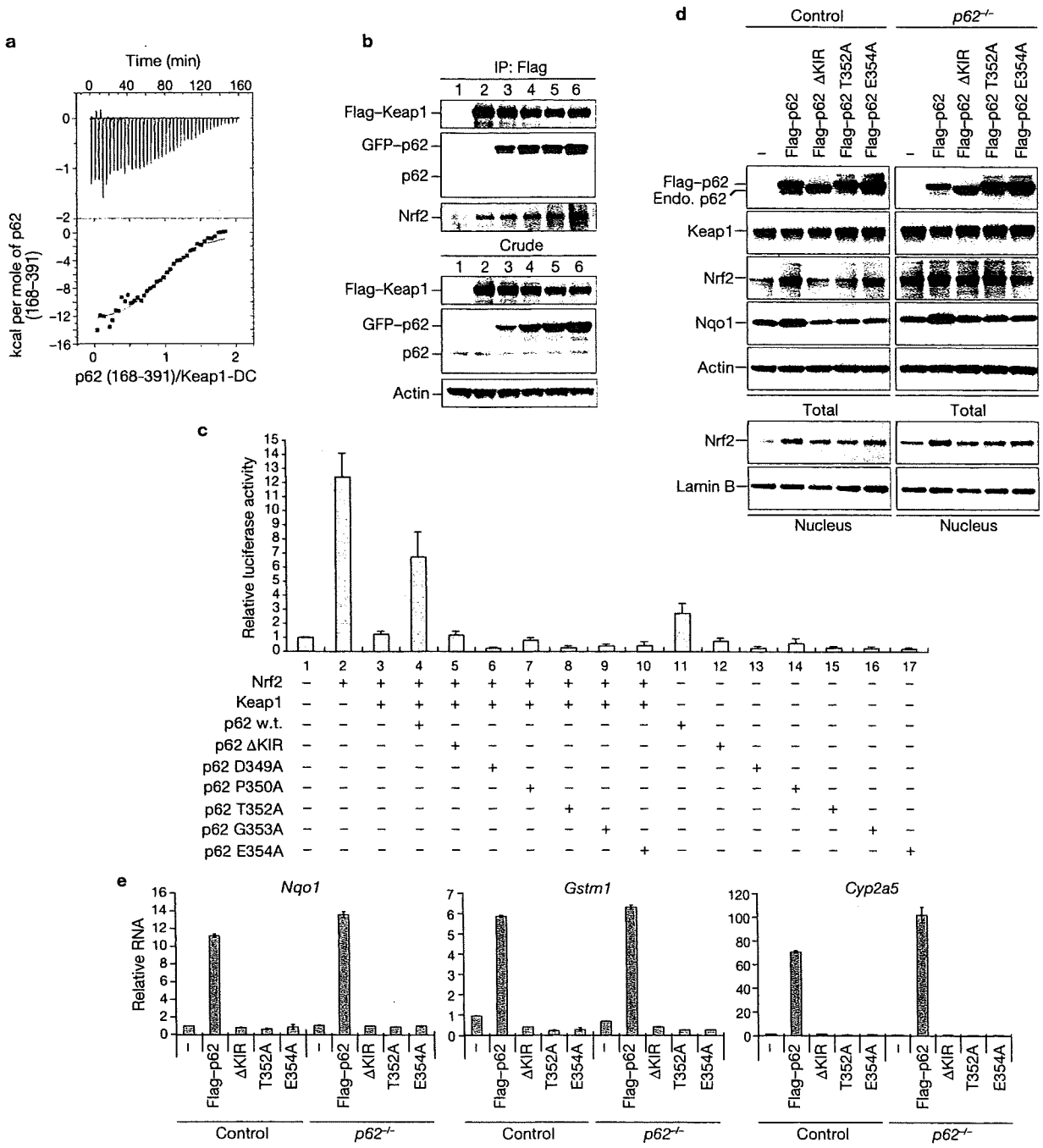

Figure 3 Competitive inhibition of the Nrf2–Keap1 pathway by p62. (**a**) A representative ITC profile of the titration of Keap1-DC with p62M7 (residues 168–391). The upper panel shows the raw ITC thermograms and the lower panel shows the fitted binding isotherms. (**b**) Immunoprecipitation assays. Flag-tagged Keap1 was co-expressed with increasing concentrations of green fluorescent protein (GFP)–p62 (lanes 3–6) in HEK293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody. The resulting immunoprecipitates were subjected to SDS–PAGE and analysed by immunoblotting with anti-Flag, anti-p62 and anti-Nrf2 antibodies. The bands corresponding to Flag–Keap1, endogenous p62, Nrf2 and actin are indicated. Data are representative of three independent experiments. (**c**) The competitive p62 activity against Keap1 was measured by luciferase assay. The expression plasmids for Nrf2, Keap1 and p62 wild-type (w.t.) or its mutants were transfected into Hepa1 cells along with pNqo1-ARE reporter plasmid and pRL-TK as an internal control. At 36 h after transfection, the luciferase activity was measured in accordance with the instructions provided by the manufacturer. Assays were performed twice in triplicate. Data are means and s.d. for six determinations. (**d**) Immunoblot analysis. Flag-tagged p62 and its mutants defective in interacting with Keap1 were overproduced in wild-type and *p62*$^{-/-}$ primary mouse hepatocytes by the adenovirus system. At 48 h after infection, total cell lysates and nuclear fractions were prepared and subjected to immunoblot analysis with the antibodies specified. The bands corresponding to Flag–p62, endogenous p62, Keap1, Nrf2, Nqo1, actin and Lamin B are shown. Data are representative of three independent experiments. Uncropped images of blots are shown in Supplementary Information, Fig. S11. (**e**) Quantification of mRNA levels of the detoxification enzymes Nqo1, Gstm1 and Cyp2a5 in hepatocytes overexpressing Flag–p62 and its mutants. Total RNAs were prepared from non-infected or infected hepatocytes and reverse transcribed into their respective cDNAs, which were used as templates in real-time PCR analysis. Values were normalized to the amount of each mRNA in the non-infected hepatocytes. The experiments were performed three times. Data are means ± s.d. for three experiments.

