Depletion of p62 reduces nuclear inclusions and paradoxically ameliorates disease phenotypes in Huntington’s model mice

Masaru Kurosawa3,4,5,8, Gen Matsumoto1,4,5,8, Yoshihiro Kino1,4,5,8, Misako Okuno4, Mizuki Kurosawa-Yamada4, Chika Washizu4, Harumi Taniguchi1,4,5, Kazuhiro Nakaso6, Toru Yanagawa7, Eiji Warabi7, Tomomi Shimogori5, Takashi Sakurai3, Nobutaka Hattori2 and Nobuyuki Nukina1,4,5,8,*

1Department of Neuroscience for Neurodegenerative Disorders, 2Department of Neurology, 3Department of Cellular and Molecular Pharmacology, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan, 4Laboratory for Structural Neuropathology, 5Laboratory for Molecular Mechanisms of Thalamus Development, RIKEN Brain Science Institute, Saitama 351-0198, Japan, 6Department of Neurology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, Tottori 683-8504, Japan, 7Faculty of Medicine, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8575, Japan and 8CREST (Core Research for Evolutionary Science and Technology), JST, Tokyo 102-0076, Japan

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INTRODUCTION

Huntington’s disease (HD) is a dominantly inherited genetic disease caused by mutant huntingtin (htt) protein with expanded polyglutamine (polyQ) tracts. A neuropathological hallmark of HD is the presence of neuronal inclusions of mutant htt. p62 is an important regulatory protein in selective autophagy, a process by which aggregated proteins are degraded, and it is associated with several neurodegenerative disorders including HD. Here, we investigated the effect of p62 depletion in three HD model mice: R6/2, HD190QG and HD120QG mice. We found that loss of p62 in these models led to longer life spans and reduced nuclear inclusions, although cytoplasmic inclusions increased with polyQ length. In mouse embryonic fibroblasts (MEFs) with or without p62, mutant htt with a nuclear localization signal (NLS) showed no difference in nuclear inclusion between the two MEF types. In the case of mutant htt without NLS, however, p62 depletion increased cytoplasmic inclusions. Furthermore, to examine the effect of impaired autophagy in HD model mice, we crossed R6/2 mice with Atg5 conditional knockout mice. These mice also showed decreased nuclear inclusions and increased cytoplasmic inclusions, similar to HD mice lacking p62. These data suggest that the genetic ablation of p62 in HD model mice enhances cytoplasmic inclusion formation by interrupting autophagic clearance of polyQ inclusions. This reduces polyQ nuclear influx and paradoxically ameliorates disease phenotypes by decreasing toxic nuclear inclusions.
linked with ubiquitin by an ubiquitin-conjugating system composed of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase). This system also ubiquitinates misfolded proteins, but misfolded proteins have a strong tendency to form inclusions that cannot be degraded by a proteasome. Macroautophagy is another degradation mechanism responsible for degradation of long-lived proteins and entire organelles. In this system, organelles and protein inclusions are surrounded by double-membraned autophagosomes, which fuse with a lysosome to degrade their contents by a rather non-selective, bulk degradation. However, another type of autophagic degradation called selective autophagy involves ubiquitin and ubiquitin-binding proteins such as p62 (3).

We previously reported that p62 is a protein that interacts with polyQ aggregates and is induced by expanded polyQ expression, suggesting that p62 is a stress-responsive protein induced by polyQ expression and proteasomal inhibition (4). p62 is also a common component of neuronal protein inclusions in neurodegenerative diseases in humans and model mice (4–8). p62 is well known as an adaptor protein that has multiple roles in the activation of the transcription factor NF-kappa B, tumorogenesis and osteoclastogenesis. p62 was recently reported to play an important role in the selective autophagy of ubiquitinated proteins. Autophagy-deficient Atg5 or 7 knockout mouse showed ubiquitin-positive inclusions (9,10) that disappeared after p62 was knocked out, suggesting that p62 could play a role in controlling intracellular inclusion body formation (11).

It was reported that autophagy inhibition increases the level of soluble UPS client proteins by slowing their clearance. This was closely associated with the accumulation of p62 (12). Furthermore, p62 knockdown in autophagy-deficient cells normalizes levels of UPS clients. From these results, p62 was suggested to be a key protein in regulating protein aggregate formation, especially in autophagy-deficient conditions. However, it remains unclear whether the decrease in p62 is beneficial or detrimental, particularly in vivo. A previous report shows that genetic inactivation of p62 in mice leads to accumulation of hyperphosphorylated tau and neurodegeneration (13). In polyQ diseases, the inclusions are mainly observed in the nuclei, where autophagy cannot work. Little is known, however, about the effect of p62 knockout for polyQ diseases in vivo. In this report, we observed the cytoplasmic accumulation of polyQ inclusions and the decrease of nuclear inclusions in polyQ model mice with the loss of p62. The detailed mechanism was investigated, in comparison with the effects of Atg5 knockout.

