known that protein aggregation, inflammation, and autophagy are intertwined[53]. Studies

271   have shown that exposure to disease-associated protein aggregates can elicit innate immune

272   response in glial cells and that LPS-induced inflammation results in enhanced aggregate

273   formation in disease models suggesting a synergistic relationship between proinflammatory

274   response, proteostasis and neurodegeneration[54,55]. Autophagy may function to downregulate

275   inflammatory signaling and TAX1BP1 may be an important link between detection and

276   monitoring of cellular protein aggregates and the inflammatory response.

277   Notably, ectopic expression of TAX1BP1 in knockout cells was able to rescue aggregate

278   clearance to WT levels and increased overexpression of TAX1BP1 in both the knockout and WT

279   cells was able to reduce aggregate levels below those observed in WT cells. Furthermore,

280   TAX1BP1 overexpression in IPSC-derived neurons was protective against huntingtin aggregate-

281   induced toxicity. TAX1BP1 thus plays a general role in promoting aggrephagy and future studies

282   aimed at increasing TAX1BP1 expression or stability *in vivo* present promising therapeutic

283   potential in addressing proteinopathies. An increased understanding of targeting specificity of

284   selective autophagy receptor proteins for protein aggregates may make autophagy-stimulating

285   approaches more specific and effective in treatment of protein misfolding diseases.

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296   **Author Contributions**

297   S.A.S. and R.J.Y. conceived the project; S.A.S. and R.J.Y. designed experiments; S.A.S and

298   H.V.S. performed experiments; G.K. performed image analysis for neuron viability; M.E.W.

299   provided iPSCs and relevant expertise, S.A.S and R.J.Y. wrote the manuscript and all authors

300   contributed to editing the manuscript.

301

302   **Competing Interests**

303   The authors declare no competing financial interests.

304 **Methods**

305 **Cell Culture and reagents**

306   HeLa and HEK293T were cultured in DMEM (Life Technologies) supplemented with

307 10% (v/v) FBS (Gemini Bio Products), 10 mM HEPES (Life Technologies), 1 mM sodium

308 pyruvate (Life Technologies), 1mM non-essential amino acids (Life Technologies) and 2 mM

309 glutamine (Life Technologies). HeLa cells were acquired from the ATCC and authenticated at

310 the Johns Hopkins GRCF Fragment Analysis Facility using STR profiling. Testing for

311 mycoplasma contamination was performed bimonthly using the PlasmoTest kit (InvivoGen).

312 Plasmids were transfected using either X-tremeGENE 9 (Roche), polyethylenimine

313 (Polysciences) or Avalanche-OMNI (EZ Bio-systems). Primary cultured cortical neurons were

314 isolated from male and female embryonic day 18 Sprague-Dawley rats and maintained in

315 Neurobasal medium (Gibco) supplemented with 2mM glutamine (Life Technologies) and B-27

316 Supplement (Gibco). All rat procedures were performed according to a protocol (#1171)

317 approved by the National Institutes of Health NINDS Institutional Animal Care and Use

318 Committee. Primary neurons were treated with MG132 on day-in-vitro (div) 10.

319   For puromycin (Invivogen), MG132 (Sigma-Aldrich) or Bafilomycin A (Sigma-Aldrich)

320 treatments, cells were treated with the indicated concentrations of drug in full media and either

321 harvested as described or washed three times in full media and returned to full media for the

322 indicated durations.

323

324 **Antibodies**

325   Rabbit mono- and polyclonal antibodies used for immunoblotting include: NDP52 (CST,

326 60732S), TAX1BP1 (CST, 5105S), Actin (CST, 4967S); GAPDH (Sigma, G9545-200UL),

327 OPTN (Proteintech, 10837-I-AP) and TAX1BP1 (Sigma, HPA024432); rabbit antibodies used

328   for immunofluorescence include TAX1BP1 (Sigma, HPA024432). Mouse Monoclonal

329   antibodies used for immunoblotting were: GFP (Roche, 11814460001), ubiquitin (Millipore,

330   MAB1510), NBR1 (Abnova, H00004077-M01) and p62 (Abnova, H00008878-M01); mouse

331   antibodies used for immunofluorescence include ubiquitin FK2 (Biomol International, PW8810-

332   0500). Secondary AlexaFluor® (ThermoFisher) conjugated antibodies were incubated for 1h RT

333   at 1:1000. DAPI counterstain (ThermoFisher) at 300nM was incubated for 10 min RT.

334

335   **TALEN and CRISPR gene knockout cell lines**

336   Construction of OPTN, NDP52, TAX1BP1, TKO and pentaKO knockout HeLa lines

337   were reported previously [17]. All knockout lines were generated using CRISPR guide RNAs

338   (gRNAs) chosen to target one or more exons common to all splicing variants of the gene of

339   interest (all information listed in Supplementary Table S1). Oligonucleotides (Operon)

340   containing CRISPR target sequences were annealed and ligated into the linearized

341   gRNA_Cloning vector which was a gift from George Church (Addgene #41824) or SpCas9-2A-

342   Puro, which was a gift from Feng Zhang (Addgene #48139). HeLa cells were cotransfected using

343   XtremeGENE9 (Roche) with either the the gRNA_Cloning vector, pCDNA YFP-C1, and the

344   hCas9 plasmid, which was a gift from George Church (Addgene #41815) or with SpCas9-2A-

345   Puro. Forty-eight hours post-transfection, YFP-positive cells were isolated either via

346   fluorescence activated cell sorting or puromycin selection and serially diluted for single colony

347   clones. Single colonies were expanded and screened for depletion of the targeted gene product

348   by immunoblotting. DNA was extracted using the Zymo gDNA Isolation Kit and genotyped

349   using primers targeting fragments of the genomic DNA from knockout clones containing the

350   putative cleavage site (primers and sequences in Supplementary Table 1).

351

Cloning and generation of stably infected cell lines

The Gateway Cloning (Life Technologies) system was used to generate pHAGE-GFP-TAX1BP1, pHAGE-N-FLAG-HA-TAX1BP1, and pHAGE-GFP-TDP43. Briefly, genes of interest were cloned into pDONR223 then recombined into destination vectors pHAGE-N-FLAG-HA or pHAGE-N-GFP using L Recombinase (Life Technologies) as per the manufacturer's protocol. Mutations in cDNA sequences were introduced using PCR site-directed mutagenesis in the pDONR223 vector. All constructs generated in this study were verified by sequencing. Original plasmids containing EGFP-HttQ43 and EGFP-HttQ74 were kindly provided by Dr. Harm Kampinga (Groningen, Netherlands). pYES2/103Q was a gift from Michael Sherman (Addgene plasmid #1385) and pEGFP-Q23 was a gift from David Rubinzstein (Addgene plasmid #40261). All huntingtin protein constructs were cloned into pLEX-EF1α-EGFP for lentiviral expression. pDONR-TDP-43 WT YFP was a gift from Aaron Gitler (Addgene plasmid #27470). Primer sequences are available upon request.

For generation of stable cell lines, lentiviruses (pHAGE- vectors) were packaged in HEK293T cells. HeLa cells were transduced with virus for 24 hours with 8 µg/ml polybrene then selected for protein expression by drug-resistance (puromycin or blasticidin) or fluorescence activated cell sorting. Generation of low- and high-expressing cells was done via infection with various dilutions of virus and expression assessed via comparison to endogenous protein levels on immunoblot.

Aggregate formation and clearance studies

For acute treatments, HeLa cells were grown on poly-D-lysine-coated (Sigma-Aldrich, P7280) coverslips and treated with either 5 µg/ml of puromycin (Invivogen) for 2 h or 1 µM MG132 (Sigma-Aldrich) for 8 h at 37°C. Cells were either fixed for imaging or assessed for

376   clearance. For clearance, cells were washed three times in DMEM–10% FBS and released into

377   drug-free medium for 3 h at 37°C. For long treatments, HeLa cells were grown on poly-D-lysine-

378   coated coverslips and treated with 1 μg/ml of puromycin or 1 μM MG132 for 18 h at 37°C.

379   Coverslips were fixed and processed for microscopy, as outlined above.

380   For huntingtin poly-Q-protein clearance assays, HeLa cells were seeded on poly-D-

381   lysine-coated coverslips and transduced the following day with lentivirus expressing either

382   HttQ23-EGFP, HttQ74-EGFP or HttQ103-EGFP for 24 hours with 8 μg/ml polybrene. Cells

383   were fixed for imaging and assessed 4 days post-infection.