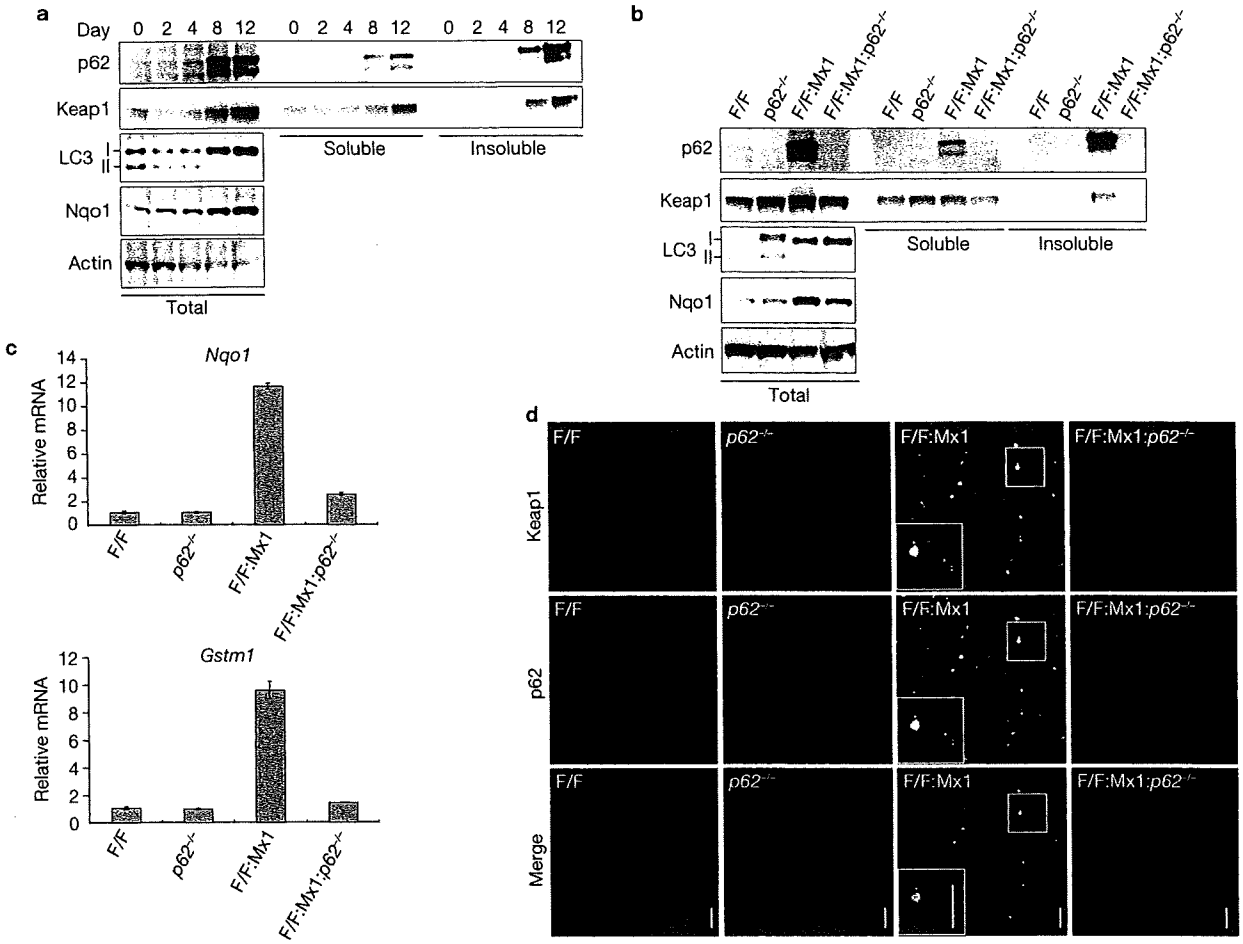

Figure 4 Formation of p62-positive and Keap1-positive inclusions in autophagy-deficient hepatocytes. **(a)** Insolubilization of Keap1 in *Atg7*-deficient hepatocytes. Liver homogenates from *Atg7*$^{F/F}$:Mx1 mice on various days after injection of poly(I)•poly(C) were separated into detergent-soluble and detergent-insoluble fractions with 0.5% Triton X-100. Each fraction was subjected to SDS–PAGE and analysed by immunoblotting with the indicated antibodies. The data displayed are representative of three separate experiments. **(b)** Immunoblot analysis of *Atg7*-deficient (*Atg7*$^{F/F}$:Mx1; *Atg7*$^{F/F}$ shown here as F/F) and *Atg7 p62*-deficient (*Atg7*$^{F/F}$:Mx1:*p62*$^{-/-}$) livers. Liver homogenates from mice of the stated genotypes at 12 days after injection of poly(I)•poly(C) were separated into detergent-soluble and detergent-insoluble fractions. Each fraction was subjected to SDS–PAGE and analysed by immunoblotting with the indicated antibodies. *Atg7*$^{F/F}$ mice[2] in which Atg7 is efficiently expressed at a level similar to that in the wild-type mice were used as control. Data shown are representative of three separate experiments. Uncropped images of blots are shown in Supplementary Information, Fig. S11. **(c)** Quantitative real-time PCR analyses of Nqo1 and Gstm1 in mouse livers. Total RNAs were prepared from livers of the indicated genotypes at 12 days after injection of poly(I)•poly(C). Values were normalized to the amount of mRNA in *Atg7*$^{F/F}$ liver. Data are means ± s.d. for three experiments. **(d)** Immunofluorescence analysis of the cellular localization of p62 and Keap1. Liver sections from mice of the indicated genotypes at 28 days after injection of poly(I)•poly(C) were immunostained with anti-Keap1 (top) and anti-p62 (middle) antibodies. Bottom: merged images of Keap1 (green) and p62 (red). Each inset in the *Atg7*-deficient liver panels is a magnified image of the boxed region. Scale bars, 20 μm.

