NEMO reshapes the protein aggregate interface and promotes aggrephagy by co-condensation with p62

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ABSTRACT

NEMO is a ubiquitin-binding protein which regulates canonical NF-κB pathway activation in innate immune signaling, cell death regulation and host-pathogen interactions. Here we identified an NF-κB-independent function of NEMO in proteostasis regulation by promoting autophagosomal clearance of protein aggregates. NEMO-deficient cells accumulate misfolded proteins upon proteotoxic stress and are vulnerable to proteostasis challenges. Moreover, a patient with a mutation in the NEMO gene resulting in defective binding of NEMO to linear ubiquitin chains, developed a widespread mixed brain proteinopathy, including α-synuclein, tau and TDP-43 pathology. NEMO amplifies linear ubiquitylation at α-synuclein aggregates and promotes the local concentration of p62 into foci. In vitro, NEMO lowers the threshold concentrations required for ubiquitin-dependent phase transition of p62. In summary, NEMO reshapes the aggregate surface for efficient autophagosomal clearance by providing a mobile phase at the aggregate interphase favoring co-condensation with p62.

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INTRODUCTION

Cellular proteostasis is maintained by an elaborate network of protein quality control components, such as molecular chaperones, the ubiquitylation machinery, the proteasome and the autophagy-lysosome pathway, to coordinate the proper triage, subcellular quarantine, and refolding or disposal of damaged proteins. In aging, proteotoxic stress along with a decline in the fidelity of protein quality control favors the accumulation of misfolded proteins, challenging the integrity of the cellular proteome. This is particularly relevant to postmitotic cells, such as neurons. In fact, the age-dependent accumulation of misfolded proteins is a characteristic feature of neurodegenerative diseases. Cells are equipped with two proteolytic machineries, the ubiquitin-proteasome system and the autophagy-lysosomal system that remove misfolded proteins depending on their subcellular localization, their structure, oligomerization state and posttranslational modifications. Both degradation pathways are critically regulated by ubiquitin in cooperation with specific ubiquitin-binding proteins that target cargo to the proteasome or to selective autophagy [1, 2, 3, 4, 5, 6]. Ubiquitylation is a highly elaborate posttranslational modification, regulating a plethora of cellular processes. Its complexity is based on different variables, such as the number of ubiquitin molecules attached to substrates, the mode of inter-ubiquitin linkage, the formation of homo- and heterotypic chains, and the posttranslational modification of ubiquitin itself, for example by phosphorylation, SUMOylation, or NEDDylation [7, 8, 9, 10]. In conventional ubiquitination, the C-terminal glycine of ubiquitin is linked to one of seven lysine residues of another ubiquitin molecule by an isopeptide bond. Alternatively, the C-terminal glycine can be linked to the N-terminal methionine of the acceptor

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82   ubiquitin, resulting in the formation of a peptide bond [11]. This type of head-to-tail linkage is

83   called linear or M1-linked ubiquitylation. Linear ubiquitin chains are exclusively generated by

84   the RING-in-between-RING (RBR) E3 ubiquitin ligase HOIP, the catalytic component of the

85   linear ubiquitin chain assembly complex (LUBAC). Within this complex, HOIP interacts with

86   HOIL-1 and SHARPIN, which bind to the ubiquitin-associated (UBA) domain of HOIP by their

87   ubiquitin-like (UBL) domain, thereby activating autoinhibited HOIP [12, 13, 14, 15, 16, 17, 18, 19, 20, 21].

88   LUBAC has mostly been studied in the context of immune signaling, NF-κB activation and cell

89   death regulation [22, 23, 24, 25, 26]. In these paradigms, the regulatory component of the IKK (inhibitor

90   of κB kinase) complex NEMO (nuclear factor-κB essential modulator), also called IKKɣ, is an

91   important player, serving both as an interactor of linear ubiquitin chains and a substrate of

92   HOIP [27, 28, 29]. Binding of NEMO to M1-linked ubiquitin chains via its UBAN (ubiquitin-binding in

93   ABIN and NEMO) domain and also ubiquitylation of NEMO upon activation of innate immune

94   receptors, such as the TNF receptor 1, induces a conformational change in NEMO and

95   activates the associated kinases IKKα and IKKβ [14, 30, 31]. The activated IKK kinases

96   phosphorylate IκBα (inhibitor of κBα), which is then modified by K48-linked ubiquitin chains

97   and degraded by the proteosome. Thereby, NF-κB heterodimers, typically p65 and p50, are

98   liberated from their binding to IκBα and translocate to the nucleus to regulate the expression

99   of NF-κB-dependent genes.

100   Amorphic or hypomorphic mutations in the NEMO-encoding *IKBKG* gene located on the X

101   chromosome are associated with *Incontinentia pigmenti* (IP), a condition that is usually lethal

102   in male fetuses and thus almost exclusively occurs in female patients with mosaic X-

103   chromosome inactivation [32, 33, 34]. IP is a rare multisystem disorder that primarily affects the

104   skin, but can also involve other ectodermal tissues including teeth, hair, nails, eyes and the

105   central nervous system [35]. IP-linked mutations comprise rearrangements, nonsense,

106   frameshift, splice site, or missense mutations [33, 34]. Although IP can be inherited in an X-linked

107   dominant fashion, between 65 and 75% of cases occur sporadically due to *de novo* mutations

108   [36]. The phenotypic heterogeneity of the clinical presentation is based on the fact that in each

109   tissue, cells expressing the wildtype *IKBKG* allele coexist with cells expressing the mutant

110   *IKBKG* allele, hampering the analysis of patients' biosamples and the establishment of patient-

111   derived cellular models. We identified a patient suffering from IP who developed early onset,

112   rapidly progressive neurodegeneration with a widespread mixed proteinopathy, including α-

113   synuclein, tau, and TDP-43 aggregates. The pathogenic Q330X NEMO mutant is

114   compromised in NF-κB pathway activation, explaining the manifestation of IP, but in addition

115   causes defective protein quality control. NEMO deficiency favors the accumulation of

116   misfolded proteins and impairs the clearance of protein aggregates by autophagy. Our study

117   revealed a critical function of NEMO in proteostasis regulation by remodeling the aggregate

118   interface and facilitating condensate formation of the autophagy cargo receptor p62.

119

120 **RESULTS**

121 **A pathogenic mutation in the *IKBKG* gene encoding NEMO is associated with a**
122 **widespread mixed proteinopathy and progressive neurodegeneration**

123 A female patient, who was diagnosed with IP during her infancy, developed parkinsonism (left-
124 sided bradykinesia, hypophonia, slowed gait) at age 48. She noted initial benefit with treatment
125 with carbidopa/levodopa but her condition rapidly progressed, forcing retirement from work as
126 a school teacher at age 52. A dopamine transporter scan was markedly abnormal, showing
127 near absence of tracer uptake within the caudate and putamen bilaterally. She then developed
128 severe cognitive impairment and died at age 56 from progressive neurodegeneration. Brain
129 autopsy revealed a widespread mixed proteinopathy, including α-synuclein, tau, and TDP-43
130 aggregates with predominant α-synuclein pathology (Fig. 1A). There was no family history of
131 neurodegenerative diseases and exome sequencing did not identify mutations in common
132 Parkinson's disease-associated genes. Genetic testing revealed a c.988 C>T nonsense
133 mutation in the *IKBKG* gene, replacing glutamine at position 330 by a premature stop codon
134 (p.Gln330X) (Fig. 1B).

135 Based on our previous finding that NEMO is recruited to misfolded huntingtin with a
136 polyglutamine expansion (Htt-polyQ) [37], we wondered whether an *IKBKG* gene mutation
137 resulting in NEMO dysfunction is causally linked to neuronal protein aggregation observed in
138 our patient. A functional characterization of NEMO Q330X in cellular models showed that this
139 mutant is defective in NF-κB signaling, explaining the clinical presentation with IP. In contrast
140 to wildtype (WT) NEMO, Q330X NEMO was not able to promote TNF-induced degradation of
141 IκBα or NF-κB transcriptional activity (Suppl. Fig. 1A-D). We observed impaired NF-κB
142 activation by Q330X NEMO also upon IL-1β receptor activation [38]. However, our previous study
143 revealed that HOIP was able to decrease Htt-polyQ aggregates independently of NF-κB
144 activation [37]. Therefore, we wondered whether NEMO is a downstream effector of LUBAC-
145 mediated protein quality control. First, we tested if endogenous NEMO is recruited to protein
146 aggregates other than Htt-polyQ. Indeed, immunohistochemistry of brain slices from patients
147 with Parkinson's disease (PD), Alzheimer's disease (AD) or Frontotemporal dementia (FTD)
148 provided evidence for both NEMO and M1-linked ubiquitin chains colocalizing at aggregates
149 formed by α-synuclein, tau, or TDP-43 (Fig. 1C, D).

150

151 **NEMO deficiency promotes protein aggregation under proteotoxic stress**

152 To analyze a possible role of NEMO in proteostasis regulation, we quantified the amount of
153 protein aggregates induced by heat stress or proteasomal inhibition in wildtype and NEMO
154 knockout (KO) mouse embryonic fibroblasts (MEFs) by Proteostat®, a red fluorescent
155 molecular rotor dye that binds to the cross-beta spine quaternary structure of aggregated

156　proteins. In the absence of NEMO, protein aggregation was significantly increased in response

157　to both heat stress and proteasome inhibition (Fig. 2A, B). In addition, NEMO-deficient cells

158　were more vulnerable to proteotoxic stress. Cell viability of NEMO KO MEFs was significantly

159　decreased in response to heat stress or proteasome inhibition compared to wildtype NEMO

160　MEFs (Suppl. Fig. 2A-C). We then made use of a luciferase-based sensor of proteostasis

161　capacity, the conformationally destabilized double mutant firefly luciferase FlucDM (R188Q,

162　R261Q) fused to EGFP [39, 40]. Imbalances in proteostasis induce misfolding and aggregation of

163　this sensor, which can be monitored by the formation of FlucDM-EGFP-positive foci and a

164　decrease in its luciferase activity. With the help of this sensor we compared the effects of

165　wildtype and Q330X NEMO under basal and proteotoxic stress conditions in a NEMO-deficient

166　background. NEMO KO MEFs transiently expressing FlucDM-EGFP and either wildtype or

167　Q330X NEMO were quantified for the abundance of EGFP-positive foci and for luciferase

168　activity. Heat stress (43°C, 20 min) increased FlucDM-EGFP foci formation and decreased its

169　luciferase activity in NEMO KO MEFs (Fig. 2C, D). Expression of wildtype NEMO significantly

170　reduced foci formation and increased luciferase activity of FlucDM-EGFP under both basal

171　and heat stress conditions, whereas Q330X NEMO had no effect (Fig. 2C, D). Interestingly,

172　NF-κB was not activated by heat stress, as shown by an NF-κB luciferase reporter assay and

173　a p65 nuclear translocation assay (Fig. 2E, F). In conclusion, NEMO plays a role in proteostasis

174　regulation that seems to be independent of NF-κB activation.

175

176　**Misfolded α-synuclein is decorated with M1-linked ubiquitin and NEMO**

177　Since the NEMO-mutant patient showed a predominant α-synuclein pathology, we employed

178　cellular models of α-synuclein (aSyn) aggregation for mechanistic studies. Recombinant A53T

179　aSyn preformed fibrils (PFFs) were added as seeds to SH-SY5Y cells stably expressing A53T

180　aSyn fused to GFP (aSyn-GFP) to induce aggregation of aSyn-GFP [41, 42, 43]. Intracellular aSyn

181　aggregates formed upon seeding showed characteristic features of pathologic aSyn, such as

182　phosphorylation at serine 129, insolubility in detergents, and non-dynamic behavior, as

183　determined by immunocytochemistry, immunoblotting and FRAP (fluorescence recovery after

184　photobleaching), respectively (Suppl. Fig. 3A-C). In addition, we characterized the aSyn seeds

185　by Thioflavin T fluorescence, dynamic light scattering, and liquid atomic force microscopy

186　(Suppl. Fig. 4A-F).

187　Consistent with our observation that M1-linked ubiquitin occurs at Lewy bodies in human brain

188　(Fig. 1C), linear ubiquitin chains co-localized with aSyn aggregates in SH-SY5Y cells after

189　seeding (Fig. 3A). Moreover, seeding of primary cortical neurons induced misfolding and serine

190　129 phosphorylation of endogenous aSyn, which stained positive for M1-linked ubiquitin (Fig.