384

385   **Subcellular fractionation**

386   Cells were washed once and scraped in cold PBS, pelleted, and lysed in RIPA buffer

387   (Thermofisher, 89900) supplemented with Complete Protease Inhibitor Cocktail (Roche), 1mM

388   EGTA, 1mM EDTA, 100mM chloroacetamide (Sigma-Aldrich) and 100mM DTT for 15

389   minutes at 4°C with end over end rotation. Five to ten percent of the total volume was collected,

390   mixed with 4X LDS (Life Technologies), boiled, and reserved for the input fraction. RIPA-

391   soluble fraction was obtained after centrifugation at 20,000 rpm for 15 min at 4°C, 4X LDS was

392   added, and samples were heated to 99°C with shaking for 10 minutes. The insoluble fraction

393   consisting of the remaining pellet was washed once with lysis buffer, spun at 20,000 rpm for 10

394   min at 4°C, then lysed in 1X LDS, 100mM DTT in PBS and heated to 99°C with shaking for 15

395   minutes. Protein concentration was determined via DC Protein Assay (BioRad). Soluble and

396   insoluble fractions were normalized to the protein concentration of the input fraction. For

397   analysis, 10-15% of RIPA-soluble fraction and 2.5-5% of insoluble fraction were loaded on 4-

398   12% Bis-Tris gels and run using MOPS buffer (Life Technologies) and visualized by western

399   blotting on PVDF. Western blotting was performed by wet transfer method in either NuPage

400 transfer buffer or Tris-glycine transfer buffer (Life Technologies). Proteins were detected using

401 horseradish peroxidase-coupled secondary antibodies (GE Healthcare Life Sciences, Piscataway,

402 NJ) and ECL Plus or ECL Prime western blotting detection reagents (GE Healthcare Life

403 Sciences). Images were acquired using an MP gel documentation system (Bio-Rad Laboratories).

404 Quantification of immunoprecipitation bands was performed using the volume tools in Image

405 Lab software (Bio-Rad Laboratories).

406

Immunoblotting

408 After the indicated treatments, cells were harvested by scraping in cold PBS and either

409 fractionated as described above or lysed in 2% SDS/1X PBS, heated to 99°C with shaking for 10

410 minutes, and spun at 20,000 rpm for 15 min at RT. Protein concentration was determined using

411 DC Protein Assay (BioRad) and 20-50 ug of protein per sample was separated on 4–12% Bis-

412 Tris gels using MOPS or MES running buffer (Life Technologies). Western blotting was

413 performed by wet transfer method in either NuPage transfer buffer or Tris-glycine transfer buffer

414 (Life Technologies). Proteins were detected using horseradish peroxidase-coupled secondary

415 antibodies (GE Healthcare Life Sciences, Piscataway, NJ) and ECL Plus or ECL Prime western

416 blotting detection reagents (GE Healthcare Life Sciences). Images were acquired using an MP

417 gel documentation system (Bio-Rad Laboratories). Quantification of immunoprecipitation bands

418 was performed using the volume tools in Image Lab software (Bio-Rad Laboratories). Human

419 tissue panel blot was purchased (NOVUS Biologicals).

420

Immunofluorescence Microscopy

422 HeLa cells were seeded on poly-D-lysine-coated (Sigma-Aldrich, P7280) coverslips and

423 treated as indicated. Following treatment, cells were fixed at room temperature in 4%

424   paraformaldehyde for 15 minutes, permeabilized and blocked with filtered IF buffer (0.1%

425   Triton X-100, 3% goat serum, 1X PBS) for 1 hour at RT. For immunostaining, cells were

426   incubated with indicated antibodies diluted in IF buffer overnight at 4˚C, washed 3 times with IF

427   buffer and incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies)

428   1:1000 in IF buffer for 1 hour at RT. Cells were then incubated with DAPI (Sigma) diluted in IF

429   buffer at 1:10,000 for 10 minutes at RT. Cells were washed 2 times with IF buffer and 1 time

430   with 1X PBS. Coverslips were mounted on slides using Prolong Gold Antifade (Life

431   Technologies). Representative images were collected with an inverted laser scanning LSM 880

432   microscope (Carl Zeiss) using a 63X/1.4 objective Plan-Apochromat (Carl Zeiss). Images were

433   collected as z-stacks captured at optimal thickness. Representative images shown are maximum

434   intensity projections unless otherwise noted.

435

436   **Cell viability and Growth curve**

437   For HeLa cell viability assays, cells were seeded in quadruplicate in white clear-bottom

438   96-well plates (Corning). The next day, cells were treated as indicated in figure legends. Cell

439   viability was measured by using the Cell Titer-Glo system (Promega) according to the

440   manufacturer's instructions. Values were normalized to the value for untreated samples for each

441   cell line. Means and standard deviations were calculated, and statistical significance was

442   calculated using one-way analysis of variance (ANOVA). One plate was measured each day

443   using a Synergy H1Hybrid Multi-Mode Reader (Biottek).

444

445   **Statistical analysis for aggregate formation and clearance assays**

446   For quantification, cells with puromycin-, MG132-, or HTT-induced foci were either

447   counted manually in a blinded fashion or using the particle analysis plug-in in ImageJ. Macros

448   created are available on request. At least 200 cells per sample were analyzed in three biological

449   replicate studies. The number of observations used in each experimental series is included in

450   Table 1. Means and standard deviations were calculated, and statistical significance was

451   assessed. For comparisons between three or more groups, a one or two way-ANOVA with

452   Tukey;s post-hoc analysis was performed using Prism V7 (GraphPad Software) as noted in the

453   figure legend. Error bars represent standard deviation (SD): *$P< 05$, **$P<.01$, ***$P<.001$,

454   ****$P<.0001$. The number of observations used in each experimental series was included in the

455   figure legend or can be found in Table 1. All test statistics (e.g. F), degrees of freedom and P

456   values are included in Table 1. Three biological replicates were performed for each experiment.

457   To determine number of foci per individual cell, CellProfiler[56], version 2.2.0 was used. All

458   CellProfiler pipelines and detailed settings to reproduce the image analysis procedures are

459   available upon request.

460

461   **Neuron Culture and Viability Assay**

462   Induced pluripotent stem cells (iPSCs), parental line WTC11, were maintained in

463   Essential 8 Flex medium (Gibco, A28583-01) supplemented with Essential 8 Flex Supplement

464   (Gibco, A28584-01). iPSCs were grown on Matrigel-coated plates (Corning, 354277) and cell

465   dissociation was performed using Accutase (StemCell Technologies, 07920). Stable expression

466   of NLS-BFP (used for nuclei counting) was obtained by infecting cells with lentivirus expressing

467   U6-NLS-BFP followed by sorting for BFP-positive cells. Stable expression of TAX1BP1 was

468   obtained by infecting NLS-BFP-expressing cells with lentivirus expressing EF1α-FLAG-

469   TAX1BP1 followed by puromycin selection.

470   For the viability assay, iPSCs grown in 6-well plates were infected with lentivirus

471   expressing EF1α-HttQ23-EGFP or EF1α-HttQ103-EGFP, the following day, media was

472   changed, and cells were allowed to recover for 1 day. Two days post-infection, cells were plated

473   in induction medium: DMEM/F12 with HEPES (Gibco, 11330032) containing N2 Supplement

474   (100X) (Gibco, 17502048), non-essential amino acids (100x) (Gibco, 11140050), glutamax

475   (100X) (Gibco, 35050061), ROCK inhibitor Y-27632 (10mM) (Tocris, 1254), and

476   doxycycline (2mg/ml) (Sigma, D9891). Induction medium was changed daily for 2 days. On

477   day 3 post-induction, cells were dissociated using Accutase, counted, and plated at 50,000

478   cells/well in poly-L-ornithine-coated (PLO) (Sigma, P3655) 96-well plates (Perkin Elmer,

479   6055302) in BrainPhys neuronal medium (StemCell Technologies, 05790) containing B27

480   supplement (50X) (Gibco, 17504044), BDNF (10μg/ml) (PeproTech, 450-02), NT-3 (10μg/ml)

481   (PeproTech, 450-03), and laminin (1μg/ml) (Gibco, 23017015). Half of the well volume was

482   removed and replaced with fresh supplemented BrainPhys media every 3 days for the duration of

483   the assay. iPSCs-derived neurons were imaged daily for 20 days. Sixteen fields of view were

484   imaged per each well of three independent biological replicates. Cells were imaged using a 20X

485   air objective (NA 0.75) with 1.5X optical zoom on a Nikon Ti-2 CSU-W1 spinning disk system

486   with a photometrics 95B camera operated by Nikon Elements software equipped with

487   temperature regulation and CO_2 control.