Information, Fig. S1), suggesting the existence of p62-dependent regulation of Nrf2.

To explore cellular regulation by p62, we used a proteomic approach[34] to screen for proteins that interact with p62, by using HEK293T cells expressing tagged p62 protein. Keap1 was identified as a p62-interacting protein (data not shown). In an independent experiment with RL34 cells expressing tagged Keap1 protein, we isolated p62 as a Keap1-associating protein (data not shown). Keap1 is a substrate adaptor protein for Cullin-3-type ubiquitin E3 ligase. Keap1 possesses four domains: the Broad complex, Tramtrack, and Bric-a-Brac (BTB, amino-acid residues 61–179); the intervening region (IVR, residues 180–314); the double glycine repeat or kelch repeat (DGR, residues 315–598); and the carboxy-terminal region (CTR, residues 599–624)[24–27] (Fig. 1a). The DGR and CTR domains are collectively called the DC domain. The BTB domain serves to dimerize Keap1, enabling ubiquitin conjugation onto specific lysine residues located within the Neh2 domain of Nrf2 (refs 35, 36). The IVR domain interacts with Cullin 3 to promote Nrf2 ubiquitylation[26], whereas the DC domain physically interacts with the Neh2 domain of Nrf2 (refs 36 37). To specify the regions of Keap1 essential for its interaction with p62, we performed an immunoprecipitation assay. Whereas Keap1 C-terminal deletion mutants (ΔDGR and ΔCTR) did not interact with either endogenous p62 or Nrf2, Keap1 amino-terminal deletion mutants (ΔNTR, ΔBTB and ΔIVR) interacted with both endogenous p62 and Nrf2, although with weaker affinities than wild-type Keap1 (Fig. 1a). The Keap1-DC domain, but not the DGR domain, bound to both p62 and Nrf2 (Fig. 1a). The CTR domain contributes to the structural fold of Keap1-DC, which is required for interaction with Nrf2 (ref. 38). These results therefore suggest that the six-bladed β-propeller structure of Keap1 is essential for its molecular recognition of p62, as is the case for Nrf2.