RESULTS

Paradoxical decrease of nuclear inclusion formation in polyQ model mice lacking p62

We first investigated pathological changes in R6/2 mice with or without p62. Anti-huntingtin (htt) antibody (EM48) revealed fewer and smaller nuclear inclusions in the brains of R6/2;p62−/− compared with those of R6/2;p62−/+ or R6/2;p62+/+ mice starting at 4 weeks of age (Fig. 1A and Supplementary Material, Fig. S1). Immunofluorescence staining of hippocampal CA1 revealed that the proportion of nuclei with inclusions in R6/2; p62−/− mice was significantly lower than that in R6/2;p62−/+ or R6/2;p62−/+ mice at each age (Fig. 1B). The average size of these nuclear inclusions became larger with age, and the inclusions in R6/2;p62−/− mice grew at a slower rate than those in R6/2;p62−/+ or R6/2;p62−/+ mice (Fig. 1C and Supplementary Material, Fig. S2). To clearly distinguish nuclear from cytoplasmic inclusions, we stained nuclear membranes with anti-Lamin B antibody (Fig. 1D).

Next, we investigated the distribution of inclusions in the striatum, which is the most affected region in HD, in R6/2;p62 and HD190QG;p62 mice (Fig. 2). In R6/2;p62 mice, anti-htt antibody (EM48) revealed significantly fewer nuclear inclusions and significantly more cytoplasmic inclusions in R6/2;p62−/− versus R6/2;p62+/+ mice starting at 4 weeks of age (Fig. 2A, C and D). In HD190QG;p62 mice, anti-htt antibody (EM48) revealed significantly fewer nuclear inclusions in HD190QG; p62−/− versus HD190QG;p62+/+ mice starting at 8 weeks of age (Fig. 2E, G and H); there were significantly more cytoplasmic inclusions in HD190QG;p62−/− versus HD190QG;p62+/+ mice (Fig. 2I). To clearly distinguish nuclear from cytoplasmic inclusions, we stained nuclear membranes with anti-Lamin B antibody (Fig. 2B and F). The expression level of mutant htt mRNA was similar in R6/2;p62−/+ and R6/2;p62−/− mice, and p62 expression was not affected by the htt transgene (Supplementary Material, Fig. S3).

When we examined the distribution of inclusions in the hippocampus, we could clearly see an increase in cytoplasmic or neurupl nuclei inclusions, especially in stratum radiatum (arrowheads) of the hippocampus of R6/2;p62−/− mice (Fig. 3A). Double immunofluorescence staining with anti-htt (N-18) and anti-MAP2 antibodies (dendritic marker) showed that part of those inclusions was localized in dendrites in the stratum radiatum of R6/2;p62−/− mice (Fig. 3B, upper panel). We further analyzed HD190QG mice, in which GFP-positive cytoplasmic inclusions are clearly observed. In HD190QG;p62−/− mice, we observed a greater number of neurupl nuclei inclusions in the molecular layer of the dentate gyrus (MolDG; arrows) as well as stratum radiatum (Rad; arrowheads) compared with HD190QG;p62+/+ mice (Fig. 3C). The inclusions in the lacunae molecular layer (LMol) of HD190QG;p62−/+ mice increased with age, but this was not observed in HD190QG;p62−/− mice. There were fewer inclusions in the hilus of dentate gyrus in HD190QG;p62−/− compared with HD190QG;p62+/+ mice at each age. Those inclusions are in the dendrites (data not shown); however, we could not reveal why this change occurred. Further investigation is necessary. Double immunofluorescence staining with anti-GFP and anti-MAP2 antibodies showed that part of those inclusions was localized in dendrites (Fig. 3D). The dendrites of CA1 pyramidal neurons are distributed throughout Rad and LMol, and the dendrites of dentate granule cells are distributed throughout MolDG, suggesting that the dendritic inclusions increased in both neurons and shifted to proximal dendrites in the case of pyramidal neurons.

We then examined whether or not the insoluble htt fragment increased by using immunoblotting to confirm the immunohistochemistry results. We used the HD190QG line because it is difficult to consistently find the htt exon 1 fragment without a tag in
SDS–PAGE, as when R6/2 brain homogenates were examined, p62 was absent in HD190QG; p62−/− mice (Fig. 4A). In the total fraction, the soluble htt-enhanced green fluorescent protein (EGFP) band showed no obvious difference between HD190QG; p62+/+ and HD190QG; p62−/− at 12 weeks of age. The hippocampal CA1 of R6/2;p62+/+ and R6/2;p62−/− was stained with EM48 and a nuclear membrane marker, Lamin B, to clearly show the nuclear inclusions. Values are means ± SEM. (*) P < 0.05, (**) P < 0.01, (***) P < 0.001), (B and C), n = 3 for each genotype (B and C). Images were captured with BZ-9000 (A) and TCS SP5 (D). Nuclei were stained with DAPI (A and D). Scale bar = 30 µm (A) and 10 µm (D).