191　3B). The presence of linear ubiquitin chains at aSyn in seeded SH-SY5Y cells was confirmed

192　biochemically by affinity purification of aSyn-GFP followed by immunoblotting using M1-

193   ubiquitin-specific antibodies [44] (Fig. 3C). Moreover, endogenous NEMO was enriched at
194   seeded aSyn aggregates (Fig. 3D) and all three LUBAC components were recruited to aSyn
195   aggregates (Fig. 3E). In contrast, the RBR E3 ubiquitin ligase ARIH1 (ariadne homolog 1),
196   used as a control, was not recruited (Fig. 3E).
197
The Q330X NEMO mutant does not bind to M1-linked ubiquitin chains and is not
198
ubiquitylated by HOIP
199
200   To gain further insight into the function of NEMO in proteostasis regulation, we compared the
201   effect of wildtype NEMO and Q330X NEMO on aSyn aggregates in the SH-SY5Y seeding
202   model. We first tested for NEMO recruitment and observed that Q330X NEMO did not co-
203   localize with aSyn aggregates, whereas wildtype NEMO was strongly enriched at aSyn
204   aggregates (Fig. 4A). Defective recruitment of Q330X NEMO to protein aggregates was
205   confirmed in SH-SY5Y cells with Htt-polyQ aggregates, indicating that this phenomenon is not
206   limited to aSyn aggregates (Fig. 4B). We previously found that NEMO is recruited to Htt-polyQ
207   aggregates by binding to linear ubiquitin chains generated by HOIP (van Well, 2019). We
208   therefore compared the capacity of wildtype NEMO and Q330X NEMO to bind linear ubiquitin
209   chains. Lysates of cells expressing either wildtype or Q330X HA-tagged NEMO were incubated
210   with recombinant tetra-M1-ubiquitin (4xM1-ub), then NEMO was affinity-purified via its HA tag
211   and immunoblotted by using M1-ubiquitin-specific antibodies. In contrast to wildtype NEMO,
212   Q330X NEMO did not bind to tetra-M1-ubiquitin, although the UBAN domain (residues 296-
213   327) is not affected by the C-terminal deletion (Fig. 4C). Moreover, Q330X NEMO was not
214   modified by linear ubiquitin chains, although the key ubiquitin acceptor lysine residues (K285
215   and K309) are present in this mutant [45]. We induced M1-ubiquitination of HA-tagged NEMO by
216   either treating the cells with TNF or overexpressing the LUBAC components HOIP, HOIL-1L,
217   and SHARPIN (Fig. 4D). The cells were lysed under denaturing conditions, NEMO was
218   immunoprecipitated via its HA tag and analyzed by immunoblotting using M1-ubiquitin-specific
219   antibodies. Whereas wildtype NEMO was M1-ubiquitylated in both conditions, Q330X NEMO
220   was not even ubiquitylated upon the overexpression of LUBAC (Fig. 4D). Next, we analyzed
221   the interaction of wildtype NEMO and Q330X NEMO with endogenous HOIP by co-
222   immunoprecipitation experiments. The NZF1 domain of HOIP interacts with the coiled coil CC2
223   region upstream the UBAN domain of NEMO [45, 46, 47]. HOIP did not co-purify with Q330X
224   NEMO, which helps to explain why Q330X NEMO is not ubiquitylated (Fig. 4E).
225   Since NEMO not only binds to M1-linked polyubiquitin but also can be covalently modified by
226   HOIP, we followed up the hypothesis that NEMO increases M1-ubiquitylation at aSyn
227   aggregates. We generated NEMO KO SH-SY5Y cells by CRISPR/Cas9 and induced
228   aggregation of transiently expressed aSyn-GFP by adding aSyn seeds. NEMO KO SH-SY5Y
229   cells were reconstituted with either wildtype NEMO or Q330X NEMO and the colocalization of

230   M1-linked ubiquitin, NEMO and aSyn aggregates was quantified by the Pearson coefficient.

231   Colocalization of M1-linked ubiquitin and aSyn was strongly reduced when Q330X NEMO was

232   expressed in comparison to wildtype NEMO (Fig. 4F, G), suggesting that NEMO amplifies M1-

233   ubiquitylation at aSyn aggregates.

234

235   **A local NF-κB signaling platform is assembled at aSyn aggregates which does not**

236   **promote a functional response**

237   It has been shown recently that LUBAC is recruited to intracellular bacteria, such as

238   *Salmonella enterica*, which can escape vacuoles and invade the cytosol [48, 49, 50]. Modification

239   of the bacterial surface with linear ubiquitin chains by HOIP induces antibacterial autophagy

240   (xenophagy) and recruits NEMO for local activation of NF-κB. We therefore tested whether an

241   NF-κB signaling platform is also assembled at aSyn aggregates. Indeed, endogenous

242   phospho-IKKα/β (p-IKKα/β), p65, and phospho-65 (p-p65) were strongly enriched at aSyn

243   aggregates (Fig. 5A). However, we did not observe nuclear translocation of the NF-κB subunit

244   p65 on day 1, 2, or 3 after seeding (Fig. 5B, C). To test whether NF-κB signaling is

245   compromised in the presence of aSyn aggregates, we treated aSyn-expressing cells with TNF

246   on day 1, 2 and 3 after seeding. Whereas almost 100% of non-seeded cells showed nuclear

247   translocation of p65 in response to TNF treatment, p65 translocation was significantly reduced

248   upon seeding with only about 50% of cells positive for nuclear p65 on day 3 after seeding (Fig.

249   5B, C). Super-resolution structured illumination fluorescence microscopy (SR-SIM) revealed

250   that p65 is trapped at aSyn aggregates upon TNF stimulation, whereas in cells without aSyn

251   aggregates, TNF induced efficient nuclear translocation of p65 (Fig. 5C). Moreover, TNF-

252   induced nuclear translocation was also impaired in SH-SY5Y cells expressing Htt-Q97,

253   suggesting that pathogenic protein aggregates interfere with NF-κB signaling (Fig. 5D). These

254   results indicated that although NF-κB is locally activated at aggregates, functional NF-κB

255   signaling is impaired most probably through sequestration of p65 and possibly other NF-κB

256   pathway components at the aggregates.

257

258   **NEMO promotes autophagosomal degradation of α-synuclein in a p62-dependent**

259   **manner**

260   Our previous data indicated that NEMO is recruited to aSyn aggregates along with other NF-

261   κB signaling components without inducing a functional NF-κB response. Thus, NEMO seems

262   to have an NF-κB-independent role in proteostasis regulation. Along this line, different protein

263   aggregates accumulate in the brain of the Q330X NEMO patient, and wildtype NEMO but not

264   Q330X NEMO reduces the fraction of cells with FlucDM-EGFP aggregates. LUBAC promotes

265   the autophagic clearance of cytosol-invading bacteria[48, 49, 50], we therefore wondered whether

266   linear ubiquitin chains can influence the degradation of protein aggregates by autophagy.

267   Intracellular aSyn can be degraded by both the proteasome and lysosomes depending on its
268   posttranslational modifications, conformational state and subcellular localization [51, 52, 53]. To
269   test whether linear ubiquitylation promotes degradation of aSyn aggregates via autophagy, we
270   transiently expressed NEMO or HOIP in SH-SY5Y cells and quantified the fraction of cells with
271   aSyn aggregates 48 h after seeding. Both wildtype NEMO and wildtype HOIP, but neither
272   Q330X NEMO nor catalytically inactive C885A HOIP, decreased the number of cells with aSyn
273   aggregates (Fig. 6A, B). Notably, inhibition of lysosomal degradation by bafilomycin A1
274   abolished the ability of NEMO and HOIP to reduce the number of cells with aSyn aggregates
275   (Fig. 6A, B). We also tested the D311N NEMO mutant, which is defective in binding to M1-
276   linked ubiquitin [54, 55]. Similarly to Q330X NEMO, D311N NEMO was impaired in modification
277   with M1- and K63-ubiquitin chains (Fig. S5A, B). Also, it was not recruited to aSyn aggregates
278   (Fig. S5A, B) and did not reduce aSyn aggregates (Fig. 6A).
279   Since NEMO and HOIP apparently promote the degradation of misfolded aSyn depending on
280   lysosomal function, we aimed at uncovering the underlying mechanism. Ubiquitylation is
281   decoded and translated into cellular effects by specific ubiquitin-binding proteins. We reasoned
282   that p62/SQSTM1 might be a promising candidate to test in our paradigm, based on its key
283   role in targeting protein aggregates for selective autophagy (aggrephagy) [5, 56, 57, 58, 59]. The
284   ubiquitin-binding UBA domain of p62, which is required to shuttle cargo to the autophagic
285   machinery, binds to M1-linked ubiquitin with the strongest affinity compared to other ubiquitin
286   linkages [60]. Moreover, p62 has been reported to interact with NEMO [61, 62] and to co-localize
287   with Lewy bodies [63, 64]. We confirmed that endogenous p62 binds to aSyn aggregates in our
288   seeding model (Fig. 6C) and that the recruitment of p62 to aSyn aggregates is dependent on
289   its UBA domain (Fig. S5C). Co-immunoprecipitation experiments using cell lysates revealed
290   that in contrast to wildtype NEMO, the Q330X NEMO mutant does not interact with
291   endogenous p62 (Fig. 6D).
292   To test for a role of p62 in mediating effects downstream of linear ubiquitylation, we analyzed
293   aSyn aggregates in p62-deficient MEFs (p62 KO MEFs) expressing aSyn-GFP. Two days after
294   seeding, about 75% of aSyn-GFP expressing p62 KO MEFs displayed aSyn aggregates.
295   Restoring p62 expression in p62 KO MEFs decreased the fraction of aggregate-positive cells
296   to about 55%, similarly to the extent of aSyn aggregation observed in wildtype SH-SY5Y cells.
297   Notably, the rescue effect of p62 was dependent on its UBA domain, since expression of p62-
298   ΔUBA had no effect on the number of cells with aSyn aggregates in p62 KO MEFs (Fig. 6E).
299   Increased expression of NEMO or HOIP in p62-deficient cells revealed a p62-dependent effect
300   of both NEMO and HOIP, seen after reconstituting p62 KO MEFs with wildtype p62 in
301   comparison to p62-ΔUBA (Fig. 6E). In addition, we observed a minor p62-independent effect
302   in reducing aSyn aggregates, most probably mediated by other ubiquitin-binding autophagy
303   receptors, such as NBR1. Notably, the p62-dependent effect of NEMO was blocked by

304   bafilomycin A1, confirming that p62 decreased aSyn aggregates downstream of NEMO by

305   autophagosomal clearance (Fig. 6F).

306   Next, we wondered whether defective p62-dependent autophagosomal degradation of aSyn

307   might explain the accumulation of aSyn aggregates in the Q330X NEMO patient. We analyzed

308   brain sections from the Q330X NEMO patient by immunohistochemistry and found that

309   colocalization of p62 with aSyn-positive aggregates was significantly reduced in comparison

310   to patients suffering from other α-synucleinopathies, such as Dementia with Lewy Bodies

311   (DLB) (Fig. 7A, B). Of note, p62 signal intensity was strongly increased in the Q330X NEMO

312   patient's brain, possibly reflecting a compensatory up-regulation of p62 expression (Fig. 7C).

313   Encouraged by these findings, we wondered whether the NEMO-dependent recruitment of p62

314   to aSyn aggregates can also be seen in cellular models. We induced aggregation of transiently

315   expressed aSyn-GFP in NEMO KO SH-SY5Y cells by adding aSyn seeds. Colocalization of

316   endogenous p62 and aSyn aggregates was quantified by the Pearson coefficient, which

317   revealed a significant decrease in NEMO KO cells compared to control cells (Fig. 7D, E). In

318   conclusion, our data from human brain samples and cellular models indicated that NEMO

319   deficiency impairs the recruitment of the selective autophagy adaptor protein p62 to aSyn

320   aggregates.

321

322   **NEMO promotes p62 condensate formation at aggregates by lowering the threshold for**

323   **ubiquitin-induced phase transition**

324   When analyzing p62 recruitment to aSyn aggregates in NEMO-deficient cells by SR-SIM, we

325   noticed a different pattern of p62 localization at aggregates. Whereas p62 mostly formed foci

326   at the aggregate surface in the presence of NEMO, it showed a more uniform distribution

327   around the aggregates in the absence of NEMO (Fig. 7D, Fig. 8A, B). Notably, ubiquitin-

328   dependent p62 condensation is required for efficient autophagic clearance of cargo [65, 66, 67, 68,

329   69]. We and others recently observed that NEMO undergoes phase separation upon binding to

330   linear ubiquitin chains [70]. Since Q330X NEMO is impaired in binding to M1-linked ubiquitin, it

331   does neither phase-separate *in vitro* nor form condensates in cells [38]. We therefore speculated

332   that NEMO by interacting with M1-linked ubiquitin chains at protein aggregates may prime the

333   aggregate surface for efficient p62 condensate formation. Fluorescence recovery after

334   photobleaching (FRAP) of Halo-tagged NEMO expressed in NEMO-KO cells indicated that

335   NEMO forms a mobile phase at aSyn aggregates (Fig. 8C). We also observed by SR-SIM

336   microscopy that endogenous p62, NEMO and M1-linked ubiquitin colocalize in condensates

337   at aSyn aggregates (Fig. 8D). This observation prompted up to study a possible effect of

338   NEMO on ubiquitin-induced p62 condensation *in vitro*. We first tested whether p62 and NEMO

339   can co-condensate. Recombinant mCherry-p62 and NEMO-GFP were mixed with or without

340   recombinant tetra- or octa-M1-linked ubiquitin. Laser scanning microscopy revealed that co-

341 condensation of p62 and NEMO occurred only in presence of tetra- or octa-M1-linked ubiquitin,
342 but not in the absence of M1-linked ubiquitin (Fig. 8E). We then studied p62 condensate
343 formation dependent on the concentration of p62 and tetra- or octa-M1-linked ubiquitin (from
344 0.5 to 10 µM each) in the presence and absence of NEMO. As illustrated by phase diagrams,
345 NEMO shifted p62 phase transition to the lowest concentration of both p62 and tetra- or octa-
346 M1-ubiquitin (Fig. 8F). Thus, by co-condensation with p62 and M1-linked ubiquitin, NEMO
347 facilitates the local concentration of p62. These results provide a mechanistic explanation for
348 the impaired autophagic clearance of protein aggregates in the absence of functional NEMO
349 observed in our cellular models and the Q330X NEMO patient brain samples.
350