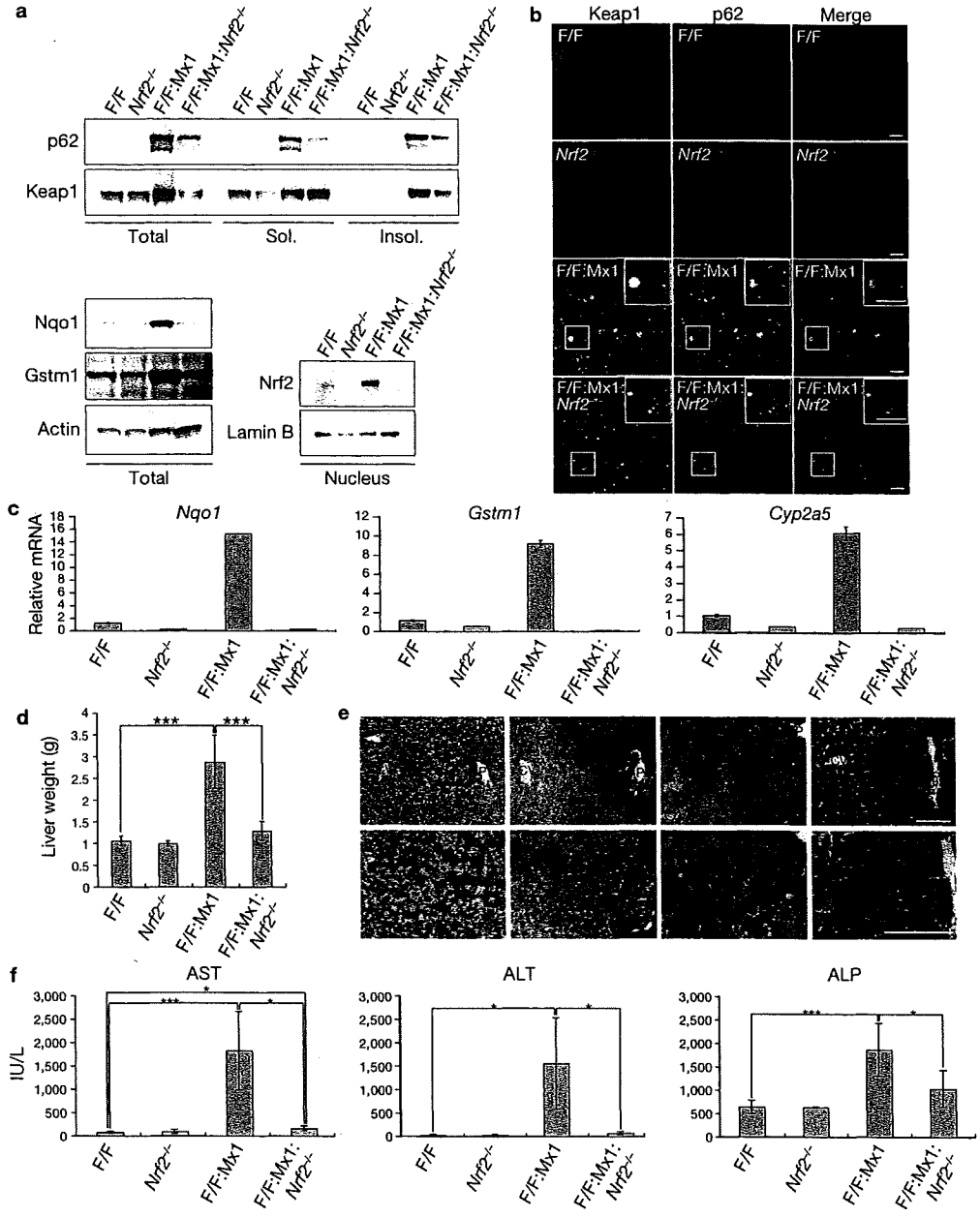

Figure 5 Amelioration of liver dysfunction in autophagy-deficient mice by the additional loss of *Nrf2*. (**a**) Immunoblotting of *Atg7*-deficient (*Atg7*$^{F/F}$:Mx1; *Atg7*$^{F/F}$ shown here as F/F) and *Atg7 Nrf2*-deficient (*Atg7*$^{F/F}$:Mx1:*Nrf2*$^{-/-}$) livers. Liver homogenates from mice of the assigned genotypes at 28 days after injection of poly(I)•poly(C) were separated into detergent-soluble and detergent-insoluble fractions. Total, soluble and insoluble fractions were subjected to SDS–PAGE and analysed by immunoblotting with the indicated antibodies (top section). Total lysates were subjected to SDS–PAGE and analysed by immunoblotting with antibodies against Nqo1, Gstm1 and actin (bottom left section). Nuclear fractions were prepared from the livers of the indicated genotypes at 28 days after injection of poly(I)•poly(C), subjected to SDS–PAGE and analysed by immunoblotting with antibodies against Nrf2 and Lamin B (as control) (bottom right section). Data were obtained from three independent experiments. Uncropped images of blots are shown in Supplementary Information, Fig. S11. (**b**) Immunofluorescence analysis of the cellular localization of p62 and Keap1. Liver sections from mice of the indicated genotypes at 28 days after injection of poly(I)•poly(C) were immunostained with anti-Keap1 (left) and anti-p62 (middle) antibodies. Right: merged images of Keap1 (green) and p62 (red). Each inset in the *Atg7*-deficient and *Atg7 Nrf2*-deficient liver panels is a magnified image of the boxed region. Scale bars, 20 μm. (**c**) Quantitative real-time PCR analyses of Nqo1, Gstm1 and Cy2a5 in mouse livers. Total RNAs were prepared from livers of the indicated genotypes at 28 days after injection of poly(I)•poly(C). Values were normalized to the amount of mRNA in the *Atg7*$^{F/F}$ liver. Data are means ± s.d. for three experiments. (**d**) Liver weight. The weights of the mouse livers of the different genotypes shown at 28 days after injection of poly(I)•poly(C) were measured. Data are means ± s.d. for five mice from each group. Three asterisks, $P < 0.001$ (Student's *t*-test). (**e**) Histological analysis of the mouse liver of the indicated genotypes. At 28 days after injection of poly(I)•poly(C), the livers were processed for haematoxylin/eosin staining. Higher-magnification views are shown in the bottom panels. CV, central vein; P, portal vein. Scale bars, 100 μm. (**f**) Liver function tests of the mice used in **d**. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured. (IU/L, international unit per liter). Data are means ± s.d. for seven mice from each group. Asterisk, $P < 0.05$; three asterisks, $P < 0.001$.