Using the agarose gel electrophoresis for resolving aggregates (AGERA) method (14), which shows htt protein complexes with high molecular weight, we did not observe a definite difference between the htt protein complexes in the total fraction from HD190QG; p62−/− versus HD190QG; p62+/+ mice (Fig. 4B). When the nuclear fraction was separated, however, HD190QG; p62−/− mice showed a decrease in htt protein complex.
Figure 2. *p62* knockout reduced nuclear inclusions and increased extranuclear inclusions in the striatum in two different HD model mice. (A) The striatum of R6/2*+*/+ and R6/2*p62−/− mice was stained with anti-htt antibody (EM48). (B) The striatum of R6/2*p62−/+ and R6/2*p62−/− mice was stained with EM48 and a nuclear membrane marker, Lamin B, to clearly show the nuclear inclusions. (C and D) The percentage of nuclei with inclusions (C) and the number of extranuclear inclusions (D) in the striatum of R6/2*p62−/+ and R6/2*p62−/− mice, analyzed by fluorescent immunostaining with EM48. (E) The striatum of HD190QG*p62−/+ and HD190QG*p62−/− mice was stained with EM48. (F) The striatum of HD190QG*p62−/+ and HD190QG*p62−/− mice was stained with EM48 and a nuclear membrane marker, Lamin B, to clearly show the nuclear inclusions. (G and H) The percentage of nuclei with inclusions (G) and the number of the extranuclear inclusions (H) in the striatum of HD190QG*p62−/+ and HD190QG*p62−/− mice, analyzed by fluorescent immunostaining with EM48. (I) Distribution of the size of extranuclear inclusions stained with anti-GFP antibody at 24 weeks of age. Values are means ± SEM. (*P < 0.05, **P < 0.01, ***P < 0.001), (C, D, G and H). n = 3 for each genotype (C, D, G, H and I). Images were captured with BZ-9000 (A and E) and TCS SP5 (B and F). Nuclei were stained with DAPI. Scale bar = 20 μm (A and E) and 10 μm (B and F).
Figure 3. p62 knockout increased extranuclear inclusions in two different HD model mice. (A) The hippocampus of R6/2 mice with and without p62 was stained with anti-htt antibody (EM48). Extranuclear inclusions appear in the stratum radiatum of R6/2; p62−/− (arrowheads), but not in R6/2; p62+/+. Hippocampal layers are indicated: Py, stratum pyramidale; Rad, stratum radiatum; LMol, lacunosum molecular layer; MoIDG, molecular layer of the dentate gyrus; GrDG, granular layer dentate gyrus; PoDG, polymorph layer of dentate gyrus. (B) Stratum radiatum of R6/2 mice with and without p62 was stained with anti-htt (N-18) antibody (Alexa 488), anti-MAP2 antibody (Alexa 546) and DAPI. Nuclear inclusions (arrows) were observed in R6/2; p62−/+ . Extranuclear inclusions existed in dendrites of R6/2; p62−/− mice (arrowhead), but not in those of R6/2; p62+/+. (C) The hippocampus of HD190QG mice with and without p62 at 24 weeks of age was stained with anti-GFP antibody (Alexa 488) and DAPI. In HD190QG; p62−/−, extranuclear inclusions appeared in the stratum radiatum (arrowheads) and the molecular layer of the dentate gyrus (arrows). On the other hand, in HD190QG; p62+/+, extranuclear inclusions appeared in the LMol. (D) Stratum radiatum of HD190QG mice with and without p62 was stained with anti-GFP antibody (Alexa 488), anti-MAP2 antibody (Alexa 546) and DAPI. Extranuclear inclusions existed in dendrites (arrowhead). Scale bar = 50 μm (A and C) and 10 μm (B and D).
complex after 12 weeks of age (Fig. 4C), corresponding to the observed histological shift of inclusions from the nucleus to the cytoplasm.

**p62 depletion extends the life span of HD model mice**

We next examined whether p62 knockout affects the life spans of R6/2, HD190QG and HD120QG mice. The life span of R6/2; p62−/− mice significantly increased with a mean survival time of 155 ± 4 days compared with 117 ± 3 days for R6/2; p62−/+ mice and 123 ± 4 days for R6/2;p62+/+ mice (Fig. 5A). The life span of HD190QG;p62−/− mice significantly increased with a mean survival time of 265 ± 11 days compared with 184 ± 7 days for both HD190QG;p62−/+ and HD190QG;p62+/+ mice (Fig. 5B). The life span of HD120QG;p62−/− mice significantly increased with a mean survival time of 415 ± 10 days.