351 **DISCUSSION**
352 Here we demonstrate that NEMO has an NF-κB-independent role in maintaining cellular
353 proteostasis. NEMO-deficient cells accumulate misfolded proteins upon proteotoxic stress and
354 are hypersensitive to proteostasis dysregulation. The crucial role of NEMO in maintaining
355 proteostasis is confirmed by the neuropathological alterations found in the Q330X NEMO-
356 mutant patient, who shows a progressive widespread mixed brain proteinopathy with
357 predominant aSyn pathology. Studying this NEMO mutant revealed that LUBAC-mediated
358 formation of linear ubiquitin chains and binding of NEMO to linear ubiquitin chains are required
359 to promote autophagosomal degradation of misfolded aSyn through the selective autophagy
360 receptor p62. We previously reported that wildtype NEMO in contrast to Q330X NEMO has
361 the propensity to form phase-separated condensates upon binding to linear ubiquitin chains
362 [38]. Here we show that through this propensity NEMO facilitates p62-dependent aggrephagy.
363 Condensate formation with ubiquitinated cargo is a crucial event in p62-mediated autophagy,
364 since it contributes to the recruitment of the autophagic machinery [65, 66, 67, 68, 69]. Whereas the
365 mechanism of autophagy initiation by p62 has been studied in great detail, little is known about
366 processes at the aggregate interface required to locally concentrate and rearrange p62. Our
367 study identified NEMO as a major player in priming the aggregate interphase for p62
368 condensation. At least two NEMO-dependent processes seem to be relevant in this context.
369 First, NEMO amplifies linear ubiquitination at protein aggregates. This is accomplished by both
370 binding to M1-linked ubiquitin and its M1-ubiquitylation by HOIP. HOIP is recruited to protein
371 aggregates by VCP/p97 and can generate free, unanchored M1-linked polyubiquitin [37],
372 suggesting that the aggregated protein not necessarily needs to be covalently modified by
373 HOIP, since NEMO would be an available substrate. Accordingly, we found M1-linked ubiquitin
374 and NEMO at various protein aggregates, including aSyn, Htt-polyQ, tau, and TDP-43.
375 Second, binding of NEMO to M1-linked ubiquitin generates a mobile phase-separated
376 aggregate surface, facilitating the local concentration of p62 by co-condensation. Indeed,

11

377 liquidity at the cargo surface seems to be an important prerequisite for efficient selective

378 autophagy [71, 72, 73, 74, 75].

379 LUBAC-mediated quality control of cellular protein aggregates shares some similarities with

380 its role in anti-bacterial autophagy. Bacteria that escape from the vacuolar compartment into

381 the cytosol, such as *Salmonella* species, are coated by ubiquitin to restrict bacterial

382 proliferation [48, 49, 50]. In this pathway, the RNF213 ubiquitin ligase ubiquitylates the bacterial

383 outer membrane component lipopolysaccharide (LPS), which is a prerequisite for the

384 subsequent recruitment of LUBAC [50]. HOIP binds to pre-existing ubiquitin at the bacterial

385 surface and assembles M1-linked ubiquitin chains on bacterial membrane proteins or on

386 ubiquitin moieties previously attached by RNF213. Linear ubiquitin chains then recruit

387 Optineurin and NEMO to the bacterial surface, which both bind to M1-linked ubiquitin with high

388 affinity via their UBAN domain. Whereas Optineurin induces selective autophagy to promote

389 the clearance of bacteria, NEMO locally activates the IKK complex and thereby transforms the

390 bacterial surface into an NF-κB signaling platform [48, 49]. LUBAC-mediated aggrephagy differs

391 from bacterial xenophagy in some substantial aspects. First, HOIP is recruited to cytosolic

392 bacteria via its NZF domain by pre-existing ubiquitin assembled by RNF213 [50], whereas

393 VCP/p97 is required to recruit HOIP to misfolded proteins by an interaction of the PIM domain

394 of VCP/p97 with the PUB domain of HOIP [37]. Second, Optineurin is required for antibacterial

395 autophagy, whereas for the clearance of misfolded aSyn p62 plays a major role, though we

396 cannot exclude an additional role of Optineurin. Third, NEMO is apparently dispensable for

397 bacterial autophagy [48] but required for autophagic degradation of aSyn. This difference may

398 be attributable to the fact that p62 is a key cargo receptor in aggrephagy. The increased

399 abundance of p62 in the brain of the Q330X NEMO patient may reflect either a compensatory

400 upregulation by transcription factors other than NF-κB, such as NRF2 [76], or an accumulation

401 of p62 due to a decreased autophagic flux. Interestingly, the *Drosophila melanogaster* NEMO

402 homolog Kenny has a LIR motif that interacts with Atg8/LC3 and promotes the autophagic

403 degradation of the IKK complex in order to prevent constitutive production of antimicrobial

404 peptides against commensal microbiota [77]. According to a mathematical model proposed by

405 Tusco et al., host-pathogen co-evolution could have been the driving force for the loss of the

406 LIR motif in mammalian NEMO [77].

407 Even though the role of LUBAC and NEMO in protein quality control seems to be independent

408 of NF-κB signaling, impaired activation of the NF-κB prosurvival pathway may contribute to the

409 toxicity of protein aggregates. Whereas prolonged and excess NF-κB activation is associated

410 with inflammation, constitutive NF-κB signaling in neurons regulates synaptic plasticity and

411 neuronal viability [78, 79, 80, 81, 82]. Moreover, induced NF-κB activation protects from neuronal cell

412 death in various stress conditions [82, 83, 84, 85]. Of note, several neurotrophic proteins, such as

413 NGF, BDNF and GDNF, signal via NF-κB to promote neuronal viability [86, 87, 88, 89, 90]. Our data

12

414   indicate that similarly to intracellular bacteria, an NF-κB signaling platform is assembled at

415   aSyn aggregates. However, this signaling platform is not functional, since p65 seems to be

416   sequestered and trapped at the aggregates so that not even an additional NF-κB-activating

417   stimulus, like TNF, promotes nuclear translocation of p65. Thus, prosurvival signals cannot

418   efficiently be transduced via NF-κB in cells with aSyn or Htt-polyQ aggregates. It is noteworthy

419   in this context that our study revisits an earlier finding linking NEMO to Parkinson's disease.

420   We previously discovered that linear ubiquitination of NEMO is a prerequisite for Parkin to

421   prevent stress-induced neuronal cell death. This activity of Parkin was associated with adding

422   K63-linked ubiquitin to NEMO, suggesting that Parkin can act as a priming E3 ligase for

423   subsequent modification of NEMO by LUBAC [91, 92]. Whether Parkin also plays a role in LUBAC-

424   mediated protein quality control is an interesting question to be addressed in further studies.

425   Finally, our study adds to the notion that different neurodegenerative diseases share common

426   pathways. The accumulation of various proteins linked to neurodegeneration in the brain of

427   the Q330X NEMO patient reflects a general neuronal proteostasis dysregulation, which at least

428   partially can be explained by defective p62-mediated selective autophagy. The presence of α-

429   synuclein, tau and TDP-43 aggregates has also been reported in some patients with *OPTN*

430   mutations affecting the UBAN domain of Optineurin, which binds linear ubiquitin chains [93].

431   Thus, neurodegeneration in these patients may also be linked to defective protein quality

432   control downstream of LUBAC. Shared pathomechanisms related to proteostasis

433   dysregulation may entail specific targets for disease-modifying strategies. Further studies need

434   to address whether the linear ubiquitination machinery can be exploited for therapeutic

435   approaches.

436

437   **MATERIALS AND METHODS**

438   **DNA constructs**

439   The following constructs were described previously: human HOIP, HOIL-1L, SHARPIN, HA-

440   HOIP, HOIL 1L-HA, SHARPIN-HA, HA-HHARI, FLAG-NEMO wt, FLAG-NEMO D311N, HA-

441   IKKβ, mCherry, NF-κB-luciferase reporter [91]; HA-HOIP C885A, Htt-Q25-GFP, Htt-Q97-GFP [37];

442   p62 and p62ΔUBA [57]. HA-NEMO wt and HA-NEMO D311N were generated using the following

443   primers: HA-NEMO-fwd: 5'-ATATGGATCC AATAGGCACCTCTGGAAGAGC-3', HA-NEMO-

444   rev: 5'-ATATGCGGCCGC CTACTCAATGCACTCCATGACATG-3' The amplified fragment

445   was digested with BamHI and NotI and cloned into pcDNA3.1-N-HA. HA-NEMO Q330X was

446   generated using the following primers: HA-NEMO Q330X-fwd: 5'- ATAT GGATCC

447   AATAGGCACCTCTGGAAGAGC-3',   HA-NEMO   Q330X-rev: 5'-

448   ATATGCGGCCGCTCACAGGAGCTCCTTCTTCTCGG-3'. The amplified fragment was

449   digested with BamHI and NotI and cloned into pcDNA3.1-N-HA. FLAG-NEMO Q330X was

450   generated using the following primers: FLAG-NEMO Q330X-fwd 5'- GCGC AAGCTT

451   ATGGACTACAAGGATGATGATGACAAG-3', FLAG-NEMO Q330X-rev 5'- ATAT TCTAGA

452   CTACAGGAGCTCCTTCTTCTCGGC-3' The amplified fragment was digested with HindIII and

453   XbaI and cloned into pEF4 -N-FLAG. HA-IκBα was generated using the following primers: HA-

454   IκBα-fwd: 5'- ATAT GGATCC ACCGAGGACGGGGACTCG-3', HA-IκBα-rev: 5'- ATAT

455   GCGGCCGC CTATAACGTCAGACGCTGGCC-3' The amplified fragment was digested with

456   BamHI and NotI and cloned into pcDNA3.1-N-HA. p62ΔUBA-HA (AA 1-388) was generated

457   using the following primers: p62ΔUBA-HA-fwd: 5'- ATAT GAATTC GCCACC ATG GCG TCG

458   CTC ACC GTG-3', p62ΔUBA-HA-rev 5'- TATAGCGGCCGC T GGC GGG AGA TGT GGG

459   TAC-3' The amplified fragment was digested with EcoRI and NotI and cloned into pcDNA3.1-

460   C-HA. aSyn A53T-GFP was generated using the following primers: α-Synuclein A53T-eGFP-

461   fwd: 5'- ATAT AAGCTT GCCACC ATG GAT GTA TTC ATG AAA GGA C-3', α-Synuclein

462   A53T-eGFP-rev: 5'- ATATGCGGCCGCGGCTTCAGGTTCGTAGTC-3' The amplified

463   fragment was digested with HindIII and NotI and cloned into pcDNA3.1-C-eGFP.

464

465   **Cell lines**

466   HEK293T cells (CRL-1573; American Type Culture Collection) were cultured in Dulbecco's

467   modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and

468   100 IU/ml penicillin 100 μg/ml streptomycin sulfate. SH-SY5Y (DSMZ number ACC 209), were

469   cultured in Dulbecco's modified Eagle's medium F-12 (DMEM/F12) supplemented with 15%

470   (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin 100 μg/ml streptomycin sulfate and 1%

471   non-essential amino acids. Mouse embryonic fibroblasts (MEFs) derived from wild-type or

472   NEMO KO mice [94] or p62 KO mice [95] were cultured in Dulbecco's modified Eagle's medium

473   (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 IU/ml penicillin 100

474   μg/ml streptomycin sulfate.

475

476   **Generation of NEMO CRISPR/Cas9 knockout (KO) SH-SY5Y cells and HOIP**

477   **CRISPR/Cas9 KO HeLa cells**

478   sgRNAs (RNF31-24147982 AGGGUGUUGAGGUAGUUUCG; RNF31-24147993

479   GAGCCGUGGACAGGGUGUUG; IKBKG-154552050 UGUGAGAUGGUGCAGCCCAG;

480   IKBKG-154552185 GAGGAGAAUCAAGAGCUCCG) were designed using the Synthego

481   website (www.design.synthego.com). 1.5 nmol sgRNAs were rehydrated in 50 μl nuclease-

482   free 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to a final concentration of 30 μM (30

483   pmol/μl). sgRNA and recombinant CAS9 were delivered as ribonucleoprotein (RNP)

484   complexes using a 4D-Nucleofector X-Unit (Lonza). Briefly, for the assembly of the RNP

485   complexes, Cas9 2NLS and sgRNAs were combined in Nucleofector™ solution at a molar

486   ratio of 9:1 sgRNA to Cas9 and incubated for 10 min at room temperature. The cells were

487   resuspended at a concentration of 150,000 cells/5 μl. 5 μl of the cell suspension was added to

488   the 25 µl of pre-complexed RNPs for a total transfection volume of 30 µl per reaction and

489   transferred to Nucleofector cartridges. Nucleofection was performed according to the

490   predefined protocol (CA-137 for SH-SY5Y- and CN-114 for HeLa cells) and cells were carefully

491   resuspended in each well of the Nucleocuvette™ with 70 µl of pre-warmed growth medium

492   and transferred to the pre-warmed 6-well and incubated in a humidified 37°C/5% CO_2

493   incubator. After 24 h the medium was replaced.