488

489   **Statistical Analysis for Neuron Viability Assay**

490   To determine iPCS-derived neuron survival, NLS-BFP-expressing nuclei were counted

491   automatically using the R EBImage package[57]. Three biological replicates per condition were

492   pooled together and the data were fitted to an exponential decay model using the R DRC

493   package[58] and the formula: (f(x) = c + (d-c)(exp(-x/e))) where (e) is the slope and (d) and (c)

494   represent the upper and the lower limits around the slope. Outliers were detected and removed

495   using interquartile range using the R Outliers package[59]. The survival score was calculated from

22

496   the fitted model, in which the slope (e) represents the survival score of each population of cells

497   counted at every time point for the designated treatment. For statistical comparison, a

498   permutation test (a.k.a randomization test) was used. In brief, the delta mean score of the groups

499   was compared to random delta mean scores of shuffled groups iterated 10,000 times. *P* value

500   was determined by calculating the number of times the delta score was higher in the shuffled

501   group than in the ground true group[58]. The number of observations used in each experimental

502   series is included in Table 2. Code is provided in Supplementary Information.

503

504   **Data Availability**

505   The datasets generated for all microscopy cell counting experiments are available as

506   supplementary files and noted in the associated figure legends. The datasets generated and

507   analyzed to assess neuron viability are available from the corresponding author on request.

508   Associated code is available as supplementary files. Figures 1D, 1F, 1J, 3B, 3D, 3F, 4C, 4D, 4E,

509   4F, 4H, 4I, 5A, 5C, 5D, 5G, 5H, and Supplementary Figure 5C have associated raw data

510   included in Tables 1 and 2. There are no restrictions on data availability.

511

512   **Code Availability**

513   Code used to generate datasets and analyze neuron viability is available as supplementary

514   files. All macros created for use with ImageJ/Fiji are available on request. CellProfiler pipelines

515   and detailed settings to reproduce the image analysis procedures are available upon request.

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658

659

660   **Figure Legends**

661   **Figure 1. TAX1BP1 depletion impairs clearance of protein aggregates. a,** Validation of

662   knockout cell lines. **b,** Experimental outline for assessing aggregate formation and clearance. **c,**

663   WT or individual knockouts for p62, NBR1, NDP52, OPTN, and TAX1BP1 cell lines were

664   exposed to 5 µg/ml puromycin for 2 h, after which cells were either fixed for imaging or washed

665   and followed for a further 3 h in full media; scale bar 10µm. **d, f,** Quantification of b: percent of

666   cells containing UB-positive foci was assessed in ~ 200 cells per condition in 3 independent

667   experiments at (d) 2 h puromycin or (f) 2 h puromycin followed by 3 h washout. Quantification is

668   displayed as mean ± s.d. from 3 independent experiments using one-way ANOVA test (**$P<0.01$,

669   ***$P<0.001$,****$P<0.0001$) comparing all to WT and Tukey's post hoc test. **e, g,** WT normalized

670   comparisons of foci formation and foci clearance in autophagy receptor knockout cell lines. **h,**

671   WT, pentaKO, or individual KO lines for each autophagy receptor were treated with 1 µM MG132

672   for 8 or 18 h, fractionated into RIPA-soluble or -insoluble fractions and immunoblotted for total

673   ubiquitin. Quantification and soluble fractions shown in Supp. Fig. 1C and 1D, respectively. **i,** WT

674   or TAX1BP1 KO cell lines were exposed to 100 nM Bafilomycin A or 1 µM MG132 for 5 h then

675   fixed for imaging. **j,** Quantification of i: percent of cells containing UB-positive foci was assessed

676   in ~ 200 cells per condition. Quantification is displayed as mean ± s.d. from 3 independent

677   experiments using one-way ANOVA test, (****$P<0.0001$) comparing all to WT and Tukey's post

678   hoc test.   The number of observations used in each experimental series and P values for all

679   comparisons are included in Table 1. All blots and microscopy images are representative of at least

680   3 independent experiments.

681

682   **Figure 2. TAX1BP1 protein responds to proteotoxic stress and associates with insoluble**

683   **protein. a, b,** WT HeLa cells were treated with either (a) puromycin or (b) MG132 as indicated,

684   lysed in 2% SDS and immunoblotted for TAX1BP1, OPTN, NBR1, p62, or NDP52. **c,** WT cells

685   treated with 1 µM MG132 for 8 or 18 h were fractionated into RIPA-soluble or -insoluble

686   fractions and immunoblotted for TAX1BP1. **d,** Human tissue panel probed for TAX1BP1 or

687   NDP52. **e,** Primary rat cortical neurons were treated with the indicated amounts of MG132 for 8

688   or 18 h, fractionated into RIPA-soluble or -insoluble fractions and immunoblotted for total UB. **f,**

689   Fractionated primary rat cortical neurons treated as in (e) were blotted for the indicated proteins.

690   All blots are representative of at least 3 independent experiments.

691

692   **Figure 3. TAX1BP1 mediates aggregate clearance. a,** WT, TAX1BP1 KO, and TAX1BP1 KO

693   with stable expression of GFP-TAX1BP1 rescue cell lines were exposed to 5 µg/ml puromycin

694   for 2 h, after which cells were either fixed for imaging or washed and followed for a further 3 h

695   in full media; scale bar 10µm. **b,** Quantification of (a): percent of cells containing UB-positive

696   foci was assessed in ~ 200 cells per condition. Quantification is displayed as mean ± s.d. from 3

697   independent experiments using one-way ANOVA test (****P<0.0001) and Tukey's post hoc

698   test. **c, d,** Stably-expressing TAX1BP1 rescue lines were created using either N-FLAG or C-

699   FLAG tag at high (H) or low (L) expression levels (See Supplementary Figure 3A, 3B) and

700   exposed to 5 µg/ml puromycin for 2 h, after which cells were either fixed for imaging (c) or

701   washed and followed for a further 3 h in full media (d) and quantified as in (b). **e,** WT,

702   TAX1BP1 KO, or TAX1BP1 KO + FLAG-TAX1BP1 (H) cell lines were exposed to 5 µg/ml

703   puromycin in the presence or absence of 100 nM Bafilomycin A, after which cells were either

704   fixed for imaging or washed and followed for a further 3 h in full media or in full media

705   containing Bafilomycin A. Larger fields of view shown in Supplementary Figure 3C. **f,**

706   Quantification of e: percent of cells containing UB-positive foci was assessed in ~ 200 cells per

707   condition in 3 independent experiments. Quantification is displayed as mean ± s.d. from 3

31

708   independent experiments using two-way ANOVA test, (****$P<0.0001$). The number of

709   observations used in each experimental series and P values for all comparisons are included in

710   Table 1. All images are representative of at least 3 independent experiments.

711

712   **Figure 4. Requirements for TAX1BP1 domains in aggrephagy a,** TAX1BP1 truncation or

713   point mutations used in this study. **b, i-x,** TAX1BP1 KO and TAX1BP1 KO with stable

714   expression of TAX1BP1 mutants were exposed to 5 µg/ml puromycin for 2 h, after which cells

715   were either fixed for imaging or washed and followed for a further 3 h in full media; scale bar

716   10µm, larger fields of view shown in Supplementary Figure 4A. **c,** Quantification of UB-foci

717   formation observed in (b). **d,** Quantification of UB-foci clearance observed in (b), percent of

718   cells containing UB-positive foci was assessed in ~ 200 cells per condition. **e,** Quantification of

719   UB-foci formation observed in TKO (OPTN/NDP52/TAX1BP1). See Supplementary Figure 4C

720   for images. **f,** Quantification of UB-foci clearance observed in TKO. See Supplementary Figure

721   4C for images, percent of cells containing UB-positive foci was assessed in ~ 200 cells per

722   condition. **g,** WT cells stably expressing high levels of FLAG-TAX1BP1 were exposed to 5

723   µg/ml puromycin for 2 h, after which cells were either fixed for imaging or washed and followed

724   for a further 3 h in full media; scale bar 10µm. **h,** Quantification of UB-foci formation observed

725   in (g) and in Supplementary Figure 4D. **i,** Quantification of UB-foci clearance observed in (g)

726   and in Supplementary Figure 4D, percent of cells containing UB-positive foci was assessed in ~

727   200 cells per condition. All quantification is displayed as mean ± s.d. from 3 independent

728   experiments using one-way ANOVA test comparing all to WT (*$P<0.05$, **$P<0.01$,

729   ***$P<0.001$, ****$P<0.0001$) and Tukey's post hoc test. The number of observations used in

730   each experimental series and P values for all comparisons are included in Table 1. All images are

731   representative of at least 3 independent experiments.