p62 comprises an N-terminal region that includes Phox and Bem1p (PB1) (residues 20–102) and a zinc finger (residues 122–167), a central region containing an LC3-recognition sequence (LRS) (residues 337–343), and a C-terminal region encompassing a ubiquitin-associated domain (UBA) (residues 391–436)[14,15,39] (Fig. 1b). To determine which domain of p62 is required for its interaction with Keap1, we divided p62 into three regions and employed a pull-down assay with the Keap1-DC domain (Supplementary Information, Fig. S2). The Keap1-DC domain was clearly detected in pull-down products both with full-length p62 and with p62M7, the p62 mutant harbouring the central region (residues 168–391). However, deletion mutants p62M3 and p62M5 showed a marked decrease in binding to the Keap1-DC domain (Supplementary Information, Fig. S2). To delineate the interaction domain more precisely, we prepared a deletion series of p62M7 (M71–M83) and performed the pull-down assay (Fig. 1b; Supplementary Information, Fig. S3a). These assays revealed that p62M83, covering residues 345–359, is essential and sufficient for the interaction between p62 and Keap1-DC (Supplementary Information, Fig. S3a). We therefore named residues 346–359 in p62M83 the Keap1-interacting region (KIR). KIR is located close to the C terminus of the LRS and is conserved across species (Fig. 1c). To further verify the Keap1–p62 interaction *in vivo*, we performed an immunoprecipitation assay. In primary mouse hepatocytes and HEK293T cells, endogenous Keap1 co-immunoprecipitated with wild-type Flag-tagged p62 and a mutant defective in oligomerization (p62 K7AD69A)[40], but failed to co-immunoprecipitate with KIR-deleted Flag–p62 (p62 ΔKIR) (Fig. 1d). The interaction between endogenous p62 and Keap1 was also verified (Fig. 1e).

Crystal structure of Keap1-DC in complex with p62-KIR

To delineate how Keap1 interacts with p62-KIR, we determined the crystal structure of Keap1-DC (residues 309–624) in complex with the p62-KIR peptide covering residues 346–359 at a resolution of 2.8 Å. Keap1-DC forms a six-bladed β-propeller structure with pseudo six-fold symmetry[37,38]. The KIR peptide binds to the bottom side of the β-propeller structure (Fig. 2a, b). A simulated-annealing $F_o - F_c$ omit map clearly indicated the electron density in the peptide bound region, in which 8 residues (V348 to L355) out of 14 were visible in p62-KIR, except for the L355 side chain (Fig. 2c).

Nrf2 is turned over rapidly through proteasomal degradation[24–27]. We previously identified the DLG and ETGE binding motifs present in the Neh2 domain of Nrf2, and these two motifs bind individually to the same binding pocket located at the bottom surface of Keap1 (refs 37, 38). One Nrf2 molecule binds to the Keap1 homodimer or to two Keap1 molecules. Because the two-site binding facilitates the ubiquitylation of lysine residues located between the DLG and ETGE motifs, the two-site substrate recognition mechanism is crucial for the rapid ubiquitylation of Nrf2 (refs 37, 38). Hydrogen-bond analysis revealed that the interactions between the Keap1-DC domain and p62-KIR (Fig. 2d, left) heavily overlap with the interactions between the Keap1-DC domain and Nrf2-ETGE (Fig. 2d, right). Eight amino-acid residues of Keap1-DC (Y334, S363, R380, N382, R415, Q530, S555 and S602) form hydrogen bonds with p62-KIR. These eight residues and two additional Keap1 residues (R483 and S508) are involved in Keap1 recognition of Nrf2-ETGE. These data strongly support the notion that p62-KIR binds to Keap1 in a manner very similar to that of the Nrf2-ETGE and Nrf2-DLG motifs[37,38].

The complex structure of Keap1-DC and p62-KIR showed that, of the eight amino-acid residues of the KIR domain, five residues (P350, S351, T352, E354 and L355) are involved in the interaction with Keap1 (Fig. 2d, left). To further verify this interaction biochemically, point mutations were created in the amino-acid residues of KIR in the mutant construct p62M80 by alanine replacement (Supplementary Information, Fig. S3a). A pull-down assay revealed that formation of the Keap1-DC–p62M80 complex was significantly decreased in the D349A, P350A, T352A, G353A and E354A mutants (Supplementary Information, Fig. S3a). Consistent with this experiment, immunoprecipitation analysis also revealed that these mutants showed a marked decrease in binding to Keap1 (Supplementary Information, Fig. S3b). The S351A mutation did not affect binding to Keap1 in either experiment, in spite of the direct interaction between S351 and Keap1 (Fig. 2d, left). The D349A mutation significantly inhibited the interaction with Keap1 (Supplementary Information, Fig. S3b), even though D349 does not interact directly with Keap1 (Fig. 2d, left). Residue D349 is involved in intra-peptide hydrogen bonding with S351 and T352, which stabilizes the type I β-turn in p62-KIR (Fig. 2e); this seems to be critical for the interaction between Keap1-DC and p62-KIR.