494   For clone screening, the cells were split into two 6-well cell culture plates, and pools were

495   analyzed by PCR and subsequent DNA sequencing. For this, primer pairs (HOIP_fwd:

496   AGTCCCACCCTCTCTCCTAG, HOIP_rev: TGTGACTGTAGCAACCTGGT, NEMO_fwd:

497   CCTGGAGCTAGGCCTTTTCA, NEMO_rev: ACTTCCTCCCCGCTAATCTG) were ordered

498   extending approx. 200-250 bp 3´and 5´ of the sgRNA binding region. To perform cell pool or

499   single clone sequencing analysis, genomic DNA was isolated using a genomic DNA extraction

500   kit (Monarch Genomic DNA Purification Kit, New England Biolabs, Frankfurt am Main,

501   Germany) and the PCR was optimized to yield a single amplicon. Following PCR product

502   purification (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel GmbH, Düren, Germany),

503   the DNA was sent for Sanger sequence analysis (Microsynth Seqlab GmbH, Göttingen,

504   Germany). The KO efficiency of the cell pools and single colony clones was determined using

505   the SYNTHEGO ICE analysis website (https://ice.synthego.com). To isolate single KO clones,

506   the KO cell pools were diluted to 1 cell/100µl and 5 cells/100µl and the dilutions were

507   distributed over several 96-well plates. 15-25 clones were grown from single cells and

508   reanalyzed using the above-mentioned process. Finally, clones with a high KO score were

509   amplified and KO efficiency was confirmed by immunoblotting.

510

511   **Recombinant expression and purification of recombinant proteins**

512   Recombinant α-synuclein expression and seeding was performed as described previously [42,

513   43]. Briefly, α-synuclein (aSyn) A53T encoded on a pT7-7 plasmid was transformed into *E. coli*

514   strain BL21 (DE3). Bacteria were grown in Terrific Broth medium supplemented with ampicillin

515   at 37°C to a density of an A600 value of 0.8-1.0, and protein expression was induced by adding

516   1 mM IPTG for 4 h at 37°C. Bacteria were harvested by centrifugation (6000 rcf, 20 min, 4 °C).

517   Pellet was resuspended in high-salt buffer (750 mM NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA,

518   protease inhibitor tablet (Roche)) and lysed by sonication at 60% power using a probe

519   sonicator (Branson sonifier 450) for a total time of 5 min (30 s pulse on, 30 s pulse off), followed

520   by boiling of the sample for 15 min. After cooling on ice, the sample was centrifuged for 20 min

521   at 6000 rcf. The supernatant was dialyzed overnight with 10 mM Tris, pH 7.6, 50 mM NaCl, 1

522   mM EDTA. Protein was concentrated with a 3.5 kDa MWCO Amicon ultracentrifuge filter

523   device (Millipore). After filtering the protein through a syringe filter (0.45 µm), the soluble

524   proteins were separated by size exclusion chromatography on a Superdex 200 column (GE

15

525   Healthcare Life Sciences). 40 2 ml fractions were collected using an ÄKTA chromatography

526   system (GE Healthcare Life Sciences). Collected fractions were analyzed by SDS-PAGE using

527   a 16% (w/v) polyacrylamide-gel followed by Coomassie staining/destaining. Fractions

528   containing aSyn A53T were combined and dialyzed over night with 10 mM Tris, pH 7.6, 25 mM

529   NaCl, 1 mM EDTA. On the next day, the combined fractions were subjected to anion-exchange

530   chromatography on two connected 5 ml HiTrap Capto Q ImpRes (GE Healthcare Life

531   Sciences) anion-exchange columns using a linear gradient, ranging from 25 mM NaCl to 1 M

532   NaCl. Forty 2 ml fractions were collected using an ÄKTA chromatography system (GE

533   Healthcare Life Sciences). Collected fractions were analyzed by SDS-PAGE using a 16% (w/v)

534   polyacrylamide-gel followed by Coomassie staining/destaining. Fractions containing aSyn

535   A53T were combined and dialyzed over night with 50 mM Tris, pH 7.5, 150 mM KCl. Protein

536   was concentrated with a 3.5 kDa MWCO Amicon ultracentrifuge filter device (Millipore) to a

537   concentration of 5 mg/ml. Aliquots of 1 ml were stored at -80 °C.

538   To prepare recombinant seeds for the induction of aSyn aggregates in SH-SY5Y cells stably

539   expressing aSyn A53T-GFP, an aliquot containing 1 ml aSyn A53T with a concentration of 5

540   mg/ml was thawed on ice and centrifuged (20,000 rcf, 30 min, 4°C). The supernatant was

541   transferred into a new tube and incubated on a thermomixer for 24 h at 37°C, 900 rpm. The

542   sample was divided into 50 µl aliquots and stored at -80°C until further use.

543   pET-Duet1-6xHis-mCherry-p62 [60] was recombinantly expressed and purified from *E. coli*

544   Rossetta (DE3) pLysS cells. Bacteria were grown in Luria broth (LB) medium until OD600 ≈

545   0.6, then induced with 0.3mM isopropylthiogalactoside (IPTG) and grown at 20°C for overnight.

546   Harvested cells were resuspended in lysis buffer 50 mM 4-(2-hydroxyethyl)-1-

547   piperazineethanesulfonic acid (HEPES) at pH 7.5, 500 mM NaCl, 10 mM imidazole, 2 mM

548    $MgCl_2$, 2 mM β-mercaptoethanol, complete protease inhibitor and DNase I and lysed by French

549   press. Lysates were cleared by ultracentrifugation at 40,000 g for 45 min at 4°C. Supernatants

550   were applied to Nickel-Nitrilotriacetic (Ni-NTA) His-Trap FF column (GE Healthcare) and

551   6xHis-tagged-mCherry-p62 was eluted via a stepwise imidazole gradient (50, 75, 100, 150,

552   200, and 300 mM). Protein-containing fractions were pooled and dialysed overnight at 4°C in

553   storage buffer containing 50 mM HEPES pH 7.5, 500 mM NaCl, 2 mM $MgCl_2$, 2 mM β-

554   mercaptoethanol. The protein was filtered using a 0.45 µm syringe, aliquoted and flash frozen

555   until further use.

556   Wildtpye NEMO-GFP and 4xM1-ubiquitin were expressed and purified as described previously

557   [38].

558   8xM1-ub was commercially bought from Enzo Life Sciences.

559

560   **Induction of α-Synuclein aggregates by α-Synuclein A53T seeds in cultured cells and**

561   **primary neurons**

562   SH-SY5Y cells stably expressing aSyn A53T-GFP or MEFs transiently expressing aSyn-
563   A53T-GFP were cultivated on glass coverslips (Laboratory Glassware Marienfeld) for
564   immunofluorescence analysis or on cell culture dishes for biochemical analysis. 24 h after
565   plating, transient transfection, or gene silencing, freshly sonicated aSyn A53T seeds were
566   added to the cells to a final concentration of 12.5 µg/ml as follows:
567   50 µl of aSyn A53T seeds were thawed at room temperature and added to 950 µl Opti-MEM
568   (Gibco) to obtain a concentration of 250 µg/ml. Sonication was performed with a probe
569   sonicator (Branson sonifier 450) for 3 min (30 % power, 10 s intervals). Sonicated seeds were
570   added to 960 µl of Opti-MEM plus 40 µl Lipofectamine 2000 (Invitrogen) in order to increase
571   uptake of seeds by the cells, to a final concentration of 125 µg/ml. After incubation for 15 min
572   at room temperature, the seed solution was added to cells to obtain a final concentration of
573   12.5 µg/ml per well in Opti-MEM. After 24 h the cells were either harvested, fixed for
574   immunofluorescence experiments, or Opti-MEM was exchanged by Dulbecco's modified
575   Eagle's medium F-12 (DMEM/F12) supplemented with 15% (v/v) FCS, 1% (v/v)
576   Penicillin/Streptomycin (Gibco) and 1% (v/v) minimum essential medium non-essential amino
577   acids (Gibco), and cells were further grown for 24 to 48 h prior to harvesting or fixing.
578   For the induction of aSyn aggregation in primary cortical mouse neurons by aSyn A53T seeds,
579   cells were plated on poly-L-lysine- and laminin-coated coverslips, and sonicated aSyn A53T
580   seeds were added on DIV 5 to a final concentration of 1 µg/ml. Primary neurons were fixed
581   with 4 % PFA 7 days after seeding and prepared for immunocytochemistry.
582

583   **Detergent solubility assay**
584   SH-SY5Y cells stably expressing aSyn A53T-GFP were grown in 3.5 cm dishes and harvested
585   in cold PBS 24, 48, or 72 h after seeding with aSyn A53T seeds. Cells were lysed 10 min on
586   ice in 1% (v/v) Triton X-100 in TBS supplemented with protease inhibitor (cOmplete, Roche)
587   and phosphatase inhibitor (PhosStop, Roche). Samples were centrifuged (15 min, 20,000 rcf,
588   4°C), the supernatants were transferred into new tubes, and 5x Laemmli sample buffer was
589   added (1 % Triton X-100-soluble fraction). The pellets were washed two times with lysis buffer,
590   solved in 1% (w/v) SDS in TBS, and boiled 15 min at 99°C. In addition, DNA was sheared by
591   passing the samples 15 times through a 23-Gauge needle. Finally, 5x Laemmli sample buffer
592   was added (1% SDS-soluble fraction). Equal amounts of the two fractions were used for
593   analysis by immunoblotting using the indicated antibodies.
594

595   **Immunocytochemistry**
596   Cells were cultivated on glass coverslips (Laboratory Glassware Marienfeld). For some
597   experiments, coverslips were coated with both poly-L-lysine (PLL) (Sigma) and laminin
598   (Sigma) or PLL only. 24–72 h after seeding with aSyn A53T seeds, the cells were fixed for 10

599   min with 4% paraformaldehyde in PBS or Tris pH 7.4, and permeabilized and blocked in 0.2%

600   (v/v) Triton X-100, 5% (v/v) goat serum in PBS or Tris for 2 h. Cells were stained with primary

601   antibodies at a dilution of 1:100 to 1:1000 in 0.2% (v/v) Triton X-100, 5% (v/v) goat serum in

602   PBS or Tris at 4°C overnight, washed 3x with PBS or Tris, and incubated with fluorescent dye-

603   conjugated secondary antibodies Alexa Fluor 488, 555, or 647 (Thermo Scientific), at a dilution

604   of 1:1000 for 1 h at room temperature. After extensive washing, cells were mounted in

605   Fluoroshield G (Thermo Scientific) with DAPI (Sigma).

606

607   **Human brain sections**

608   *Post mortem* brain samples from the Q330X NEMO patient, from DLBD patients and from

609   respective controls were provided by the Department of Pathology, University of California,

610   San Francisco, California, USA. PD brain samples were provided by the Charité

611   Universitätsmedizin Berlin, Germany. *Post mortem* brain samples of frontal isocortex from

612   neuropathologically confirmed AD, DLBD, FTLD and tauopathy patients were obtained from

613   the Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg,

614   Germany. Ethical review for the use of anonymized human post-mortem tissues was

615   performed according to the guidelines of the local ethical committees. Available data are

616   listed in the supplementary Table1.

617

618   **Immunohistochemistry**

619   For peroxidase immunohistochemistry, paraffin-embedded brain sections (5 μm) were

620   deparaffinized with xylene and ethanol and briefly washed with deionized water. Brain sections

621   were cut from archived paraffin tissue blocks obtained during routine brain autopsy after whole-

622   brain fixation in formalin for at least two weeks. Antigen retrieval through microwaving in 100

623   mM citrate buffer pH 6.0 was followed by blocking of endogenous peroxidase with 5% H_2O_2 in

624   methanol. Then sections were transferred in PBS with 0.02% Brij35 and blocked with 2% FBS

625   in PBS. Incubation with the primary antibody was performed overnight at 4°C. After rinsing with

626   0.02 % Brij35 in PBS, antibody binding was detected and enhanced by DCS Super Vision 2

627   HRP-Polymer-Kit (DCS, Germany) using the chromogen DAB. Counterstaining with

628   hematoxylin for cellular structures was performed. Microscopic images were obtained with a

629   BX50 microscope and Cell-D software (Olympus).

630   For immunofluorescence histochemistry, paraffin-embedded human brain sections were

631   deparaffinized and antigen retrieval was performed as described above. Sections were

632   blocked and permeabilized with 10% (v/v) goat serum, 0.3% (v/v) Triton X-100 in PBS for 1 h.

633   Primary antibodies at a dilution of 1:50 (α-synuclein), 1:100 (Tau, TDP-43, M1Ubi and NEMO)

634   or 1:250 (p62) in 10% (v/v) goat serum, 0.3% (v/v) Triton X-100 in PBS were incubated at 4°C

635   for 48 h. After the brain sections were washed 3x with PBS and blocked with 2% (w/v) BSA in

636   PBS for 1 h, they were incubated with fluorescent dye-conjugated secondary antibodies Alexa

637   Fluor 488 and 555 (Thermo Scientific) at a dilution of 1:1000 in 2% (w/v) BSA in PBS at room

638   temperature for 1 h. After extensive washing, sections were mounted with Prolong Gold

639   including DAPI (Thermo Fisher Scientific). Confocal images were obtained using a Zeiss

640   ELYRA PS.1 equipped with an LSM880 (Carl Zeiss, Oberkochen). Super-resolution and

641   confocal images were processed using the ZEN2.1 software (Carl Zeiss, Oberkochen).