732

Figure 5. TAX1BP1 mediates aggrephagy of cytotoxic aggregation-prone proteins. a, WT,

TAX1BP1 knockout, or TAX1BP1 knockout with stably-expressed TAX1BP1 rescue cell lines

were exposed to proteotoxic stressors as indicated on Day 1, then followed for 6 days during

which viability was measured by quantification of ATP production. Relative viability represents

normalized luminescence displayed as mean ± s.d. from 3 independent experiments; significance

was assessed using two-way ANOVA test (****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$)

with Tukey's post hoc test. P values and normalized viability measurements shown on graphs are

for day 6 comparisons. The individual measurements for each time point and conditions used in

each experimental series and P values for all comparisons are included in Table 1. **b,** WT,

TAX1BP1 knockout, or rescue cells uninfected or infected with virus expressing HttQ23-EGFP,

HttQ74-EGFP, or HttQ103-EGFP were assessed 4 days post-infection. **c,** Quantification of

HttQ74-EGFP aggregates observed in (b), **d,** Quantification of HttQ103-EGFP aggregates

observed in (b), percent of cells containing GFP-positive foci was assessed in ∼ 200 cells per

condition in 3 independent experiments. Quantification is displayed as mean ± s.d. from 3

independent experiments using one-way ANOVA test (**$P<0.01$,***$P<0.001$,****$P<0.0001$)

and Tukey's post hoc test. The number of observations used in each experimental series is

included in Table 1. All images are representative of at least 3 independent experiments. **e,**

Immunofluorescence labeling of endogenous TAX1BP1 in cells infected with HttQ23-EGFP or

HttQ103-EGFP. Maximum intensity projections shown. **f,** A single 1μm slice is shown from

images taken of immunofluorescence labeling of endogenous TAX1BP1 in cells infected with

HttQ103-EGFP as in **(e)**. Scale bars 10μm. **g, h,** Graphs show line fitted to the number of BFP-

positive nuclei counted daily for iPSC-derived neurons with or without stable TAX1BP1

overexpression infected with HttQ23-EGFP (g) or HttQ103-EGFP (h). Ribbon represents 95%

33

756   confidence interval around the fitted line. Beeswarm box plots compare survival scores

757   determined by performing permutation analysis using the means of all slopes (center line =

758   median, box limits = first to third quartile, whiskers = minimum and maximum). The number of

759   observations used in each experimental series is included in Table 2.

760 **Supplementary Figure Legends**

761 **Supplementary Figure 1. a,** Schematic illustration of protein domain architectures of

762 mammalian autophagy receptors OPTN, NDP52, TAX1BP1, p62, and NBR1. PB1, Phox and

763 Bem1 domain; ZZ, ZZ-type zinc finger domain; NLS1 and NLS2, nuclear localization signals 1

764 and 2; NES, nuclear export signal; LIR, LC3-interacting region; KIR, Keap-interacting region;

765 UBA, ubiquitin-associated domain; CC, coiled-coil domain; FW, four tryptophan domain;

766 SKICH, SKIP carboxyl homology domain; ZF, Zinc-finger domain; UBAN ubiquitin binding in

767 ABIN and NEMO domain. The size of the receptors (in number of amino acids) is indicated. **b,**

768 Representative image of segmentation analysis performed using CellProfiler. **c, d,** Quantification

769 of (b) using CellProfiler: number of foci per cell in WT or TAX1BP1 KO cells in 3 independent

770 experiments at (c) 2 h puromycin or (d) 2 h puromycin followed by 3 h washout (For box plots,

771 center line = median, box limits = first to third quartile, whiskers = minimum and maximum). **e,**

772 WT and individual knockouts for p62, NBR1, NDP52, OPTN, and TAX1BP1 cell lines were

773 exposed to 1 µM MG132 for 8 or 18 h, after which cells were either fixed for imaging or washed

774 and followed for a further 3 h in full media; scale bar 10 µm. **f,** Quantification of Figure 1H

775 determined by densitometry and normalized first to soluble GAPDH and subsequently to WT

776 levels within each fraction. **g,** WT and individual KO lines for each autophagy receptor were

777 treated with 1 µM MG132 for 8 or 18 h, fractionated into RIPA-soluble or -insoluble fractions

778 and immunoblotted for total UB. Soluble fractions shown here, insoluble fractions shown in

779 Figure 1H. All blots and microscopy images are representative of at least 3 independent

780 experiments.

781

782 **Supplementary Figure 2. a,** Primary rat cortical neurons treated with 1 µM MG132 for 18 h,

783 after which cells were fixed for imaging and stained with antibodies for TAX1BP1 and UB;

35

784   scale bar 10µm. **b,** Neurons derived from human induced pluripotent stem cells (iPSCs) treated

785   with 1 µM MG132 for 20 h, fixed and stained with antibodies targeting TAX1BP1 and UB; scale

786   bar 20 µm. All images are representative of at least 3 independent experiments.

787

788   **Supplementary Figure 3. a,** GFP- or FLAG-tagged TAX1BP1 was stably reintroduced into

789   TAX1BP1 KO cells via viral infection. TAX1BP1 expression levels were titered for use in

790   rescue experiments: L = low expression, H = high expression. **b,** Cell lines in (a) were exposed

791   to 5 µg/ml puromycin for 2 h, after which cells were either fixed for imaging or washed and

792   followed for a further 3 h in full media; scale bar 10µm. **c,** Full field of view images associated

793   with Figure 3E, F showing WT, TAX1BP1 KO, or TAX1BP1 KO + FLAG-TAX1BP1 (H) cell

794   lines exposed to 5 µg/ml puromycin in the presence or absence of 100 nM Bafilomycin A, after

795   which cells were either fixed for imaging or washed and followed for a further 3 h in full media

796   or in media containing Bafilomycin A. **d**, WT cells exposed to 5 µg/ml puromycin for 2 h in the

797   presence or absence of Bafilomycin A, after which cells were either fixed for imaging or washed

798   and followed for a further 3 h in full media with or without Bafilomycin A; scale bar 10µm. All

799   images are representative of at least 3 independent experiments.

800

801   **Supplementary Figure 4. a,** Full field of view images of all TAX1BP1 stable mutant expression

802   cell lines exposed to 5 µg/ml puromycin for 2 h, after which cells were either fixed for imaging

803   or washed and followed for a further 3 h in full media. Associated with Figure 4B, C, D. **b,**

804   Validation of knockout cell lines. **c,** TKO (triple knockout: TAX1BP1, OPTN, NDP52) cell line

805   with stable expression of TAX1BP1 mutants exposed to 5 µg/ml puromycin after which cells

806   were either fixed for imaging or washed and followed for a further 3 h in full media; scale bar

807   10µm. **d,** WT cells stably expressing low levels of FLAG-TAX1BP1 were exposed to 5 µg/ml

36

808   puromycin for 2 h, after which cells were either fixed for imaging or washed and followed for a

809   further 3 h in full media; scale bar 10µm. All images are representative of at least 3 independent

810   experiments.

811

812   **Supplementary Figure 5. a,** Constructs used in this study. **b,** Expression of GFP control or

813   GFP-TDP-43. **c,** WT, TAX1BP1 knockout, or TAX1BP1-rescue cells transfected with DNA

814   expressing EGFP-TDP-43 at the indicated concentrations on Day 1, then followed for 6 days

815   during which viability was measured by quantification of ATP production. Relative viability

816   represents normalized luminescence displayed as mean ± s.d. from 3 independent experiments;

817   significance was assessed using two-way ANOVA test (****$P<0.0001$, ***$P<0.001$, **$P<0.01$)

818   with Tukey's post hoc test. P values and normalized viability measurements shown on graphs are

819   for day 6 comparisons. The individual measurements for each time point and conditions used in

820   each experimental series and P values for all comparisons are included in Table 1. **d,** A single

821   1µm slice is shown from images taken of immunofluorescence labeling of endogenous

822   TAX1BP1 in cells infected with HttQ103-EGFP; scale bar 10µm. **e,** Proteotoxic stress, induced

823   by translational stress, proteasome inhibition, or expression of aggregate-promoting proteins

824   causes misfolded or damaged proteins to assemble into toxic oligomers or aggregates. In WT

825   cells (green panel), the proteasome and aggrephagy both work to remove potentially toxic

826   protein products. If the proteasome is overwhelmed, aggregated protein is shunted to the

827   autophagy pathway. In the absence of TAX1BP1 (red panel), aggrephagy is deficient - once the

828   proteasome has become overwhelmed by misfolded or aggregated protein, there is decreased