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**Figure 4.** Immunoblot analysis of soluble and aggregated htt in total and nuclear fraction from HD190QG mice with and without p62. Brain samples were subjected to subcellular fractionation as described in Materials and Methods. (A) Total fractions were analyzed by western blot for soluble htt. There is no change in the amount of soluble htt (arrowheads) detected by anti-GFP or 1C2 antibody from HD190QG with and without p62 at 4, 12 and 24 weeks of age. HD190QG mice have mutant htt transgenes with two different lengths of polyQ stretches. The sizes of these two bands were slightly different among HD190QG mice, because these mice show CAG-repeat length instability. The sizes of polyQ lengths of HD model mice used in this figure are described in Supplementary Material, Table S2. Knockout of p62 was confirmed with anti-p62 antibody. (B) Total fractions were analyzed by AGERA. There was no difference between the htt protein complexes in the total fraction from HD190QG;p62−/− mice and that from HD190QG;p62−/+ . (C) Nuclear fractions were analyzed by AGERA. In nuclear fractions, the amount of aggregated htt from HD190QG;p62−/− mice was reduced at 12 and 24 weeks of age compared with that from HD190QG;p62−/+. β-Tubulin was used as a marker for control protein (A–C) and Lamin B was used as a marker for nuclear membrane protein (B and C).
Figure 5, p62 knockout increased life span in three different HD model mice and body weight in R6/2 and HD190QG mice, but not in HD120QG mice. (A) Mean survival time of R6/2; p62−/− was extended by 26% compared with that of R6/2; p62+/+ . (B) Mean survival time of HD190QG; p62−/− was extended by 44% compared with that of HD190QG; p62+/+. (C) Mean survival time of HD120QG; p62−/− was extended by 27% compared with that of HD120QG; p62+/+. (D) There was no difference in mean survival time among p62−/−, p62+/+ and p62+/− mice. These survival data were analyzed by the Kaplan–Meier method followed by a log-rank test with Bonferroni correction (significance level 0.05/3 = 0.0167). Mean ages ± SEM are provided. (∗∗∗P < 0.001). (E, F and H).
compared with 294 ± 13 days for HD120QG; p62−/+ mice and 326 ± 25 days for HD120QGp62−/+ mice (Fig. 5C). There was no significant difference in life span among p62+/+, p62−/+ and p62−/− mice (Fig. 5D). p62 knockout significantly extended the life spans of HD model mice, especially in HD190QG, which has longer polyQ proteins than HD120QG and R6/2. These data suggest that p62 knockout may extend life span in a polyQ length-dependent manner, and that the extended life span is not due to the effect of p62 knockout itself.

HD model mice normally show a progressive decrease in body weight. We next examined whether p62 knockout affects the body weight of R6/2, HD190QG and HD120QG mice. A delay in body weight loss was observed in p62 knockout of R6/2 and HD190QG, but not in HD120QG, mice (Fig. 5E–G). The body weight of R6/2; p62−/− mice was significantly greater at 11 weeks of age compared with that of R6/2; p62−/+ mice (**P < 0.001) (Fig. 5E). The body weight of HD190QG; p62−/− mice was significantly greater at 18 weeks of age compared with that of HD190QG; p62−/+ mice (P < 0.05) (Fig. 5F). p62−/− mice were reported to develop mature onset obesity (15, 16). At 20 weeks, the average body weight of p62−/− mice without htt transgene became significantly greater than that of p62−/+ and p62−/+ mice (Fig. 5H).

Nuclear inclusions decrease and extranuclear inclusions increase in autophagy-deficient HD model mice

As stated above, we showed that nuclear inclusions in HD model mice decreased and life spans increased after depletion of p62, a protein that has an important role in selective autophagy. To examine whether these phenotypes result from deficiency of selective autophagy, we generated R6/2 mice with conditional knockout of Atg5, an essential gene for autophagosome formation. A transgenic mouse expressing Cre recombinase under the control of the synapsin I promoter (SynCre), which is expressed in neuronal cells as early as E12.5 (17), was used to generate Atg5−/−; SynCre; R6/2 mice. Transgene expression in male SynCre mice occurs in germline recombination in progeny (18). The hippocampal CA3 region, in which Cre recombinase is expressed in almost all neuronal cells (19), was stained with EM48 (Fig. 6A), and the proportion of nuclei with inclusions was examined. There were significantly fewer nuclear inclusions in Atg5−/−; SynCre; R6/2 compared with R6/2 mice (Fig. 6B). Next, we used magnified immunofluorescence images with EM48, the anti-ubiquitin antibody, and p62-C antibody to characterize these inclusions. In contrast to EM48-positive nuclear inclusions in R6/2 mice (Fig. 6D—n), nuclear inclusions decreased and extranuclear inclusions increased in the number of Atg5−/−; SynCre; R6/2 (Fig. 6D—d and e). These extranuclear inclusions were subdivided into EM48-positive, ubiquitin-positive, and p62c-positive inclusions (Fig. 6D—e, arrows) and EM48-negative, ubiquitin-positive, and p62c-positive inclusions (Fig. 6D—e, arrowheads). The former inclusions could result from nucleation of mutant htt proteins followed by ubiquitination, and the latter inclusions could result from the accumulation of ubiquitinated proteins into the sequestosome that is a targeting unit for autophagosome entry. The change of inclusion formation in autophagy-deficient R6/2 mice was similar to that in p62 knockout R6/2 mice. We previously reported that selective autophagy of the ubiquitinated proteins is enhanced by S403-phosphorylation of p62 (20). Therefore, we used immunohistochemistry in R6/2, Atg5−/−; SynCre and Atg5−/−; SynCre; R6/2 mice to determine whether p62 that colocalizes with ubiquitinated inclusions is S403-phosphorylated. Both types of extranuclear inclusions colocalized with S403-phos-p62 (Supplementary Material, Fig. S4A—c), but nuclear inclusions in R6/2 mice did not (Supplementary Material, Fig. S4A—i). We further examined the life spans of these mice. Despite fewer nuclear inclusions in Atg5−/−; SynCre; R6/2 mice, the life span of Atg5−/−; SynCre; R6/2 mice was significantly shorter, with a mean survival time of 86 ± 2 compared with 108 ± 4 days for Atg5−/+; SynCre; R6/2 and 119 ± 10 days for Atg5−/+; SynCre; R6/2 mice (Fig. 6C).