642

643   **Fluorescence Microscopy**

644   Fluorescence microscopy was performed using a Zeiss ELYRA PS.1 system equipped with an

645   LSM880 (Carl Zeiss, Oberkochen) and a 20x/0.8, 63x/1.4 oil or 100x/1.46 oil immersion

646   objective or a C2+ system (Nikon). Super-resolution images were generated by the Structured

647   Illumination (SIM) technology using 405, 488, 561 and 647 nm widefield laser illumination. SIM

648   confocal images were processed using the ZEN Black software (Carl Zeiss, Oberkochen),

649   image data were exported using the ZEN Blue software for further use. For the analysis of

650   stained human brain sections, laser scanning microscopy was performed using the 405, 488,

651   561 and 647 nm laser illumination set in individual channels to avoid cross-talk. The pinhole

652   was adjusted to generate optical section of 2-5 μm, the acquisition settings were kept constant

653   throughout the experiment. For the analysis of p62 positive aSyn aggregates in human brain

654   sections, a 2x2 tile scan using the 20x/0.8 objective acquired and subsequently stitched with

655   ZEN Black software.

656   *Colocalization studies*

657   To investigate the colocalization of aSyn, NEMO and M1-ub, the respective cell lines were

658   transiently transfected with pcDNA3.1 aSyn A53T-GFP and seeded with aSyn seeds as

659   described above. After 48 h the cells were fixed and stained with the respective antibodies as

660   described in under Immuncytochemistry. To quantify the colocalization of NEMO and aSyn,

661   M1-ub and NEMO, and M1-ub and aSyn, samples were imaged with constant laser settings

662   and respective signal thresholds were set to specific signal intensities and kept constant

663   throughout the experiment. A contour was drawn around each protein aggregate and the

664   Pearson colocalization coefficient within the contour was plotted for each comparison.

665   *Analysis of condensate formation*

666   Fluorescent imaging laser scanning microscopy was performed using an LSM880 (Carl Zeiss,

667   Oberkochen) with a 63× oil immersion objective. A 63× NA 1.4 oil immersion objective was

668   used to record a z-stack of 67.5 × 67.5 × 10 and 0.330 μm for each optical section. The argon

669   laser power was set to 0.006% at 488 nm with pixel dwell time of 5.71 μs. During all

670   measurements, laser power, gain, and field of view were kept constant. The Z-stacks were

671   then processed to obtain maximum intensity projections.

672

Immunoprecipitations

Cells were lysed in 1% (v/v) Triton X-100 in PBS supplemented with 30 mM NEM (Sigma), protease inhibitor (cOmplete, Roche) and phosphatase inhibitor (PhosStop, Roche). The lysates were cleared by centrifugation (20,000 × g, 4°C, 15 min). Samples were incubated overnight with anti-HA magnetic beads (Thermo Scientific) or for 5 h with GFP-Trap magnetic agarose (Chromotek) at 4°C under rotation. Beads were washed five times with 1% (v/v) Triton X-100 in PBS. Immunopurified proteins were eluted by adding 2x Laemmli sample buffer and boiling for 10 min at 95°C. Samples were analyzed by immunoblotting using the indicated antibodies.

P65 translocation assay

SH-SY5Y cells stably expressing α-Synuclein A53T-GFP were plated on cover slips, and after 24h they were treated with α-Synuclein A53T seeds (+ seeds) or PBS as a control (- seeds). One, two, and three days post-seeding, cells were treated with 25 ng/ml TNF (PeproTech) for 15 min as indicated and fixed with PFA. Samples were analyzed by immunocytochemistry using an antibody for p65. Nuclear translocation was quantified using the green, red, and DAPI channel of a fluorescence microscope (Nikon Eclipse E400).

Quantification of aSyn A53T-GFP aggregates

After preparation of the samples on coverslips, they were analyzed using the green, red, and DAPI channel of a fluorescence microscope (Nikon Eclipse E400). Cells containing aggregates or fibrillar structures of aSyn A53T-GFP were counted positive, cells with neither aggregates nor fibrillar structures but with cytosolic and nuclear GFP staining were counted negative. When plasmids were transiently transfected during sample preparation, only cells positive for the transfected construct were considered. For quantification of the percentage of cells with aggregates five independent experiments were performed, and at least 150 cells were analyzed per condition for each replicate.

Sedimentation assay

An aliquot of both aSyn A53T seeds and aSyn A53T monomers was thawed at room temperature. 10 µl of each sample was centrifuged for 10 min at 20,000 rcf, respectively. The supernatant was transferred into a new tube and 10 µl of 2x Laemmli sample buffer was added (sup). The pellets were resuspended in 200 µl PBS and centrifuged again (20,000 rcf, 10 min). Supernatants were discarded and 10 µl PBS and 10 µl 2x Laemmli sample buffer were added to the pellets (pellet). Sup and pellet fractions of both seeds and monomeric aSyn A53T were analyzed by SDS-PAGE and Coomassie staining/destaining.

Thioflavin T assay

An aliquot of both aSyn A53T seeds and aSyn A53T monomers was thawed at room temperature. For each sample 4 technical replicates were prepared: For each technical replicate either 5 µl of seeds, monomeric aSyn A53T, or PBS were pipetted into a black 96-well plate (Berthold), respectively. Samples were incubated with 95 µl of a 25 µM Thiovlavin T (Sigma-Aldrich) in PBS solution for 45 min at room temperature. Fluorometry was performed using a microplate reader (Cytation 5, BioTek, excitation 442 nm, emission 485 nm).

Atomic Force Microscopy (AFM)

AFM measurements were conducted on a Bruker Bioscope RESOLV, using Peak Force Tapping Mode at 2 kHz resonant frequency. For liquid AFM measurements a ScanAsyst Fluid+ probe from Bruker Nano was run at 0.5 nN setpoint and a peak force amplitude of 90 nm. Freshly cleaved MICA from PLANO was used as a substrate for in situ measurements. Beam alignment was done in the buffer solution. 4 µl of the target solution was drop cast on the MICA substrate, shortly after (max. 10 s) the volume was filled with 2 ml buffer solution. For dry AFM measurements a ScanAsyst Air probe from Bruker Nano was run at 1.3 nN setpoint and a peak force amplitude of 150 nm. A silicon wafer from PLANO was used as a substrate for dry measurements. 4 µl of the target solution was spin coated on the Si-substrate at 1000 rpm and air counter flux.

Dynamic Light Scattering (DLS)

DLS measurements were performed on a Malvern Instruments Zetasizer Ultra (633 nm laser source), in a 20 µl quartz cuvette (ZEN2112). The displayed data were recorded over three cycles in back-scattering mode. The total acquisition time was 2 s. Attenuation and position where fixed by the device automatically. The refractive index and absorbance of polystyrene was used.

Analysis of Proteotoxic Stress

Immunocytochemistry

MEFs were cultivated on glass coverslips (Laboratory Glassware Marienfeld). 24 h after seeding, cells were heat stressed at 42°C for 1 h or treated with 0.5 µM MG-132 for 48 h. Cells were fixed for 15 min with 4 % paraformaldehyde in PBS pH 7.4 and permeabilized with 0.5% (v/v) Triton X-100, 3 mM EDTA pH 8.0 and 5% goat serum in PBS for 30 min at room temperature and then stained with Proteostat® (Enzo Life Sciences, Inc.) at a dilution of 1:2000 in 10x assay buffer (ENZO) for 20 min. Cells were then mounted in Fluorshield with DAPI (Sigma). Fluorescence microscopy was performed using a Zeiss ELYRA PS.1 equipped with an LSM880 (Carl Zeiss, Oberkochen) with a 63 x oil immersion objective. Super-resolution

21

747   images were generated by the Structured Illumination (SIM) technology. SIM confocal images

748   were processed using the ZEN2.1 software (Carl Zeiss, Jena). Data represent the mean ± SD

749   from n = 4 biological replicates. At least 250 cells were assessed per condition. Statistical

750   analysis was carried out using the two-way ANOVA followed by Bonferroni´s multiple

751   comparison test to determine significant differences between samples (significance levels: *p

752   ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

753   *Trypan blue dye exclusion*

754   Cells were seeded in a 6-well dish at a density of ~500,000 cells. After 24 h of seeding, cells

755   were either heat stressed at 46°C for 1 h or treated with MG-132 for 16 h (2 µM), or 24 h (2

756   µM), or 48 h (0.5 µM) and and trypsinized. The cell suspension was centrifuged for 5 min at

757   100 x g and the cell pellet was then resuspended in PBS. 1 part of cell suspension was mixed

758   with 1 part of trypan blue dye. Trypan blue dye only permeates damaged cell membranes.

759   Therefore, the unstained (viable) and stained (nonviable) cells were counted separately using

760   the hemocytometer. To obtain the total number of viable cells per ml of aliquot, the total number

761   of viable cells were multiplied by 2 (the dilution factor for trypan blue). To obtain the total

762   number of cells per ml of aliquot, the total number of viable and nonviable cells were added up

763   and multiplied by 2. The percentage of viable cells were calculated as follows: Cell viability (%)

764   = (total number of viable cells per ml of aliquot/total number of cells per ml) *100. Data

765   represent the mean ± SD from n ≥ 5 biological replicates. Statistical analysis was carried out

766   using the two-way ANOVA followed by Bonferroni´s multiple comparison test to determine

767   significant differences between samples (significance levels: *p ≤ 0.05; **p ≤ 0.01; ***p ≤

768   0.001).

769   *Immunoblotting*

770   Proteins were size-fractionated by SDS-PAGE (16% or 8% polyacrylamide) and transferred to

771   nitrocellulose by electroblotting. The nitrocellulose membranes were blocked with 5% non-fat

772   dry milk in TBST (tris-buffered saline (TBS) containing 0.1% Tween 20) for 30 min at room

773   temperature and subsequently incubated with the primary antibody against Poly (ADP-ribose)

774   polymerase (PARP) or active caspase-3 in TBST for 16 h at 4°C. After extensive washing with

775   PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary

776   antibody for 1 hour at room temperature. Following washing with PBST, the antigen was

777   detected with the enhanced chemiluminescence detection system.

778   **Phase separation assay**

779   Protein aliquots were thawed on ice. Using Vivaspin 500 columns with 30 or 10 kDa molecular

780   weight cut off (Sartorius Stedim Biotech), the buffer was exchanged to 10 mM Tris, pH 7.4, 1

781   mM DTT by centrifuging five to eight times for 9 min at 12,000 g, 4°C. After buffer exchange,

782   protein was collected and finally centrifuged at 20,000 g for 10 min at 4°C to remove

783   aggregates The final protein concentration was determined by NanoDrop 2000. TEV protease

784 was added to the samples and incubated 1 h for complete cleavage of MBP and 6xHis before

785 microscopy. After the reaction, 10 µl of reaction mix was spotted on ibidi coverslip bottom

786 dishes. The samples were then imaged as maximum intensity projection of a z-stack obtained

787 using laser scanning microscopy.

788

789 **Antibodies**

790

Antibody	source	Identifier
Mouse monoclonal anti-HA	BioLegend	Cat# 901502, RRID: AB_2565007
Rabbit polyclonal anti-IKBKG/NEMO	Sigma-Aldrich	Cat# HPA000426, RRID: AB_1851572
Mouse monoclonal anti-IKKγ/NEMO	Cell Signaling Technology	Cat#2695, RIDD: N/A, clone: DA10-12
Rabbit anti-IKKγ/NEMO	Abcam	Ab178872
Rabbit monoclonal anti-linear ubiquitin	Millipore	Cat# MABS199, RRID: AB_2576212
Human monoclonal anti-linear ubiquitin	Genentech	clone: 1F11/3F5/Y102L, [44]
Mouse monoclonal anti-GFP	Thermo Fisher Scientific	Cat# 14-6674-82, RRID: AB_2572900
Mouse monoclonal anti-α-synuclein	BD Biosciences	Cat# 610787, RRID: AB_398108
Rabbit monoclonal anti-α-synuclein, (phospho Ser129)	Abcam	Cat# ab51253, RRID: AB_869973
Mouse anti-α-synuclein	Santa Cruz Biotechnology	Sc-12767
Mouse anti-phospho-tau	Invitrogen	Cat# MN1020
Mouse anti-TDP-43	Proteintech	Cat# 60019-2-Ig
Rabbit anti-TDP-43	Proteintech	Cat#12892-1-AP
Mouse monoclonal anti β-actin	Sigma-Aldrich	Cat# A5316, RRID: AB_476743
Rabbit polyclonal anti-phospho-IKK α/β (Ser176, Ser180)	Thermo Fisher Scientific	Cat# 710676, RRID: AB_2532752
Rabbit monoclonal anti-NF-κB p65	Cell Signaling Technology	Cat# 8242, RRID: AB_10859369
Rabbit monoclonal anti-phospho-NF-kB p65 (Ser536)	Thermo Fisher Scientific	Cat# MA5-15160, RRID: AB_10983078
Rabbit polyclonal anti-p62 / SQSTM1	MBL International	Cat# PM045, RRID: AB_1279301
Mouse monoclonal anti- beta III Tubulin - Neuronal Marker	Abcam	Cat# ab78078, RRID: AB_2256751
Rabbit polyclonal anti-HOIP	Bethyl	Cat# A303-560A, RRID: AB_10949139
Mouse monoclonal anti-IκBα	Cell Signaling Technology	Cat# 4814, RRID: AB_390781
Mouse monoclonal anti-DYKDDDDK Tag (FLAG)	Cell Signaling Technology	Cat# 8146, RRID: AB_10950495
Rabbit monoclonal anti-cleaved caspase-3	Cell Signaling Technology	Cat# 9664
Rabbit polyclonal anti-PARP	Cell Signaling Technology	Cat# 9542
Rabbit monoclonal anti-K63 Ubiquitin	Millipore	Cat# 05-1308, RRID: AB_1587580
Rabbit monoclonal anti-K48 Ubiquitin	Cell Signaling Technology	Cat# 8081, RRID: AB_10859893
Mouse monoclonal anti-pan-Ubiquitin	Santa Cruz Biotechnology	Cat# sc-8017, RRID: AB_628423

791

800

801 **AUTHOR CONTRIBUTIONS**

802 **Nikolas Furthmann:** Conceptualization; Data curation; Formal analysis; Validation;

803 Investigation; Visualization; Methodology; Writing—original draft;

23

Lena Angersbach: Conceptualization; Data curation; Formal analysis; Validation; Investigation; Visualization; Methodology;

Verian Bader: Conceptualization; Data curation; Formal analysis; Validation; Investigation; Visualization; Methodology; Writing—original draft;

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Carsten Saft: Resources;

Gisa Ellrichmann: Resources;

Arend Koch: Resources;

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Marcus Glatzel: Resources; Writing – review & editing

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CONFLICT OF INTEREST

RBD is a scientific advisor for Immagene B.V.