Because the tertiary structure of the complex indicates that p62-KIR associates with eight amino-acid residues in the basic surface of Keap1-DC, we introduced mutations in the residues of Keap1-DC (Y334, S363, R380, N382, R415, S508, Q530, S555 and S602) that are critical in the interaction with the KIR domain, and executed a GST pull-down assay (Supplementary Information, Fig. S3c). All except one of these GST–Keap1-DC mutants were markedly impaired in their ability to pull down maltose-binding protein (MBP)–p62M80 (Supplementary Information, Fig. S3c); the exception was R483A, which is not involved in the interaction with KIR (Fig. 2d). Further immunoprecipitation analysis confirmed that these residues in Keap1 are essential for efficient interaction with endogenous p62 and Nrf2 (Supplementary Information, Fig. S3d). These biochemical results are in good agreement with the crystal structure analysis.

Competitive inhibition of the Nrf2–Keap1 pathway by p62

Previous structural and kinetics studies demonstrated that the ETGE motif has a high affinity but the DLG motif has a low affinity for the same basic surface of Keap1 ($K_{a,ETGE} = (1.90 \pm 0.40) \times 10^8\ M^{-1}$, compared with $K_{a,DLG} = (1.00 \pm 0.00) \times 10^6\ M^{-1}$) (ref. 41). If the affinity of the p62 KIR domain for the basic surface in Keap1 were higher than or comparable to that of the ETGE or DLG motif, p62 might serve as an endogenous protein inducer of Nrf2 target genes by competitive binding inhibition of the Nrf2–Keap1 complex. It was therefore important to determine the binding-dissociation constant of Keap1–p62. Assessment of the binding energy by isothermal titration calorimetry (ITC) showed that the affinity of p62M7 (residues 168–391) for Keap1-DC ($K_a = (5.4 \pm 0.3) \times 10^5\ M^{-1}$) was similar to that of the DLG motif (Fig. 3a) and that, unlike with Nrf2, the binding stoichiometry was 1:1. This result suggests that overproduction of p62 counteracts the interaction between Nrf2-DLG and Keap1, but not that between Nrf2-ETGE and Keap1. In support of this notion, whereas the amounts of p62 forming a complex with Flag–Keap1 increased in proportion to the p62 expression level, the levels of Nrf2 interacting with Keap1 were hardly affected by overexpression of p62 (Fig. 3b). Meanwhile, p62 overproduction led to a marked decrease in Nrf2 ubiquitylation (see Supplementary Information, Fig. S4) and consequent Nrf2 stabilization (see Fig. 3d), suggesting that p62 inhibits Nrf2-DLG, but not Nrf2-ETGE, from interacting with Keap1.

So far, structural and kinetic analyses have strongly argued that p62 competitively inhibits the Keap1–Nrf2 interaction, leading to

the stabilization of Nrf2 and the expression of cytoprotective genes. To verify this concept further, we performed luciferase assays with a reporter plasmid harbouring the canonical Nrf2 recognition motif referred to as the ARE (antioxidant-responsive element). The transactivation activity of Nrf2 was suppressed by simultaneous expression of Keap1 (Fig. 3c, lanes 2 and 3), but this repression by Keap1 was inhibited by overexpression of p62 (Fig. 3c, lane 4). Mutants of p62 defective in interaction with Keap1 failed to counteract the repression of Nrf2 activity by Keap1 (Fig. 3c, lanes 5–10). Even in the absence of Keap1 transfection, p62 overexpression activated reporter gene expression (Fig. 3c, lane 11), perhaps by targeting the interaction of endogenous Keap1 with Nrf2; however, p62 mutants did not show this activity (Fig. 3c, lanes 12–17). This antagonistic effect of p62 to Keap1 seems to be independent of the self-oligomerization ability of p62 because the oligomerization-defective mutant p62 K7AD69A similarly counteracted the repression by Keap1 (Supplementary Information, Fig. S5a).