p62 knockout impairs protein degradation in cytoplasm but not in nucleus

To assess whether the depletion of p62 affects protein degradation in the cytoplasm and nucleus, we prepared mouse embryonic fibroblast (MEF) p62−/+; MEF p62−/− or MEF p62−/+ expressing GFP-fused human wild-type p62 (G-p62) transfected with HD106Q-red fluorescent protein (RFP) or HD106Q-NLS-RFP. In cells transfected with HD106Q-RFP, inclusions mainly existed in the cytoplasm due to the nuclear export signal in the exon 1 of htt (21). The ratio of cells with inclusions to transfected cells was greater in MEF p62−/− than in MEF p62−/+; however, the proportion of cells with inclusions in MEF p62−/+ + G-p62 did not reach to that in MEF p62−/+ (Fig. 7A and B). This was due to the fact that the expression level of endogenous p62 in MEF p62−/+ is higher than that of transfected G-p62 (Supplementary Material, Fig. S6). On the other hand, in the cells transfected with HD106Q-NLS-RFP, inclusions appeared in the nucleus and there was no significant difference in the proportion of cells with nuclear inclusions among MEF p62−/−, MEF p62−/+ and MEF p62−/+ + G-p62. These data suggest that p62 knockout impairs protein degradation in the cytoplasm but not in the nucleus.

DISCUSSION

In this study, we report that genetic ablation of p62 in three kinds of HD model mice paradoxically ameliorates the disease phenotypes. The life span of HD model mice lacking p62 was extended by 26, 44 and 27%, compared with those with p62 (Fig. 5A–C). These extended life spans are not due to the effect of p62 knockout itself, because there was no significant difference in life span among p62−/+ and p62−/+ and p62−/− mice. Regarding the body weight of these mice, significant delays in body weight loss were observed in p62 knockout in R6/2 after 11 weeks of age and HD190QG mice after 18 weeks of age (Fig. 5E and F). On the other hand, the weight of p62−/− mice after 20 weeks of age was significantly greater than that of p62−/+ mice (Fig. 5H). Therefore, the delays in body weight loss in R6/2;p62−/− and HD190QG;p62−/− mice were not due to the increased body weight after p62 knockout; the significant increase in body weight in p62−/− mice was observed after those in R6/2;p62−/− and HD190QG;p62−/− mice. Immunohistochemical analysis revealed significantly fewer nuclear inclusions in HD model mice without p62, compared with HD model mice with p62 (Fig. 1). This was also confirmed biochemically in HD190QG using the
AGERA method (Fig. 4). It has been proposed that nuclear mutant htt may be toxic (22,23); therefore, the extended life span may be explained by a decrease in toxic nuclear mutant htt.

We also performed microarray analysis to examine whether transcriptional abnormalities in R6/2 changed by p62 depletion. When comparing R6/2; p62+/+ with R6/2; p62−/−, we only...
found 12 genes with differential expression (five up-regulated and seven down-regulated; Supplementary Material, Fig. S5C and E). Moreover, we performed quantitative PCR of genes with previously known alteration in HD mice (Supplementary Material, Fig. S5A and B) (24–26), and we only found one gene, Agxt2l1, the expression level of which recovered by p62 depletion in R6/2; p62$^{−/−}$. Despite the decrease in nuclear inclusions, specific transcriptional changes were not observed in our experiment. In a previous study of environmental enrichment in R6/1 mice, no specific transcriptional changes were found, despite a decrease in nuclear inclusions (27). The effect of invisible aggregates on the transcriptional abnormality of early affected genes should be investigated further.