FIGURE LEGENDS

841 **Figure 1. NEMO is associated with pathological protein aggregates.**

842 **A. Widespread mixed brain proteinopathy in a patient expressing mutant Q330X NEMO.**

843 Both low and high magnification images show the presence of aggregated proteins, such as

844 α-synuclein, hyperphosphorylated tau, TDP-43, and amyloid beta in different brain regions.

845 Many structures resembling Lewy body and Lewy neurites in pigmented neurons in the

846 substantia nigra pars compacta (SNpc) are positive in immunostaining for α-synuclein,

847 hyperphosphorylated tau, and ubiquitin.

848 **B. Domain structure of wildtype (WT) human NEMO and mutant Q330X NEMO.** DD,

849 dimerization domain; CC1, coiled coil 1 domain; CC2, coiled coil 2 domain; UBAN, ubiquitin

850 binding in ABIN and NEMO; LZ, leucine zipper; ZF, zinc finger.

851 **C, D. M1-linked ubiquitin and NEMO colocalize with α-synuclein, tau, and TDP-43**

852 **aggregates in human brain.** Immunofluorescent stainings of cortical or midbrain sections

853 from patients with Parkinson's disease (PD), Alzheimer´s disease (AD), or frontotemporal

854 dementia (FTD). Brain sections were stained with antibodies against M1-ubiquitin (C), NEMO

855 (D), and α-synuclein (PD), tau (AD), or TDP-43 (FTD). Scale bar, 10 µm.

856

857 **Figure 2. NEMO protects from proteotoxic stress.**

858 **A, B. NEMO-deficient cells are prone to protein aggregation under proteotoxic stress.**

859 **A.** NEMO wildtype (WT) and knockout (KO) mouse embryonic fibroblasts (MEFs) were heat

860 stressed (42°C, 1 h) or treated with the proteasomal inhibitor MG-132 (0.5 µM , 48 h) and then

861 stained by Proteostat® to detect protein aggregates. Scale bar: 20 µm **B.** Cells positive for

862 aggregates were quantified. All data are displayed as mean ± SD based on 4 independent

863 experiments, analyzed by two-way ANOVA followed by Bonferroni´s multiple comparison test.

864 At least 250 cells were assessed per condition.

865 **C, D. Wildtype NEMO but not Q330X NEMO decreases misfolding of the folding sensor**

866 **FlucDM-EGFP-luciferase**. NEMO KO MEFs transiently expressing FlucDM-EGFP-luciferase

867 and either wildtype (WT) NEMO or Q330X NEMO were subjected to a heat stress (HS, 43˚C,

868 20 min) 48 h after transfection or left untreated. **C.** The cells were then analyzed by

869 immunocytochemistry and fluorescence microscopy. Shown is the fraction of NEMO-

870 expressing cells with EGFP-positive foci. Data represent mean ± SEM based on 3 independent

871 experiments. At least 900 transfected cells have been analyzed per condition. Statistics: one-

872 tailed Mann Whitney U-tests *$p \geq 0.05$. **D.** In parallel, luciferase activity of control and heat

873 stressed cells were analyzed luminometrically. Data represent mean ± SEM based on 7

874 independent experiments. Statistics: One-way ANOVA with Bonferroni's Multiple Comparison

875 posthoc test; ***$p \leq 0,001$.

876 **E, F. Transient heat stress does not activate NF-κB signaling. E.** HEK293T transiently

877 expressing an NF-κB luciferase reporter construct were heat stressed for 20, 40, or 60 min

878 (42°C) and 8 h later luciferase activity was quantified. As a positive control, one set of cells

879 was treated with TNF (10 ng/ml, 8 h). Data are shown as normalized mean ± SD based on 4

880 independent experiments. **F.** SH-SY5Y cells were heat stressed (42°C) for the indicated time

881 and then nuclear translocation of the NF-κB subunit p65 was analyzed by

882 immunocytochemistry and fluorescence microscopy using antibodies against p65. As a

883 positive control, one set of cells was treated with TNF (25 ng/ml, 15 min).

884

885 **Figure 3. NEMO and LUBAC components are recruited to aSyn aggregates.**

886 **A. M1-linked ubiquitin is enriched at aSyn aggregates formed in the cellular aSyn**

887 **seeding model.** SH-SY5Y cells stably expressing aSyn A53T-GFP were treated with aSyn

888 A53T seeds, fixed 72 h after seeding, and analyzed by immunocytochemistry and fluorescence

889 SR-SIM using M1-ubiquitin-specific antibodies. Scale bar, 20 and 5 μm.

890 **B. M1-linked ubiquitin colocalizes with pS129-aSyn-positive neurites in primary**

891 **neurons.** Primary cortical mouse neurons were treated with aSyn A53T seeds at day 5 *in vitro*

892 to induce aggregation of endogenous aSyn, fixed 7 days (rows 1 and 2) or 10 days (rows 3

893 and 4) after seeding, and analyzed by immunocytochemistry and fluorescence SR-SIM using

894 antibodies against pS129-aSyn, M1-linked ubiquitin, and βIII-Tubulin. Scale bar, 20 and 5 μm.

895 **C. M1-linked ubiquitin co-immunoprecipitates with aSyn.** SH-SY5Y cells stably expressing

896 A53T-GFP aSyn were treated with aSyn A53T seeds for 72 h, lysed in 1% Triton X-100 in

897 PBS, and aSyn-GFP was immunoprecipitated using GFP-trap beads. An immunoprecipitation

898 with anti-HA beads was used to control for nonspecific binding. The pellet was analyzed by

899 immunoblotting for M1-linked ubiquitin and GFP. The input was immunoblotted against aSyn

900 and β-actin.

901 **D. Endogenous NEMO is enriched at aSyn aggregates.** SH-SY5Y cells stably expressing

902 aSyn A53T-GFP were treated as described in A and analyzed by immunocytochemistry and

903 fluorescence SR-SIM using antibodies against NEMO and M1-linked ubiquitin. Scale bar, 20

904 and 5 μm.

905 **E. LUBAC components are recruited to aSyn aggregates.** SH-SY5Y cells stably expressing

906 α-Synuclein A53T-GFP were transiently transfected with plasmids encoding either HA-HOIP,

907 HA-HOIL 1L, or HA-SHARPIN, or HA-HHARI as a control. One day after transfection, the cells

908 were treated with aSyn A53T seeds, fixed 48 h after seeding, and analyzed by

909 immunocytochemistry and fluorescence SR-SIM using antibodies against the HA-tag. Scale

910 bar, 20 and 5 μm.

911

912 **Figure 4. In contrast to Q330X NEMO, WT NEMO is recruited to pathological protein**

913 **aggregates and increases the abundance of M1-linked ubiquitin**

914 **A. In contrast to WT NEMO, Q330X NEMO is not present at aSyn aggregates.** SH-SY5Y
915 cells stably expressing aSyn A53T-GFP were transiently transfected with either HA-tagged WT
916 NEMO (left panel) or HA-tagged Q330X HA-NEMO (right panel). After 24 h, the cells were
917 treated with aSyn A53T seeds, fixed 48 h after seeding, and analyzed by immunocytochemistry
918 and fluorescence SR-SIM using an antibody against the HA-tag. Scale bar, 20 and 5 µm.

919 **B. Q330X NEMO is not recruited to Htt-Q97-GFP aggregates.** SH-SY5Y cells were
920 transiently transfected with Htt-Q97-GFP and either HA-tagged WT NEMO (left panel) or HA-
921 tagged Q330X NEMO (right panel). Cells were fixed after 72 h and analyzed by
922 immunocytochemistry and fluorescence SR-SIM using antibodies against the HA-tag. Scale
923 bar, 20 and 5 µm.

924 **C. Q330X NEMO does not bind to M1-linked ubiquitin.** HEK293T cells were transiently
925 transfected with either WT HA-NEMO or Q330X HA-NEMO. After 24 h, the cells were lysed
926 and lysates were incubated with recombinant M1-linked tetra-ubiquitin (4xM1-ub) for 2 h at
927 4°C. NEMO was immunoprecipitated using anti-HA beads. Immunoprecipitated NEMO was
928 immunoblotted using antibodies against M1-linked ubiquitin. The input was immunoblotted for
929 M1-linked ubiquitin, NEMO and β-actin.

930 **D. Q330X NEMO is not M1-ubiquitinated upon TNF treatment or increased LUBAC**
931 **expression.** HEK293T cells were transiently transfected with WT HA-NEMO or Q330X HA-
932 NEMO +/- LUBAC (HOIP + HOIL-1L + SHARPIN) as indicated. After 24 h, one set of cells was
933 treated with TNF (25 ng/ml, 30 min) to stimulate linear ubiquitination. After cell lysis under
934 denaturing conditions, NEMO was immunoprecipitated using anti-HA beads.
935 Immunoprecipitated NEMO was immunoblotted using antibodies against M1-linked ubiquitin.
936 The input was immunoblotted for NEMO and β-actin.

937 **E. In contrast to WT NEMO, Q330X NEMO does not co-immunoprecipitate with**
938 **endogenous HOIP.** HEK293T cells were transiently transfected with either WT HA-NEMO or
939 Q330X HA-NEMO. After cell lysis, NEMO was immunoprecipitated using anti-HA beads. Anti-
940 c-myc beads were used to control for nonspecific binding. Immunoprecipitated NEMO was
941 immunoblotted for endogenous HOIP. The input was immunoblotted for HOIP, NEMO, and β-
942 actin.

943 **F, G. M1-ubiquitin chains at aSyn aggregates are increased by WT but not Q330X NEMO.**
944 CRISPR/Cas9 NEMO KO SH-SY5Y cells were transiently transfected with aSyn A53T-GFP
945 and either WT NEMO or Q330X NEMO. One day after transfection, the cells were treated with
946 aSyn A53T seeds, and fixed 48 h after seeding and analyzed by immunohistochemistry and
947 fluorescence SR-SIM using anti-antibodies against aSyn, M1-ubiquitin and NEMO.
948 Colocalization of M1-ubiquitin and aSyn aggregates (F) or M1-ubiquitin, NEMO and aSyn
949 aggregates (G) was quantified using the Pearson colocalization coefficient. **F**. Data are
950 displayed as mean ± SEM. n = 16, 16. Statistics: two-tailed Mann-Whitney U-test ***p ≥ 0.001.

27

951 **G.** Data are displayed as mean ± SEM. n = 18, 23. Statistics: two-tailed student´s t-test. ***p ≥
952 0.001.

953

954 **Figure 5. A local NF-κB signaling platform is assembled at aSyn aggregates that does**
955 **not promote nuclear translocation of p65.**

956 **A. IKKα/β and p65 are recruited to aSyn aggregates.** SH-SY5Y cells stably expressing aSyn
957 A53T-GFP were treated with aSyn A53T seeds, fixed on day 3 after seeding, and analyzed by
958 immunocytochemistry and fluorescence SR-SIM using antibodies against phospho-IKKα/β,
959 p65, phospho-p65, and M1-linked ubiquitin. Scale bar, 20 and 5 µm.

960 **B, C. Assembling of an NF-κB signaling platform at aSyn aggregates does not result in**
961 **p65 nuclear translocation and impairs TNF-induced NF-κB activation. B.** Samples were
962 prepared as described in A, treated with TNF for 15 min (25 ng/ml) on day 1, 2, or 3 days after
963 seeding, fixed and quantified for nuclear translocation of p65 by immunocytochemistry and
964 fluorescence confocal microscopy using antibodies against p65. For the seeded samples, only
965 cells with aSyn aggregates were used for quantification. Data represent the means ± SD of
966 five independent experiments. Statistics: One-way ANOVA followed by Tukey's multiple
967 comparison test. **C.** Representative immunofluorescence images of the experiment described
968 in B (day 2 after seeding). Scale bar, 20 µm.