829   backup clearance via aggrephagy, and insoluble protein accumulates, leading to toxicity and cell
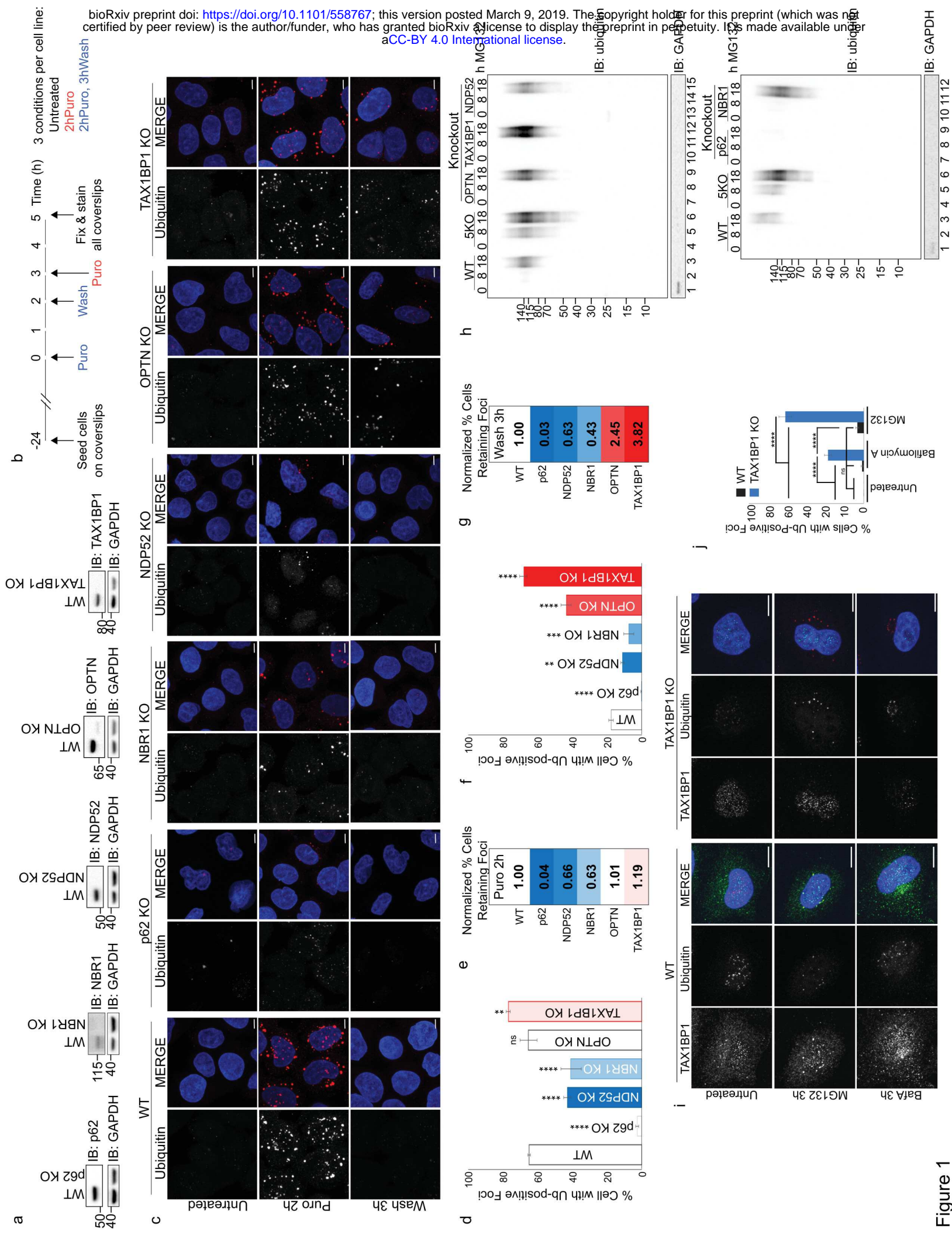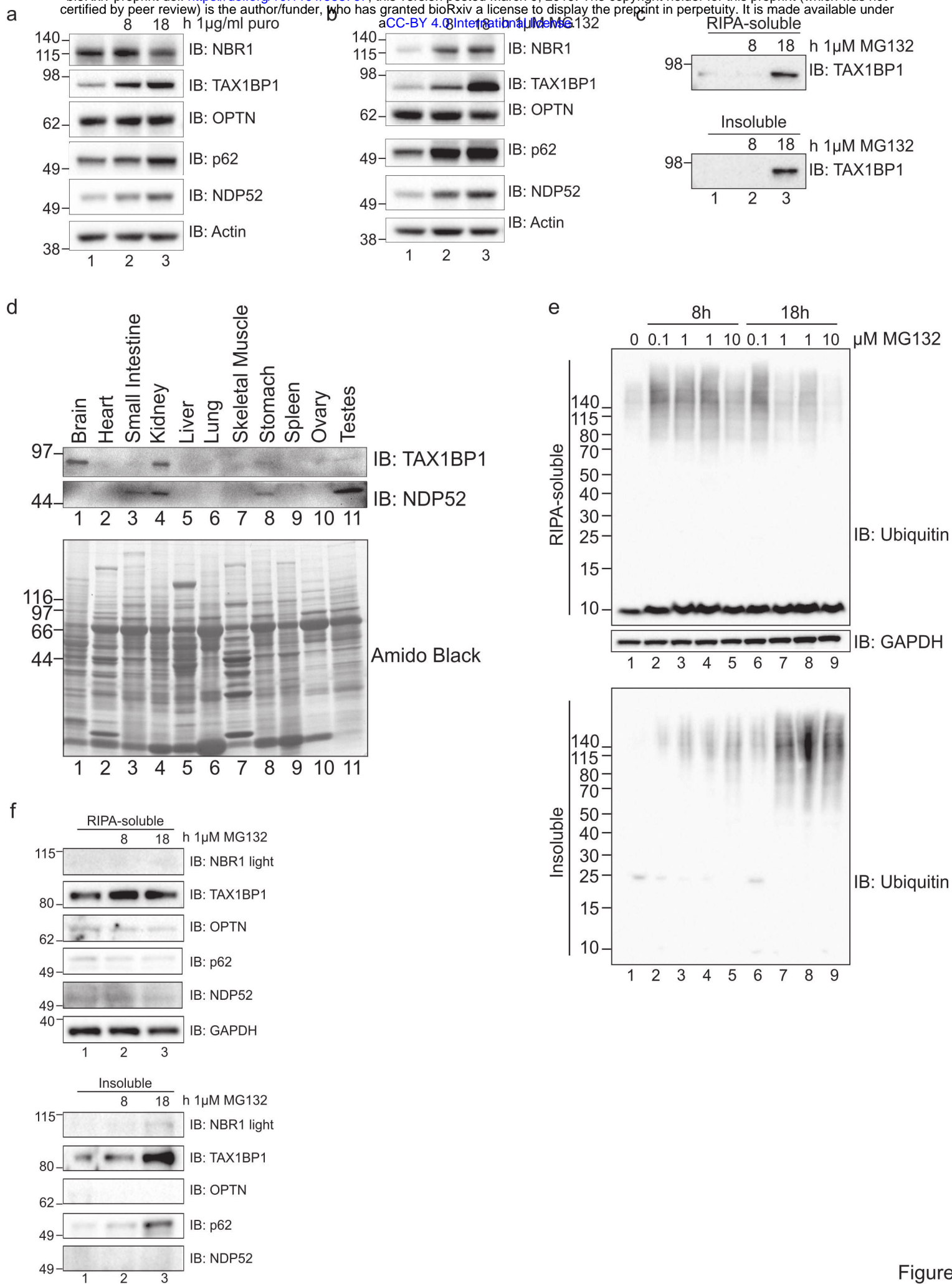