Autophagic degradation machinery works in the cytoplasm. Thus, we assumed that the decrease in nuclear inclusions correlated with an increase in cytoplasmic inclusions, which increased due to reduced protein clearance by loss of p62-dependent selective autophagy in the cytoplasm. To confirm this, we also examined R6/2 mice with Atg5 depletion, which impairs autophagy in general. Similar to HD model mice without p62, nuclear inclusions almost disappeared in neurons in the region where Atg5 was deficient (Fig. 6A and B), but the life span of R6/2 mice with Atg5 depletion was shorter than that of R6/2 mice without Atg5 depletion (Fig. 6C). Both p62 and Atg5 depletion induced decreases in nuclear inclusions. p62 controls selective autophagy and Atg5 controls more general autophagy including selective autophagy. Ubiquitinated substrates are degraded through selective autophagy, and p62 depletion results in accumulation of those selected substrates. However, Atg5 depletion might induce accumulation of more substrates in general, resulting in more toxic effects that could overcome the positive effect of decreased nuclear inclusions.

Cytoplasmic degradation of mutant htt was affected by p62 depletion, but its nuclear degradation was not (Fig. 7). Therefore, an increased accumulation of cytoplasmic mutant htt and a decrease in nuclear inclusions in vivo might be due to accelerated inclusion formation in the cytoplasm and a decrease of htt translocation into the nucleus after p62 depletion.

Similar findings to our study were observed in R6/2 mice with super-long CAG-repeat expansions (28). These mice also showed decreased nuclear inclusions, increased extranuclear inclusions and extended survival. It is thought that R6/2 mice have an upper threshold for forming nuclear inclusions, and when polyQ length is beyond the threshold, nuclear entry of mutant htt is blocked, resulting in decreased toxic nuclear inclusions and extended survival (28). This suggests that the decreased clearance of mutant htt due to the deficiency of p62 or Atg5 leads to increased cytoplasmic mutant htt and its
inclusions, even for the usual expanded polyQ length, resulting in decreased nuclear inclusions and extended survival as observed in R6/2 mice with super-long CAG-repeat expansions.

It has recently been reported that, in a transgenic mouse model of spinal and bulbar muscular atrophy (SBMA), another polyQ disease in which a mutant androgen receptor (AR) with an expanded polyQ repeat is toxic, p62 depletion exacerbated motor phenotypes and the neuropathological outcome; overexpression of p62 protected the mice against AR toxicity (29). p62 depletion led to increased diffuse nuclear staining, whereas overexpressed p62 led to a decrease in diffuse staining and increased nuclear inclusions, suggesting a protective role of p62 and nuclear inclusions. These results were opposite to our results. This discrepancy may be due to the different tendencies for nuclear localization between mutant AR and mutant htt. AR easily translocates into the nucleus in the presence of androgen, but htt is basically a cytoplasmic protein. As shown in our study (Fig. 7), mutant htt with nuclear localization signal (NLS) showed no difference in nuclear inclusion formation with or without p62. For mutant htt without NLS, p62 depletion increased the cytoplasmic inclusions. In the case of mutant AR, it is stable with client proteins in the cytoplasm, but with androgen signal, mutant AR is freed from client proteins and moves into the nucleus as rapidly as mutant htt with NLS. Thus, there is no effect of p62 on cytoplasmic inclusion formation in SBMA model mice. Regarding the effect of p62 in the nucleus, we could not find the oligomers of mutant htt by immunohistochemistry as observed in SBMA mice and could not identify the shift from oligomers to nuclear inclusions. Using AGERA analysis in HD190QG without p62 (Fig. 4C), we found few higher molecular-weight polyQ complexes in nuclear fraction, which may include oligomers. This could be explained, however, by the decrease of mutant htt in the nucleus due to cytoplasmic deposit. So, in conclusion, it is difficult to confirm that nuclear inclusion formation is protective. Rather it seems to be toxic, though we could not exclude the possible existence of an invisible oligomer in the nucleus.

In summary, we observed that p62 depletion paradoxically ameliorates the phenotypes of HD model mice due to an increase in cytoplasmic inclusions and a decrease in nuclear inclusions. This result suggests that mutant htt is degraded through autophagy in the cytoplasm, and loss of this quality control system increases cytoplasmic inclusions and decreases nuclear inclusions. The regulation for this system has been considered a possible therapeutic target, although this paradoxical effect requires further investigation.

MATERIALS AND METHODS

Mice

Three HD model mice (R6/2, HD190QG and HD120QG), p62 knockout mice and Atg5 conditional knockout mice were used in this study. Heterozygous Htt exon 1 transgenic mice of the R6/2 strain (121–133 CAG-repeats) were used for our in vivo study (30). The HD190QG mice harbor two mutant truncated N-terminal htt containing around 120 and 190 CAG repeats fused with EGFP in its genome (31). HD120QG mice were generated by selecting mice with shorter CAG repeats from the HD190QG line. HD120QG mice show a milder phenotype compared with HD190QG mice. The polyQ lengths were slightly different among HD190QG and HD120QG mice, because these mice show CAG repeat length instability. Precise sizes of polyQ lengths of HD model mice used in each experiment are described in Supplementary Material, Table S2. p62 knockout mice used in this study were described previously (11). Atg5 conditional knockout mice were kindly provided by Professor Noboru Mizushima (9). R6/2 and Synapsin I-Cre (SynCre) transgenic mice [B6.Cg-Tg(Syn1-cre)671Jxm/J] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) (17). All mouse experiments were approved by the Animal Experiment Committee of the RIKEN Brain Science Institute.