969 **D. TNF-induced p65 nuclear translocation is impaired in cells with Htt-polyQ**
970 **aggregates.** SH-SY5Y cells were transiently transfected with GFP-tagged Htt-25Q or Htt-Q97
971 and either vector (co), wildtype HOIP or catalytically inactive C885A HOIP, as indicated. On
972 day 3 after transfection, the cells were treated with TNF (20 ng/ml, 20 min) and nuclear
973 translocation of p65 was analyzed by as described in B. Expression of HOIP was analyzed by
974 immunoblotting, actin was used as input control. Data represent the means ± SD of three
975 independent experiments each performed in triplicates. At least 600 transfected cells were
976 assessed per condition. Statistics: One-way ANOVA with Tukey's Multiple Comparison
977 posthoc test; ***p ≤ 0,001.

978

979 **Figure 6. NEMO decreases the number of cells with aSyn aggregates in a p62-dependent**
980 **manner.**

981 **A. WT NEMO but neither Q330X NEMO nor D311N NEMO decreases the number of cells**
982 **with aSyn aggregates.** SH-SY5Y cells stably expressing aSyn A53T-GFP were transiently
983 transfected with either HA-NEMO, Q330X HA-NEMO, D311N HA-NEMO, or mCherry as a
984 control and treated with aSyn A53T seeds 24 h after transfection. The cells were treated 16 h
985 after seeding with Bafilomycin A1 (Baf, 25 nM). 40 h after seeding, the cells were fixed and
986 analyzed by immunocytochemistry and fluorescence microscopy using anti-HA antibodies.
987 The fraction of HA- or mCherry-positive cells containing aSyn-GFP aggregates was quantified.

988   Data are shown as mean ± SD based on 5 independent experiments. At least 750 cells per

989   condition were quantified. Statistics were applied to the entire dataset. For better comparability,

990   the control is shown for each NEMO construct. Statistics: One-way ANOVA followed by

991   Tukey's multiple comparison test. ***p ≤ 0,001.

992   **B. Catalytically active HOIP decreases the number of cells with aSyn aggregates.** SH-

993   SY5Y cells stably expressing aSyn A53T-GFP were transiently transfected with either WT HA-

994   HOIP, catalytically inactive C885A HA-HOIP, or mCherry as a control, and treated as

995   described in A. The fraction of HA- or mCherry-positive cells containing aSyn-GFP aggregates

996   was quantified. Data are shown as mean ± SD based on 5 independent experiments. At least

997   750 cells per condition were quantified. Statistics were applied to the entire dataset. For better

998   comparability, the control is shown for each NEMO construct. Statistics: One-way ANOVA

999   followed by Tukey's multiple comparison test. ***p ≤ 0,001.

1000   **C. p62 is present at aSyn aggregates.** SH-SY5Y cells stably expressing aSyn A53T-GFP

1001   were treated with aSyn A53T seeds, fixed 72 h after seeding, and analyzed by

1002   immunocytochemistry and SR-SIM fluorescence microscopy using anti-p62 antibodies. Scale

1003   bar, 20 and 5 µm.

1004   **D. p62 co-immunoprecipitates with WT NEMO but not with Q330X NEMO.** HEK293T cells

1005   were transiently transfected with either WT HA-NEMO, Q330X HA-NEMO, or WT FLAG-

1006   NEMO to control for unspecific binding. After cell lysis, HA-tagged proteins were

1007   immunoprecipitated using anti-HA-beads. Immunoprecipitated proteins were immunoblotted

1008   using antibodies against p62, M1-linked ubiquitin, and NEMO. The input was immunoblotted

1009   for p62, NEMO, and β-actin.

1010   **E. NEMO and HOIP reduce the number of aSyn aggregates in a p62-dependent manner,**

1011   **which requires the UBA domain of p62.** p62 KO MEFs were transiently transfected with

1012   aSyn A53T-GFP, HA-NEMO, HA-HOIP or mCherry as a control, and p62 or p62ΔUBA, as

1013   indicated. One day after transfection, the cells were treated aSyn A53T seeds, and fixed 48 h

1014   after seeding. The fraction of cells containing aSyn aggregates was quantified as described in

1015   A. Statistics: One-way ANOVA followed by Tukey's multiple comparison test. **p ≤ 0,01, ***p

1016   ≤ 0,001.

1017   **F. The p62-dependent effect of NEMO on aSyn aggregates is sensitive to lysosomal**

1018   **inhibition.** p62 KO MEFs were transiently transfected with aSyn A53T-GFP and HA-NEMO,

1019   or HA-NEMO and p62, or mCherry as a control, as indicated. The next day, cells were treated

1020   with aSyn A53T seeds, and after 16 h treated with Bafilomycin A1 (Baf, 25 nM). 40 h after

1021   seeding, cells were fixed and the fraction of cells containing aSyn aggregates was quantified

1022   as described in A. Statistics: One-way ANOVA followed by Tukey's multiple comparison test.

1023   **p ≤ 0,01, ***p ≤ 0,001.

1024

Figure 7. p62 binds to aSyn aggregates in a NEMO-dependent manner.

A, B, C. Colocalization of p62 and aSyn aggregates is decreased in the Q330X NEMO patient brain despite increased p62 expression. A. Paraffin-embedded brain sections from control, DLBL (Dementia with Lewy Bodies) or the Q330X NEMO patient brain were analyzed by immunohistochemistry and fluorescence SR-SIM using antibodies against aSyn and p62. Scale bar, 200 μm **B.** Foci staining positive for both aSyn and p62 were quantified in 28-30 fields of view per brain section. **C.** Foci staining positive for p62 only were quantified in 10 fields of view per brain section. Data are displayed as mean ± SEM. Statistics: Kruskal-Wallis test followed by Tuckey's multiple comparison test. ***$p \geq 0.001$.

D, E. Colocalization of p62 and aSyn aggregates is decreased in NEMO KO SH-SY5Y cells. D. CRISPR/Cas9 NEMO KO or WT SH-SY5Y cells were transiently transfected with aSyn A53T-GFP. One day after transfection, the cells were treated aSyn A53T seeds, and fixed 48 h after seeding and analyzed by immunohistochemistry and fluorescence SR-SIM using anti-antibodies against aSyn and p62. Scale bar = 10 μm **E.** Colocalization of aSyn-GFP and endogenous p62 was quantified using the Pearson colocalization coefficient. Data are displayed as mean ± SEM. n = 18-23 Statistics: two-tailed student´s t-test. **$p \geq 0.01$.

Figure 8. NEMO promotes p62 local concentration by co-condensation with M1-linked ubiquitin.

A, B. Foci-like concentration of p62 at aSyn aggregates is reduced in NEMO-deficient cells. **A.** CRISPR/Cas9 NEMO KO or WT HeLa cells were transiently transfected with aSyn A53T-GFP. One day after transfection, the cells were treated with aSyn A53T seeds, fixed 48 h after seeding and analyzed by immunohistochemistry and fluorescence 3D SR-SIM using anti-antibodies against p62. Scale bar, 1 μm. Coverage of aSyn-GFP aggregates by p62 was analyzed using the Imaris 10.0 surface modules and a surface-to-surface MatLab Plugin. **B.** The p62 coverage of the reconstructed aSyn-GFP surface was quantified and plotted as percentage of the total aSyn-GFP aggregate surface. Data are displayed as mean ± SEM. n = 10-12 Statistics: two-tailed student´s t-test. **$p \geq 0.01$.

C. NEMO forms a mobile phase at aSyn aggregates. CRISPR/Cas9 NEMO KO HeLa cells were transiently transfected with aSyn A53T-GFP and NEMO-Halo7. One day after transfection the cells were incubated with aSyn A53T seeds for 48 h. For labelling of the Halo tag, the cells were incubated for 30 min with 2.5 μM TMR dye and washed for 30 min with cell culture medium prior to live cell imaging. Fluorescence recovery after photobleaching (FRAP) was performed by 5 consecutive bleaching pulses using the 561 nm laser at 100% intensity within a defined region of interest (white circle) at a aSyn-GFP protein aggregate (green outline). Fluorescence recovery was measured for 5 min and plotted as a percentage of baseline fluorescence. n = 9. Scale bar, 1 μm.

1062   **D. p62 colocalizes with NEMO and M1-linked ubiquitin at aSyn aggregates.** HeLa cells

1063   were transiently transfected with aSyn A53T-GFP. One day after transfection, the cells were

1064   treated with aSyn A53T seeds, and fixed 48 h after seeding and analyzed by

1065   immunohistochemistry and fluorescence SR-SIM using anti-antibodies against p62, NEMO

1066   and M1-ubiquitin (all at endogenous expression). Shown are representative images of p62 co-

1067   localizing with NEMO (left panel) and p62 colocalizing with M1-ubiquitin (right panel) at aSyn-

1068   GFP aggregates. Scale bar, 1 μm.

1069   **E. p62 and NEMO co-condensate in the presence of M1-linked polyubiquitin.** 2.5 μM

1070   recombinant mCherry-p62 (red) mixed with 5 μM recombinant wildtype NEMO-GFP (green)

1071   were supplemented with M1-linked ubiquitin to induce phase separation. Top lane: No M1-

1072   linked ubiquitin added. Middle lane: 2.5 μM M1-linked tetra-ubiquitin (4×M1-ub). Bottom lane:

1073   1.0 μM M1-linked octa-ubiquitin (8xM1-ub). Shown are laser scanning microscopy images.

1074   Scale bar, 10 μm.

1075   **F. NEMO reduces the threshold concentrations required for ubiquitin-dependent p62**

1076   **phase separation.** Phase diagrams depicting concentration-dependent phase separation of

1077   p62 and M1-linked octa- or tetra-ubiquitin (8×M1-ub or 4×M1-ub) with or without 5 μM

1078   recombinant wildtype NEMO. p62 was incubated in presence of recombinant M1-linked

1079   ubiquitin at the concentrations indicated and analyzed by laser scanning microscopy. Black

1080   empty circles: no phase separation; red/yellow solid circles: phase separation.

1081

1082   **Figure S1. The Q330X NEMO mutant is defective in NF-κB signaling.**

1083   **A, B. The Q330X mutation disrupts binding of NEMO to IκBα but not to IKKβ.** HEK293T

1084   cells were transiently transfected with wildtype FLAG-NEMO or Q330X FLAG-NEMO and HA-

1085   IκBα (A) or HA-IKKβ (B) as indicated. One day after transfection, the cells were lysed and HA-

1086   tagged proteins were immunoprecipitated using anti-HA-beads followed by immunoblotting

1087   using antibodies against NEMO. The input was immunoblotted for NEMO, IκBα (A) or IKKβ

1088   (B) and β-actin.

1089   **C. WT NEMO but not Q330X NEMO rescues defective IκBα degradation in NEMO KO**

1090   **MEFs.** WT and NEMO KO MEFs were transiently transfected with WT FLAG-NEMO, Q330X

1091   FLAG-NEMO or luciferase as a control. One day after transfection, the cells were treated with

1092   TNF (25 ng/ml, 15min) as indicated or left untreated and analyzed by immunoblotting using

1093   antibodies against IκBα, NEMO and β-actin.

1094   **D. In contrast to WT NEMO, Q330X NEMO does not promote NF-κB transcriptional**

1095   **activity.** WT and NEMO KO MEFs were transiently transfected with an NF-κB luciferase

1096   reporter plasmid and WT FLAG-NEMO, Q330X FLAG-NEMO, or EGFP as a control. 24 h after

1097   transfection, the cells were lysed and luciferase activity was measured luminometrically using

1098   a plate reader. Data represent the means ± SEM of three independent experiments consisting

31

1099 of three technical replicates each. Statistics: Student's t-test. Lower panel: Cell lysates were

1100 immunoblotted using antibodies against NEMO and β-actin (input control).

1101

1102 **Figure S2. NEMO KO MEFs are more vulnerable to proteotoxic stress.**

1103 **A. NEMO deficiency decreases cell viability upon proteasomal inhibition.** WT and NEMO

1104 KO MEFS were treated with MG-132 for 16 h (2 µM), 24 h (2 µM), or 48 h (0.5 µM), or kept

1105 untreated. Cell viability was quantified using Trypan blue dye exclusion. Data are displayed as

1106 mean ± SD and were analyzed by two-way ANOVA followed by Bonferroni´s multiple

1107 comparison test, n=6.

1108 **B. NEMO deficiency increases apoptotic cell death upon proteasomal inhibition.** WT and

1109 NEMO KO MEFS were treated with MG-132 (2 µM, 24 h) and then analyzed by immunoblotting

1110 using antibodies against PARP and active caspase-3. The input was immunoblotted for β-

1111 actin.

1112 **C. NEMO deficiency decreases cell viability upon heat stress.** WT and NEMO KO MEFS

1113 were subjected to a heat stress (46°C, 1 h), followed by overnight recovery. Cell viability was

1114 quantified using Trypan blue dye exclusion. Data are displayed as mean ± SD and were

1115 analyzed by two-way ANOVA followed by Bonferroni´s multiple comparison test, n=5.