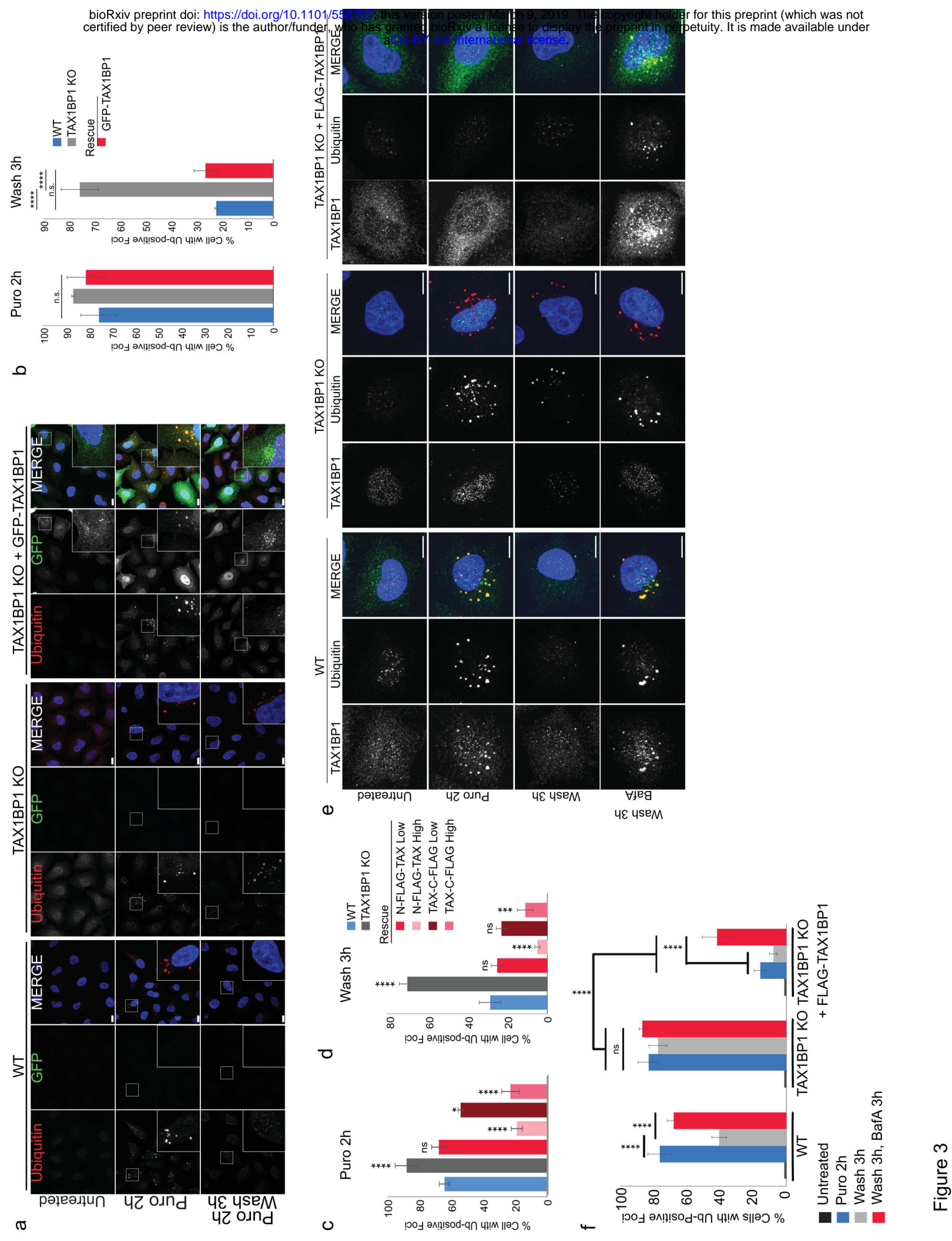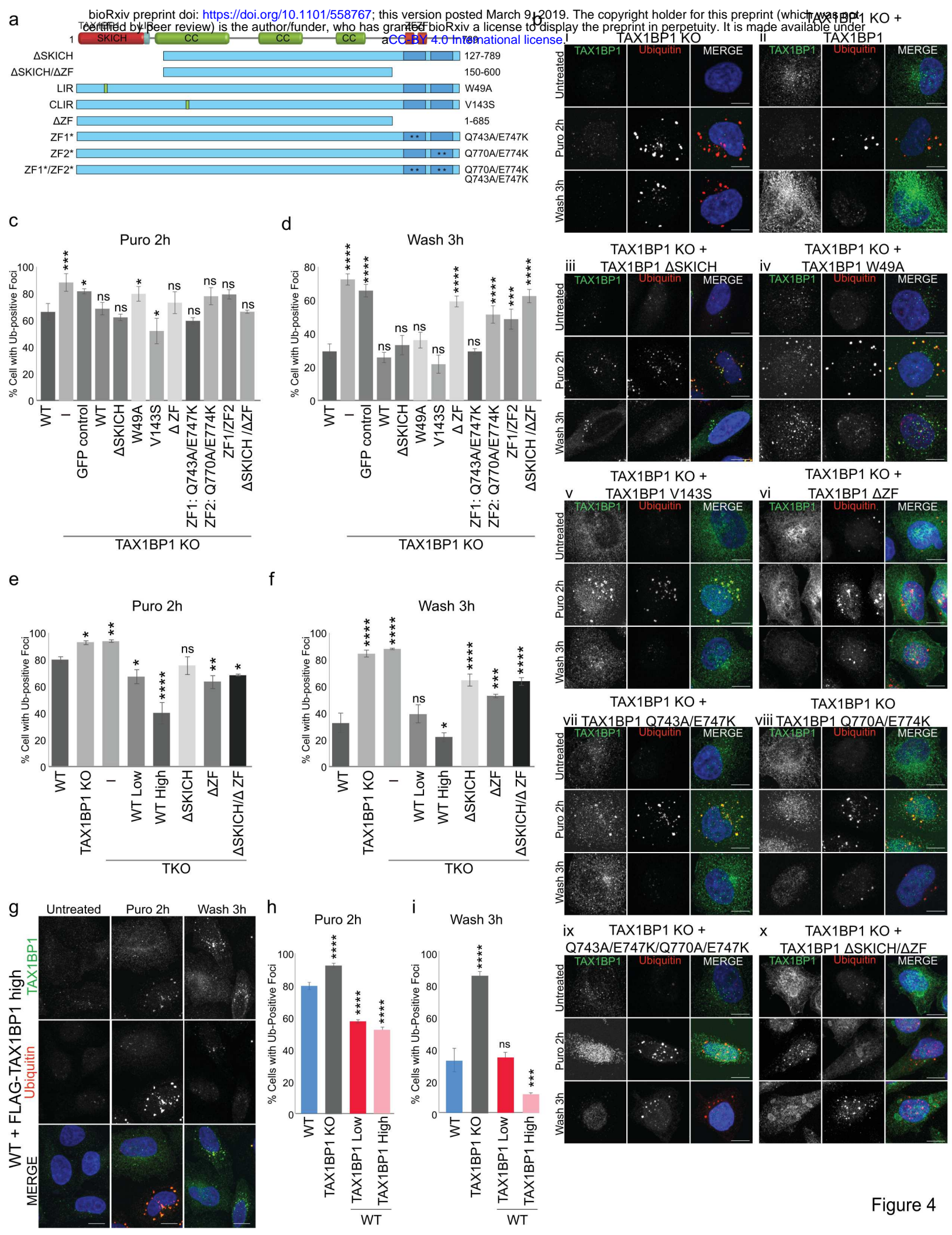
830   death.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