Generation of htt transgenic/p62 conditional knockout mice and genotyping

The ovaries of R6/2 mice were transplanted into B6CBAF1 female mice and the ovaries of HD190QG or HD120QG mice were transplanted into BDF1 female mice. Those female mice were mated with homozygous p62 knockout male mice of the B6 strain. The female offspring (R6/2; p62−/−, HD190QG; p62−/− or HD120QG; p62−/−) were mated with heterozygous p62 knockout male mice to produce all the genotype combinations. Genotyping PCR and determination of CAG-repeat number of HD model mice were performed as described previously (31). Genotyping PCR of p62 knockout mice was performed with two primer pairs: 5′-CTCTGATGTCTTCTCCCAATGC-3′/ 5′-TAGATACCTAGGTGACCTG-3′ and 5′-CTTACGAGT CCTTTCAC-3′/5′-TCTCCTGTCGCCAGAGATAG-3′.

Generation of htt transgenic/Atg5 conditional knockout mice and genotyping

The ovaries of R6/2 were transplanted into B6CBAF1 female mice. Those female mice were mated with Atg5f/f male mice. Female offspring (R6/2;Atg5f/f) were mated with Atg5f/f;SynCre to produce all the genotype combinations. Genotyping PCR of Atg5f/f alleles and the Cre-recombinase transgene was performed as described previously (9).

Antibodies

The following antibodies were used: mouse anti-htt: EM48 (MAB5374, Chemicon, Temecula, CA, USA), anti-htt: N-18 (sc-8767, Santa Cruz Biotechnologies, Dallas, TX, USA), mouse anti-polyQ; 1C2 (MAB1574, Chemicon), guinea pig anti-p62 (p62-C, GP62-C, Progen, Heidelberg, Germany), rabbit anti-p62 (PM045, MBL, Nagoya, Japan), rat anti-S403-phosphorylated p62 (S403-phos-p62, D343-3, MBL), chicken anti-GFP (ab13970, Abcam, Cambridge, UK), rabbit anti-GFP (A6455, Molecular Probe, Eugene, OR, USA), rabbit anti-ubiquitin (Z0452, DAKO, Copenhagen, Denmark), mouse anti-MAP2 (M9942, Sigma-Aldrich, Saint Louis, MO, USA), goat anti-Lamin B (M-20, Santa Cruz Biotechnologies), anti-β-tubulin (T4026, Sigma-Aldrich) and Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA).

Immunohistochemistry and immunofluorescence staining

Mice were anesthetized and immediately fixed by perfusion through the left ventricle with 4% paraformaldehyde in PBS.
A brain was collected, postfixed with the same fixative overnight and processed for embedding. Paraffin-embedded brain sections (5 μm thickness) were deparaffinized, autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 5 min and immunostainings were performed as described in our previous study (32–34). The primary antibodies were diluted as follows: 1:500 for anti-htt (EM48), anti-GFP, anti-ubiquitin and anti-S403-phos-p62, 1:1000 for anti-p62-C and 1:300 for anti-MAP2 and anti-htt (N-18). Alexa-conjugated secondary antibodies were diluted at 1:300.

Imaging and counting inclusions
Images were obtained with a BZ9000 digital microscopic system (KEYENCE, Osaka, Japan) and a TCS SP5 confocal system (Leica, Heidelberg, Germany). Inclusions were stained with anti-htt antibody (EM48) and anti-GFP antibody. Nuclei were stained with DAPI. The percentages of nuclei with inclusions were calculated and averaged from three mice of each genotype.

Preparation of total and nuclear fraction
Homogenization and fractionation of mice cerebrum were performed as described previously (31). Mouse brains were homogenized in nine volumes (v/w) of 0.25 M sucrose-buffer A (50 mM Tris–HCl, pH 7.4, 5 mM MgCl2, 2 mM dithiothreitol and 1 mM phenylmethylsulfonyl), supplemented with complete EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) in a digital homogenizer (As One, Osaka, Japan) using eight strokes at 1000 rpm. A portion of the homogenate was briefly sonicated and used as the total lysate, and the rest of the homogenate was centrifuged at 1000 g for 10 min at 4°C. The pellets were resuspended and homogenized in 5 ml of 2.1 M sucrose-buffer A by five strokes at 1000 rpm with a digital homogenizer. Nuclei were sedimented by centrifugation at 8000 g for 80 min at 4°C. The pellet containing the nuclei was resuspended in 0.5 ml of 10 mM Tris–HCl (pH 7.4) and 2 mM MgCl2 with the protease inhibitors.