1116

1117 **Figure S3. aSyn A53T seeds induce aggregation of pS129-positive aSyn-GFP**

1118 **aggregates that colocalize with M1-linked ubiquitin.**

1119 **A. aSyn-GFP aggregates are phosphorylated at S129 and are modified by M1-linked**

1120 **ubiquitin.** SH-SY5Y cells stably expressing aSyn A53T-GFP were treated with aSyn A53T

1121 seeds (+ seeds) or PBS as a control, fixed 24 h, 48 h, or 72 h after seeding, and analyzed by

1122 immunocytochemistry and fluorescence SR-SIM using antibodies against pS129-aSyn and

1123 M1-linked ubiquitin. Scale bar, 20 µm.

1124 **B. aSyn A53T seeds induce aggregation and phosphorylation of aSyn-GFP at S129.** SH-

1125 SY5Y cells stably expressing aSyn A53T-GFP were treated with aSyn A53T seeds or PBS as

1126 a control and harvested 24 h, 48 h, or 72 h after seeding. The cells were subjected to a

1127 detergent solubility assay and analyzed by immunoblotting using antibodies against pS129-

1128 aSyn, aSyn, and β-actin.

1129 **C. aSyn A53T seeds induce the formation of immobile aSyn-GFP aggregates.** SH-SY5Y

1130 cells stably expressing aSyn A53T-GFP were treated with aSyn A53T seeds and GFP

1131 aggregates in living cells were analyzed by FRAP (fluorescence recovery after photobleaching)

1132 72 h after seeding. An area in the middle and at the edge of an aSyn aggregate was

1133 photobleached. Representative images are shown in the upper panel. Scale bar, 5 µm.

1134 Normalized intensities of the FRAP experiments are shown in the lower panel. All data are

1135 displayed as mean, n=3.

1136

1137 **Figure S4. Validation of aSyn A53T seeds.**

1138 **A. Sedimentation assay for monomeric aSyn A53T and aSyn A53T seeds.** *In vitro* formed

1139 aSyn A53T seeds were separated from soluble aSyn A53T monomers by centrifugation.

1140 Supernatant (sup) and pellet fractions were analyzed by SDS-PAGE and Coomassie staining.

1141 **B. aSyn A53T seeds bind to Thioflavin T.** Fluorometry of monomeric aSyn, aSyn seed or

1142 PBS as a control was performed using a microplate reader. Data are shown as mean ± SD,

1143 n=4 technical replicates for each sample.

1144 **C. Derived hydrodynamic diameter of aSyn A53T seeds measured by dynamic light**

1145 **scattering (DLS).** The size distribution of freshly sonicated aSyn A53T seeds dispersed in

1146 PBS was evaluated from the derived intensity, considering the number distribution of colloids.

1147 A mean diameter of 27.7 nm with a standard deviation of 5.7 nm was derived from a Gaussian

1148 fit.

1149 **D. Analysis of aSyn A53T seeds by atomic force microscopy (AFM).** 3D rendered image

1150 of a single aSyn A53T seed adsorbed on MICA from a freshly sonicated dispersion of aSyn

1151 A53T seeds in PBS, measured by liquid AFM in peak force mode. Rendering was done on a

1152 240x240 nm height sensor image by NanoScope Analysis software from Bruker. The

1153 measured feature exhibits a vertical length of 59 nm, a horizontal length of 41 nm and a

1154 maximum height of 10 nm.

1155 **E. aSyn size distribution of LAFM height measurements on MICA in PBS solution.**

1156 Obtained 2D mapping of the measured surface (Top: size 6.6 x 6.6 µm). Particle size

1157 distribution was derived by particle analysis mode of the Bruker Nanoscope software: Height

1158 threshold was set to > 5 nm. Statistical distribution was evaluated with a Log normal function:

1159 Mean particle diameter 32.1 ± 10.2 nm.

1160 **F. aSyn size distribution of AFM height measurements on Si-wafer after 4 µl**

1161 **dropcasting.** Obtained 2D mapping of the measured surface (Top: size 6.6 x 6.6 µm). Particle

1162 size distribution was derived by particle analysis mode of the Bruker Nanoscope software:

1163 Height threshold was set to > 16 nm. Statistical distribution was evaluated with a Lognormal

1164 function: Mean particle diameter 28.7 ± 10.0 nm.

1165

1166 **Figure S5. Impaired recruitment of D311N NEMO and p62ΔUBA to aSyn aggregates.**

1167 **A. D311N NEMO is not recruited to aSyn aggregates.** SH-SY5Y cells stably expressing

1168 aSyn A53T-GFP were transiently transfected with D311N HA-NEMO. After 24 h, the cells were

1169 treated with aSyn A53T seeds, fixed 48 h after seeding, and analyzed by immunocytochemistry

1170 and fluorescence SR-SIM using an antibody against the HA-tag. Scale bar, 20 and 5 µm.

1171 **B. Modification of NEMO Q330X with ubiquitin is impaired**. HEK293T cells were transiently

1172 transfected with plasmids encoding HA-tagged wildtype NEMO, Q330X NEMO, or D311N

1173   NEMO. Cells were lysed under denaturing conditions and the HA-tag was immunoprecipitated
1174   using anti-HA agarose; anti-myc-agarose served as a control for unspecific binding. Samples
1175   were analyzed by immunoblotting with antibodies specific for M1-, K63-, K48-linked, or pan-
1176   ubiquitin.

1177   **C. Recruitment of p62 lacking the UBA domain aSyn aggregates is impaired**. SH-SY5Y
1178   cells stably expressing aSyn A53T-GFP were transiently transfected with plasmids encoding
1179   either HA-tagged p62 or p62ΔUBA as indicated. After 24 h, the cells were treated with aSyn
1180   A53T seeds, fixed 48 h later, and analyzed by immunocytochemistry and fluorescence SR-
1181   SIM using an antibody against the HA-tag. Scale bar, 20 and 5 µm.

1182

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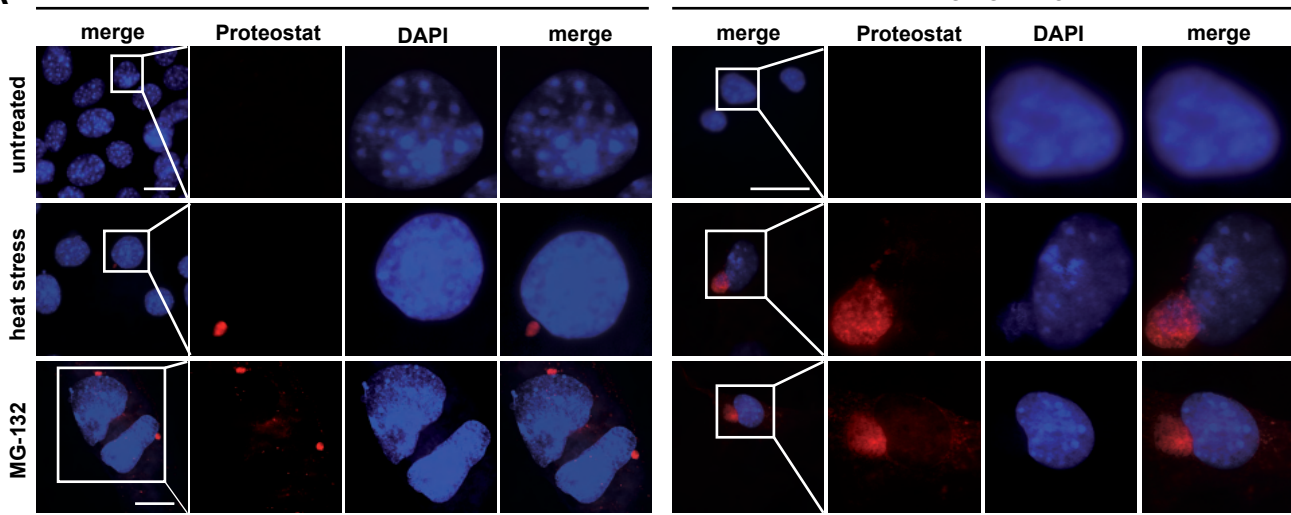1462   factor-stimulated PC12 cells. *J Neurosci* **20**, 7556-7563 (2000).

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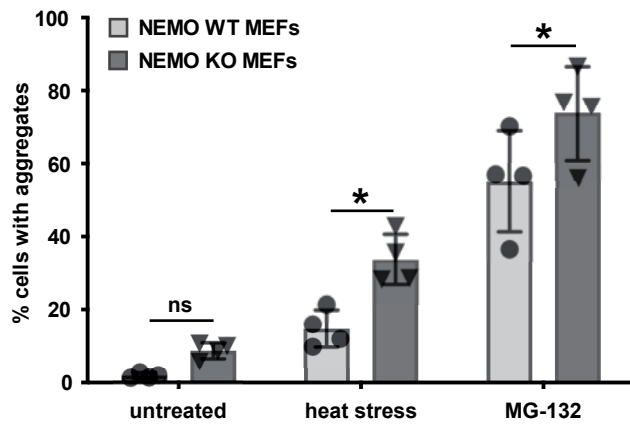1465   272-289 (1998).

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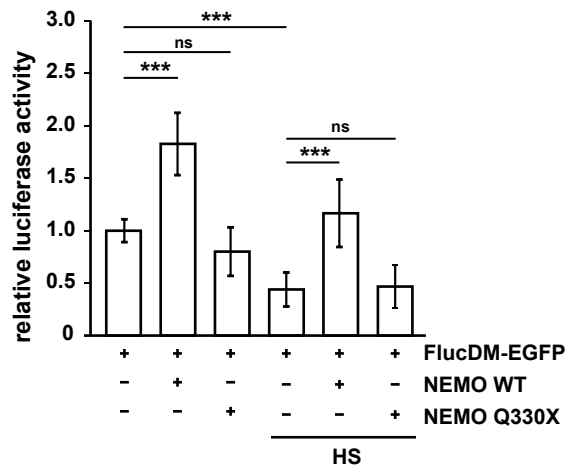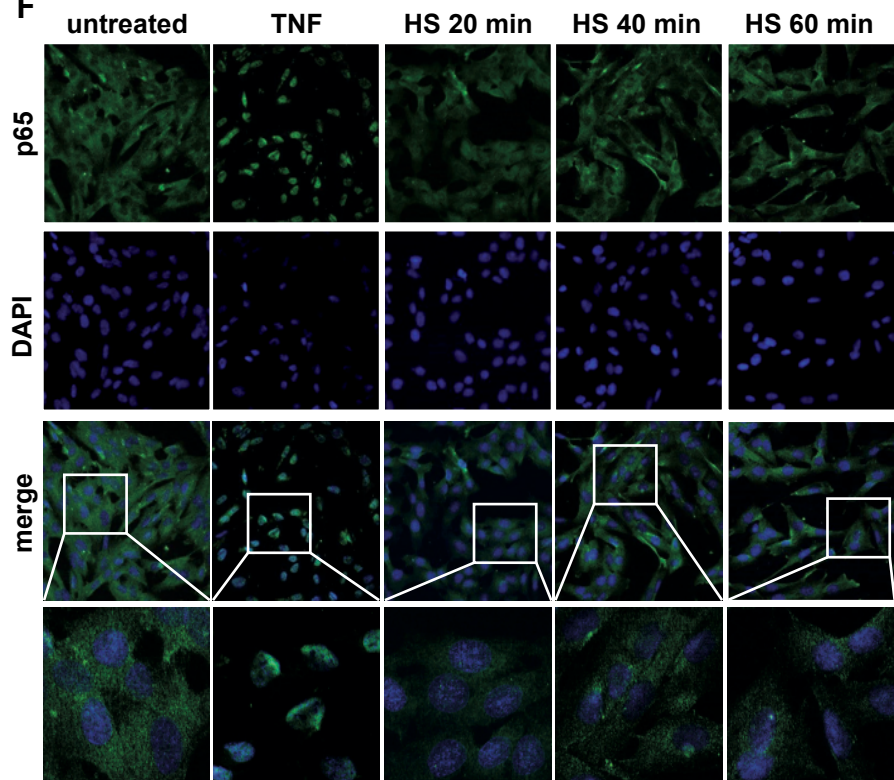1486   formation in autophagy-deficient mice. *Cell* **131**, 1149-1163 (2007).
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1488

Figure 1

Figure 2

Figure 3

Figure 4

A

| merge | aSyn-GFP | WT NEMO | DAPI | merge | merge | aSyn-GFP | Q330X NEMO | DAPI | merge |

B

| merge | Htt-Q97 | WT NEMO | DAPI | merge | merge | Htt-Q97 | Q330X NEMO | DAPI | merge |

C

4xM1-ub + + +
HA-NEMO WT − + −
HA-NEMO Q330X − − +

IP: HA

kDa
120
85
M1-ub
50
35

Input
35 — 4xM1-ub
50 — NEMO
35
50 — ß-actin

D

HA-NEMO WT + − + − + −
HA-NEMO Q330X − + − + − +
LUBAC − − + + − −
TNF − − − − + +

IP: HA

kDa
250
130
100
M1-ub
55

Input
50 — NEMO
35
50 — ß-actin

E

HA-NEMO WT − + + −
HA-NEMO Q330X − − − +

kDa
120 — HOIP

IP: HA + − + +
IP: myc − + − −

Input
120 — HOIP
50 — NEMO
35
50 — ß-actin

F

M1-ub colocalizing with
NEMO at aSyn-positive foci

Pearson colocalization coefficient [A.U.]

1.0
0.5
0.0
-0.5

***

NEMO WT + −
NEMO Q330X − +

G

M1-ub colocalizing with
aSyn-positive foci

Pearson colocalization coefficient [A.U.]

1.0
0.5
0.0
-0.5

***

NEMO WT + −
NEMO Q330X − +

Figure 5

A

B

C

D

Figure 6

Figure 7

Figure 8