Supplementary Figure 4

a

GFP-TDP43

Glycine rich domain

WT full length protein
- soluble expression in nucleus
- some cytosolic when overexpressed

HttQ23-EGFP

Htt 1-17   23Q   GFP

Soluble & diffuse

HttQ74-EGFP

Htt 1-17   74Q   GFP

Forms cytosolic aggregates

HttQ103-EGFP

Htt 1-17   103Q   GFP

Forms cytosolic aggregates

b

GFP

GFP-TDP43

c

WT
TAX1BP1 KO
Rescue

0.125ug TDP-43

35.6
28.4
23.4

0.25ug TDP-43

30.2
19.6
9.2

d

HTTQ103   TAX1BP1   MERGE

e

Proteotoxic Stress:
Puromycin, MG132, aggregate-forming protein

Oligomer/Aggregate Formation
TAX1BP1 localizes to aggregates

WT

TAX1BP1 KO

UPS failure

UPS failure

Autophagy failure

Misfolded protein degraded by the proteasome and aggrephagy

If the UPS is overwhelmed, aggregates are shunted towards aggrephagy

Aggregates are cleared

In the absence of TAX1BP1, aggrephagy cannot compensate for UPS failure
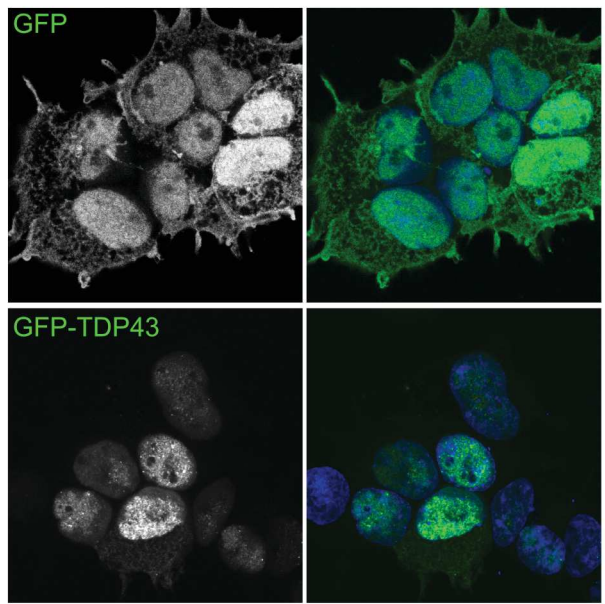
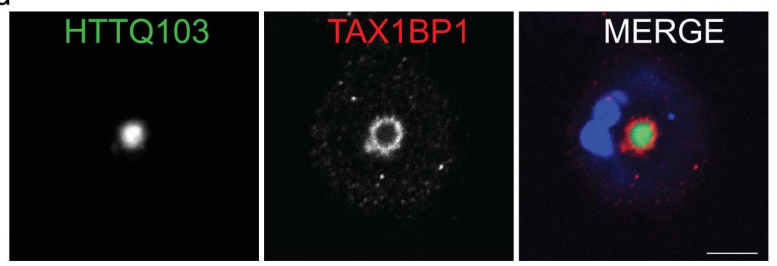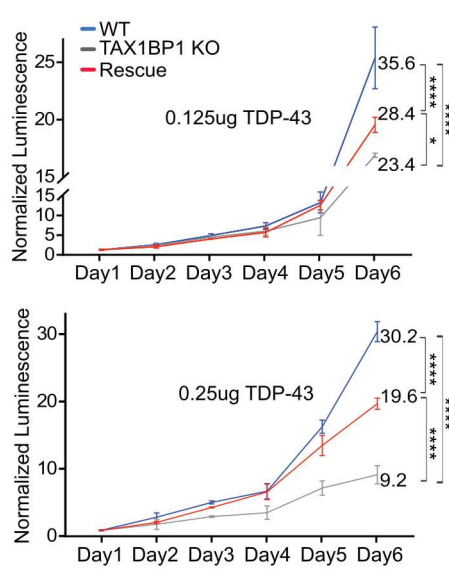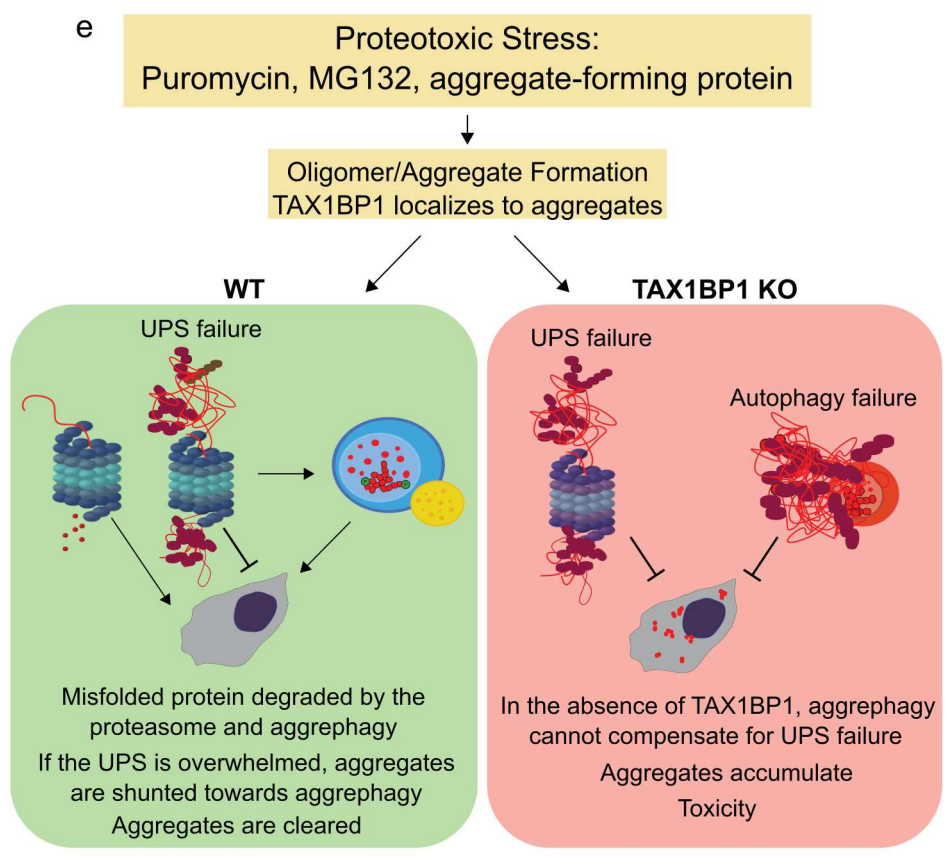Aggregates accumulate

Toxicity

Supplementary Figure 5

```
1    R code for Neuron Viability Analysis

2    ---
3    title: ""Selective autophagic clearance of protein aggregates is
4    mediated by the autophagy receptor, TAX1BP1" "
5    output: html_notebook
6    ---

7    Images of the size 1200*1200 were segmented using the following
8    segmentation code.

9    ```{r}
10   args = (commandArgs(TRUE)) image.tif=args[1] library("dplyr")
11   library("data.table") library("gtools") library("EBImage")
12   library("outliers")

13   frame<-readImage(image.tif, type = "tif")
14   x<-1200
15   y<-1200
16   dapi<-frame[1:x,1:y,2] minDapi<-min(as.vector(dapi)) maxDapi<-
17   max(as.vector(dapi)) dapin<-normalize(dapi,
18   ft=c(0,1),c(minDapi,maxDapi)) dapi_normal<- dapin*(66)

19   nmask2 = thresh(dapi_normal, 50, 50,0.062) mk3 = makeBrush(11, shape=
20   "diamond") nmask2 = opening(nmask2, mk3)
21   nmask2 = fillHull(nmask2)

22   nseg = bwlabel(nmask2) #binery to object chackpoint<-
23   computeFeatures.shape(nseg) nmask = watershed(distmap(nmask2),1)
24   #display(nmask)

25   nf = computeFeatures.shape(nmask) nr = which(nf[,2] < 60)
26   nseg = rmObjects(nmask, nr) #display(nseg)

27   #rm(nf,nmask)
28   nn = max(nseg) chackpoint<-computeFeatures.shape(nseg) seg_GFP<-
29   paintObjects(nseg,toRGB(dapi*20),opac=c(1,

30   0.2),col=c("Green",NA),thick=F,closed=FALSE) chackpoint<-
31   computeFeatures.shape(nseg) df<-as.data.frame(chackpoint) xy<-
32   computeFeatures.moment(nseg)[,c('m.cx','m.cy')] df<-cbind(df,xy)
33   seg_GFP<-paintObjects(nseg,toRGB(dapi*20),opac=c(1,

34   0.2),col=c("Green",NA),thick=F,closed=FALSE) df.combine<-
35   as.data.frame(matrix(0,nrow(xy),5)) colnames(df.combine)<-
36   c("x","y","Area_real","Areal_roundess","ratio") df.combine$x<-xy[,1]

37   df.combine$y<-xy[,2] df.combine$Area_real<-df[,1] #area of sample
38   df.combine$Areal_roundess<-pi*(df[,3])^2 df.combine$ratio<-
39   df.combine[,4]/df[,1]
40   nr = which(df.combine[,5] > 1 )
41   gsegg = rmObjects(nseg, nr)
42   #rm(nseg,df)
43   nr = which(df.combine[,5] < 0.6 )
```

```
44    #rm(df.combine)
45    gsegg = rmObjects(gsegg, nr) bfp.number<-computeFeatures.shape(nseg)
46    seg_GFP<-paintObjects(gsegg,toRGB(dapi*60),opac=c(1,

47    0.2),col=c("Green",NA),thick=F,closed=FALSE)
48    #writeImage(seg_GFP,paste("processed",image.tif,sep = "_"))
49    write.table(nrow(bfp.number), file=args[2], row.names=FALSE, sep =
50    ";")

51    ```

52    The procedure was done on the nih biowulf server using the nih
53    parallel procedure unix command swarm.
54    swarm -g 16 --time 08:00:00 -f file.swarm --module R/3.5
55    Swarm file was creating by the following code:

56    ```{r}
57    dir.folder<-dir() dir.folder<-dir.folder[1:22] for (j in
58    1:length(dir.folder)){

59        setwd(dir.folder[j])

60    file.copy("/spin1/users/kanferg/Images/Tax1bp_Shireen/per_well_Image/r
61    unn
62    .R",paste0("/spin1/users/kanferg/Images/Tax1bp_Shireen/per_well_Image/
63    ",d ir.folder[j]))

64    tifsS<-dir()[grep(".tif$",dir())]

65    for (i in 1:length(tifsS)){ name<-gsub(".tif",".txt",tifsS[i])

66        file.create(name)
67    }

68    name.folder<-paste0(dir.folder[j]) #seq(1,length(tifsS))

69    mydata<-
70    paste0("/spin1/users/kanferg/Images/Tax1bp_Shireen/per_well_Image/",na
71    me. folder)

72    #tifsS<-dir()[grep(".tif$",dir())] sink(paste0(name.folder,".swarm"))
73    cat("#! /bin/bash")
74    cat("\n")

75    cat("#swarm -f first.swarm --module R/3.5")
76    cat("\n")
77    for (i in 1:length(tifsS)){ name<-gsub(".tif",".txt",tifsS[i])
78    cat("Rscript"," ",paste(mydata,"/runn",".R",sep =""),"

79    ",paste(tifsS[i])," ",paste(name)) cat("\n")

80    } sink()

81    setwd("..")
```