Immunoblotting
The lysates or fractions were boiled in SDS sample buffer and subjected to western blot analysis as described previously (34).

Agarose gel electrophoresis for resolving aggregates
Agarose was dissolved in gel buffer (100 mM Tris and 100 mM glycine) by boiling, and then the 1% agarose solution was cooled to a moderate temperature and poured into the gel plates of PAGEL (ATTO, Tokyo, Japan), of which the sides and the bottom had been tightly sealed with a plastic tape. Immediately, a plastic comb was inserted at the top. After cooling to room temperature, the gel was used immediately or stored at 4°C. Before setting the gel into the apparatus for electrophoresis, the plastic tape was removed. Electrophoresis was performed using an apparatus for PAGEL (AE-6530P, ATTO) at 50 V for 1 h at room temperature with running buffer (100 mM Tris, 100 mM glycine and 0.1% SDS). When necessary, HiMark pre-stained protein standard (Life Technologies, Carlsbad, CA, USA) was loaded as a size marker. For western analysis, proteins were transferred onto a polyvinylidene fluoride membrane for 90 min at 40 V, 4°C or 150 mA at room temperature. Immunodetection was performed as in conventional western analysis.

DNA transfection into MEF p62−/− cells and counting inclusions
MEF p62−/− and p62+/+ cells were generated from p62−/− and p62+/+ mice, and immortalized with SV40T antigen (kind gift from Dr Yusuke Yanagi, Kyushu University). G-p62 (20) was cloned into a pJTI vector (Jump-in system; Invitrogen). pJTI-G-p62 was then stably transfected into the subcloned immortalized MEF p62−/− cells. G-p62 expression in the single colony of MEF p62−/− + G-p62 cells was confirmed by western blotting using p62 antibody. The htt exon 1 with 106 CAG repeats (HD106Q) was cloned into the N-terminal of monomeric RFP (HD106Q-RFP), and three repeats of NLS sequence (GGATCCACCAAAAAAGAAGAGAAAGGTAG ATCT) were introduced at a BamHI site between HD106Q and RFP (HD106Q-NLS-RFP). CAG-repeat numbers and entire htt exon 1 sequences were confirmed. HD106Q-RFP or HD106Q-NLS-RFP plasmids were transfected into MEF p62−/−, MEF p62−/− + G-p62 or MEF p62+/+ cells with lipofectamine 2000 (Invitrogen). Cells were fixed with formaldehyde 3 days after transfection and RFP were visualized by fluorescent microscopy using Keyence BZ-9000 equipped with a 20 x dry objective lens. RFP-transfected cells and inclusions in five different microscopic fields were separately counted using the Keyence software.

Statistical analysis
We used unpaired Student’s t-test for comparison between two sample groups. One-way ANOVA followed by the Tukey test was used for multiple comparisons. For survival rate, we plotted the survival distribution curve with the Kaplan–Meier method followed by a log-rank test and with Bonferroni correction. We generated these data with Prism5 (GraphPad Software, La Jolla, CA, USA). We considered the difference between comparisons to be significant when P < 0.05 for all the statistical analyses except for the survival analysis.

AUTHORS’ CONTRIBUTIONS
M.K., G.M. and Y.K. performed the main experiments. M.O., M.K.-Y., C.W. and H.T. conducted some supportive experiments. T.Y. and E.W. provided p62 knockout mice and K.N. performed the initial experiments. M.K., T.Sh., T.Sa., N.H. and N.N. analyzed data. M.K. and N.N. designed experiments and wrote the manuscript. N.N. organized the whole project.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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We thank Professor Noboru Mizushima (University of Tokyo) for Aag5 knockout mice, Dr Yusuke Yanagi (Kyushu University) and Dr Yusuke Yanagi, Kyushu University). G-p62 (20) was cloned into a pJTI vector (Jump-in system; Invitrogen). pJTI-G-p62 was then stably transfected into the subcloned immortalized MEF p62−/− cells. G-p62 expression in the single colony of MEF p62−/− + G-p62 cells was confirmed by western blotting using p62 antibody. The htt exon 1 with 106 CAG repeats (HD106Q) was cloned into the N-terminal of monomeric RFP (HD106Q-RFP), and three repeats of NLS sequence (GGATCCACCAAAAAAGAAGAGAAAGGTAG ATCT) were introduced at a BamHI site between HD106Q and RFP (HD106Q-NLS-RFP). CAG-repeat numbers and entire htt exon 1 sequences were confirmed. HD106Q-RFP or HD106Q-NLS-RFP plasmids were transfected into MEF p62−/−, MEF p62−/− + G-p62 or MEF p62+/+ cells with lipofectamine 2000 (Invitrogen). Cells were fixed with formaldehyde 3 days after transfection and RFP were visualized by fluorescent microscopy using Keyence BZ-9000 equipped with a 20 x dry objective lens. RFP-transfected cells and inclusions in five different microscopic fields were separately counted using the Keyence software.

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