To examine whether p62 overexpression reinforces Nrf2 stability, we infected wild-type and *p62*$^{-/-}$ mouse primary hepatocytes with adenovirus Flag-tagged wild-type p62 and p62 mutants (ΔKIR, T352A and E354A) severely attenuated in their interactions with Keap1 (see Fig. 1d for ΔKIR; see Supplementary Information, Fig. S3a, b, for T352A and E354A). In both wild-type and mutant hepatocytes, overproduction of Flag–p62, but not of the mutants, triggered an increase in both total Nrf2 (Fig. 3d, upper panel) and the level of Nrf2 in the nuclear fractions (Fig. 3d, lower panel; Supplementary Information, Fig. S6). Because overproduction of Flag–p62, but not of the mutants, in primary mouse hepatocytes significantly inhibited Nrf2 ubiquitylation (Supplementary Information, Fig. S4), the Nrf2 stabilization can be attributed to Nrf2 being outcompeted by p62 for Keap1 binding. Consistent with this conclusion, the expression of Nrf2 target genes, such as *Nqo1*, *Gstm1* and *Cyp2a5*, was induced by the expression of Flag–p62, but not by expression of the mutants deficient in interaction with Keap1 (Fig. 3d, e; Supplementary Information, Fig. S6). In contrast, another p62 mutant (K7AD69A), which was defective in self-oligomerization but able to interact with Keap1 (see Fig. 1d) activated Nrf2 target genes to a modest but significant extent (Supplementary Information, Fig. S5b, c).

Keap1 is sequestered in inclusion bodies in a p62-dependent manner

The next series of experiments served to clarify whether the Nrf2–Keap1 interaction is affected by the intracellular accumulation of p62 or p62-positive and ubiquitin-positive inclusion bodies, which is known to occur in autophagy-deficient liver[16]. These studies were conducted in *Atg7*$^{F/F}$:Mx1 mice in which *Atg7* can be depleted in the liver by intraperitoneal injection of polyinosinic acid•polycytidylic acid (poly(I)•poly(C))[2]. Consistent with our previous work[16], immunoblot analysis revealed that p62 started to accumulate at day 4, was abundant in both the detergent-soluble and insoluble fractions at day 8 and further increased in both fractions at day 12 after injection of poly(I)•poly(C) (Fig. 4a). This pattern correlated well with the decrease in conversion of LC3-I to LC3-II, which signifies defective autophagosome formation[42] (Fig. 4a). On accumulation of p62, Keap1 was detected abundantly, especially in the detergent-insoluble fraction (Fig. 4a). The quantity of Nqo1 also increased gradually in *Atg7*-deficient livers (Fig. 4a), suggesting that Keap1 was inactivated by the loss of autophagy.

To determine whether the accumulation of Keap1 in the insoluble fraction of autophagy-deficient livers depends on the presence of p62, we used *Atg7*:*p62*-double-knockout mice (*Atg7*$^{F/F}$:Mx1:*p62*$^{-/-}$ or *Atg7 p62* DKO)[16]. The concurrent loss of *Atg7* and *p62* in the liver significantly suppressed the accumulation of Keap1 in the insoluble fraction that was observed in *Atg7*-deficient livers (Fig. 4b). This indicates that Keap1 is inactivated in a p62-dependent manner. Indeed, the high mRNA and protein levels of Nrf2 target genes in *Atg7*-deficient livers returned to almost normal levels after the additional loss of p62 (Fig. 4b, c). Immunofluorescence microscopy showed that the numerous Keap1-positive inclusions of various sizes seen in *Atg7*-deficient hepatocytes were absent from *Atg7 p62*-DKO hepatocytes (Fig. 4d). Double immunofluorescence microscopy showed that most of the Keap1-containing inclusions observed in *Atg7*-deficient hepatocytes were also positive for p62 (Fig. 4d). Taken together, these results indicate that under conditions of suppressed autophagy, a significant population of Keap1 is trapped by the excessive upsurge in p62, leading to sequestration of Keap1 into p62-positive and ubiquitin-positive inclusions.

Liver injury in autophagy-deficient mice is alleviated by loss of *Nrf2*

Although the excessive build-up of p62 seems to be the main cause of the pathogenic changes seen in the livers of autophagy-deficient mice, the molecular events involved in the pathogenic onset are still unknown. The data described so far suggest that p62-dependent regulation of the Nrf2–Keap1 pathway is involved in the pathological changes found in autophagy-deficient mice. To address this point, we crossed *Atg7*$^{F/F}$:Mx1 mice with *Nrf2*$^{-/-}$ mice[21] to produce *Atg7*:*Nrf2*-double-knockout mice (*Atg7*$^{F/F}$:Mx1:*Nrf2*$^{-/-}$ or *Atg7 Nrf2* DKO). Immunoblot analysis revealed that although the amount of p62 in *Atg7 Nrf2*-DKO livers was lower than that in *Atg7*-deficient livers, it was markedly higher than in control and *Nrf2*-knockout mouse livers (Fig. 5a). About half of the p62 in *Atg7 Nrf2*-DKO livers and *Atg7*-deficient livers was recovered in the insoluble fraction (Fig. 5a). The lower p62 level in *Atg7 Nrf2*-DKO mouse livers might be attributable, at least in part, to the loss of Nrf2 regulation for *p62* gene transcription[43]. Keap1 was also fractionated into the insoluble fraction in *Atg7*-deficient and *Atg7 Nrf2*-DKO mouse livers in proportion to the amount of insoluble p62 (Fig. 5a). The Keap1-positive aggregates detected in *Atg7*-deficient hepatocytes were also found in *Atg7 Nrf2*-DKO hepatocytes, although the aggregates were smaller and were almost completely co-localized with p62 (Fig. 5b). Electron microscopy showed accumulation of organelles and inclusion bodies in *Atg7 Nrf2*-DKO hepatocytes, similar to that observed in *Atg7*-deficient hepatocytes (Supplementary Information, Fig. S7). However, in contrast to *Atg7*-deficient livers, the induction of antioxidant proteins and detoxifying enzymes was completely abrogated in *Atg7 Nrf2*-DKO livers (Fig. 5a, c), confirming the Nrf2-dependent transcriptional induction of cytoprotective enzymes in autophagy-deficient livers.