2

```
82    }

83    mydata<-"/data/kanferg/Ward_lab/Plate_4/" sink("second.swarm")

84    cat("#! /bin/bash")
85    cat("\n")
86    cat("#swarm -f second.swarm --module R/3.5") cat("\n")
87    for (i in 1000:1496){

88    name<-gsub(".tif",".txt",tifsS[i])

89    cat("Rscript"," ",paste(mydata,"runn",".R",sep =""),"
90    ",paste(tifsS[i])," ",paste(name))

91    cat("\n") }

92    sink()
93    ```
94    Segmented object number per well table are aggregated to a list

95    ```{r} HttQ23_1<-as.data.frame(list.observation$E02) HttQ23_2<-
96    as.data.frame(list.observation$F02) HttQ23_3<-
97    as.data.frame(list.observation$G02) HttQ103_1<-
98    as.data.frame(list.observation$E03) HttQ103_2<-
99    as.data.frame(list.observation$F03) HttQ103_3<-
100   as.data.frame(list.observation$G03)

101   HttQ23.tax_1<-as.data.frame(list.observation$E08) HttQ23.tax_2<-
102   as.data.frame(list.observation$F08) HttQ23.tax_3<-
103   as.data.frame(list.observation$G08) HttQ103.tax_1<-
104   as.data.frame(list.observation$E09) HttQ103.tax_2<-
105   as.data.frame(list.observation$F09) HttQ103.tax_3<-
106   as.data.frame(list.observation$G09)

107   vector.name<-
108   c("HttQ23_1","HttQ23_2","HttQ23_3""HttQ103_1","HttQ103_2","HttQ103_3",
109   "Ht
110   tQ23.tax_1","HttQ23.tax_2","HttQ23.tax_3","HttQ103.tax_1","HttQ103.tax
111   _2" ,"HttQ103.tax_3")
112   list.df<-
113   list(HttQ23_1,HttQ23_2,HttQ23_3HttQ103_1,HttQ103_2,HttQ103_3,HttQ23.ta
114   x_1
115   ,HttQ23.tax_2,HttQ23.tax_3HttQ103.tax_1,HttQ103.tax_2,HttQ103.tax_3)
116   ```

117   Generate fitted curves using exponential decay model

118   ```{r} make.list<-function(input.list){

119   df.list<-as.data.frame(input.list) ls.temp<-list()
120   for (i in 1:16){

121   vec.cellnumber<-as.vector(t(df.list[i,1:15])) day<-1:15
122   df<-as.data.frame(cbind(day,vec.cellnumber)) colnames(df)<-
```

```
123    c("day","cellNumber") drc.plot<-try(drm(df$cellNumber~df$day,data =
124    df, fct =

125    EXD.3(),na.action = na.omit))
126    if (inherits(drc.plot,"try-error")){

127    next } else {

128    sl<-summary(drc.plot)
129    slope<-sl$coefficients[[3]]
130    slope.se<-sl$coefficients[[6]] pv.slope<-sl$coefficients[[9]]
131    ls.temp[[i]]<-as.data.frame(cbind(slope,slope.se,pv.slope))

132    } }

133      return(ls.temp)

134    } ```

135    Transformation of exponential decay fitted slope into ggplot2
136    compatible form

137    ```{r} drc.table<-function(input){

138    #browser() df.ctrl.drc<-as.data.frame(matrix(0,0,2))
139    colnames(df.ctrl.drc)<-c("day","cellNumber") colnames(input)<-c(1:38)
140    input<-input[,c(2:20)] mean.c<-median(input[,1])
141    li<-list(NULL) ctrl.temp<-rep(1,length(input[,1]))

142        li[[1]]<-ctrl.temp
143      for (i in 1:19){

144    ctrl.temp<-(input[,i])/mean.c

145    li[[paste0(i)]]<-ctrl.temp }

146    vec.num<-as.vector(li[[paste0(1)]]) vec.day<-rep(1,length(vec.num))
147    df.ctrl.drc<-cbind(vec.day,vec.num)

148    for (j in 1:19){ vec.num<-as.vector(li[[paste0(j)]]) vec.day<-
149    rep(j,length(vec.num)) df.ctrl.drc.temp<-cbind(vec.day,vec.num)
150    df.ctrl.drc<-rbind(df.ctrl.drc,df.ctrl.drc.temp)

151    }

152    df.ctrl.drc<-as.data.frame(df.ctrl.drc)

153      return(df.ctrl.drc)
154    }

155    ```
156    Eliminate Outliers Using Interquartile Range
```

```
157   ```{r}
158   lll103<-list()
159   for (i in 1:length(unique(drc.input.table.df$day))){

160   d.temp<-filter(drc.input.tabledf,day==i) outliers<-
161   scores(d.temp$survival_rate,type = c("iqr")) ind<-which(outliers == 0)
162   if (length(ind) > 0) {

163       d.temp<-d.temp[ind,]
164     }

165   lll103[[i]]<-mean(d.temp[,2]) }

166   ```
167   Preforming permutation analysis on the calculated slops

168   ```{r} df.permut.tax<-
169   filter(df.anova.slope.pv,df.anova.slope.pv$name=="set1")
170   nrow(df.permut.tax)
171   #df.permut.tax<-df.permut.tax[1:32,] df.permut.notax<-
172   filter(df.anova.slope.pv,df.anova.slope.pv$name=="set2")
173   nrow(df.permut.tax)
174   #df.permut.notax<-df.permut.notax[1:32,]
175   # test intalisation
176   nsim<-10000 combine.df.slope<-
177   c(df.permut.notax$slope,df.permut.tax$slope) combine.df.name<-
178   c(df.permut.notax$name,df.permut.tax$name) dif.obse<-
179   mean(df.permut.notax$slope)-mean(df.permut.tax$slope) diffres<-
180   rep(NA,nsim)
181   for (i in 1:nsim){

182   suffled.lables<-sample(combine.df.name, replace = F)

183   diffres[i]<-mean(combine.df.slope[suffled.lables == "set1"]) -
184   mean(combine.df.slope[suffled.lables == "set2"])
185   }

186   #calculate the two sided pvalue
187   pv<- length(diffres[abs(diffres) >= abs(dif.obse)])/nsim print(pv)
188   ```

189
```

5

Supplementary Table S1

Previously reported Lazarou et al. 2014

Name	Gene	Clone #
Single Knockout Lines		
OPTN KO	OPTN	24
NDP52 KO	NDP52	5
TAX1BP1 KO	TAX1BP1	3
Triple Knockout Line		
TKO	OPTN	6, (parent 24)
	NDP52	6
	TAX1BP1	6

Name	Gene	Clone #	Exon Targeted	CRISPR sequence		Editing Results
Single Knockout Lines						
p62 KO	p62	11	3	GGCGCCTCCTGAGCACACGG	1	1 basepair insertion
					2	1 basepair deletion
		Restriction enzyme		HpyCH4III cuts into 168, 99, 3 bp		
		PCR product (bp)				
		270				
	Genotyping Primers					
Forward	ACAGTGACGACAGAGGGGGA					
Reverse	AATGCGAGCTTGGTGTGCC					
NBR1 KO	NBR1	4	5	GCCAGAGGATCCTGCAGTGC	1	1 basepair insertion
					2	19 basepair deletion
					3	13 basepair deletion
		Restriction enzyme		BtsI cuts into 175, 74 bp		
		PCR product (bp)				
		249				
	Genotyping Primers					
Forward	ACCAACTGCAGATGCAAGTCC					
Reverse	AGAAACCTGTTCAGCTTTATTTA					