We further examined the liver damage in these mice. Increased liver weight (Fig. 5d), disorganization of lobular structures (Fig. 5e, upper panels), hepatocytic hypertrophy (Fig. 5e, lower panels), an increased proportion of infiltrating cells (data not shown), and higher serum levels of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase (Fig. 5f) were observed in *Atg7*-deficient livers[2]; these were significantly suppressed by the additional loss of *Nrf2*. These results strongly suggest that persistent activation of Nrf2 in autophagy-deficient conditions may be one of the main causes of liver injury.

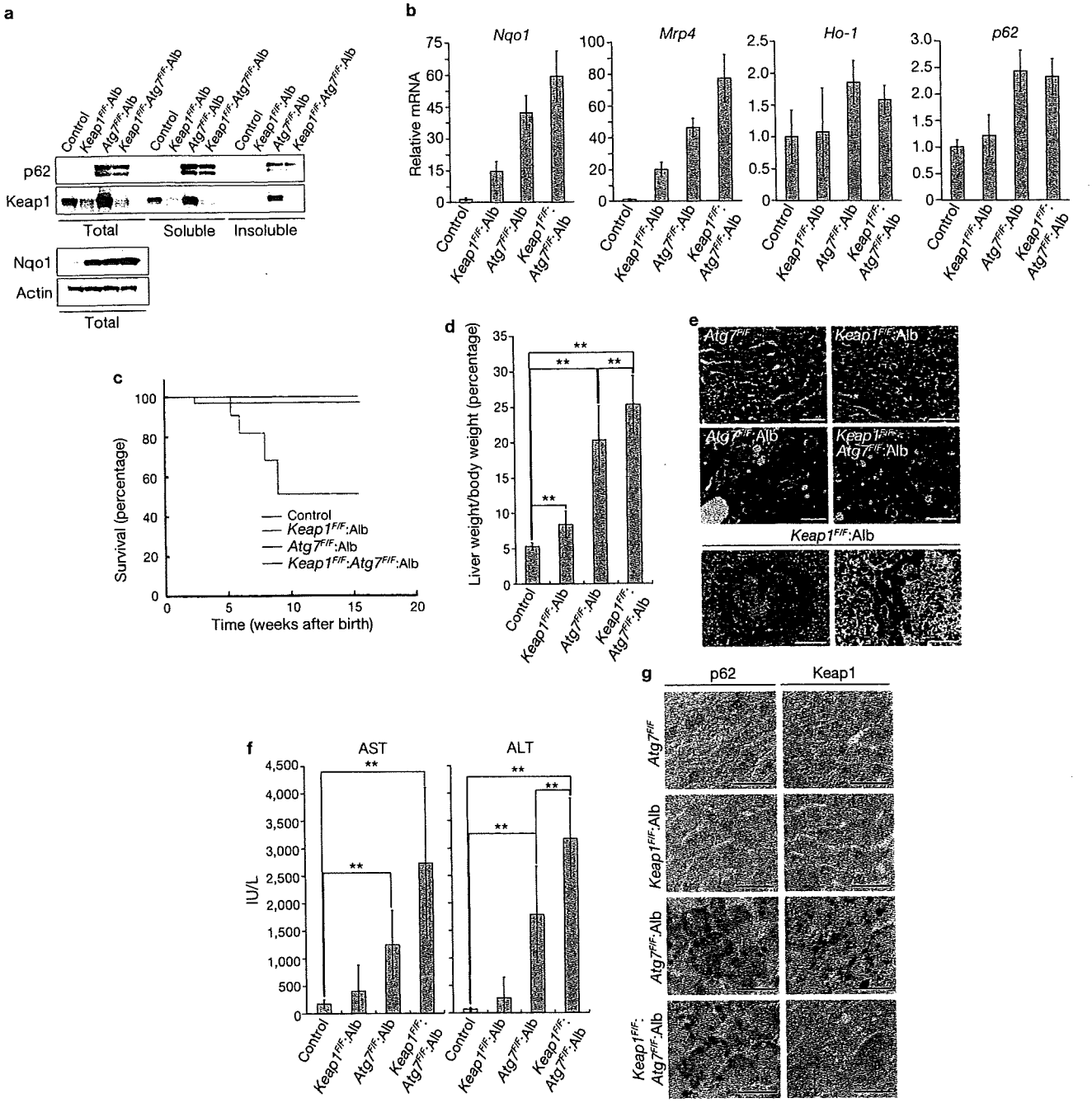

Figure 6 Exacerbation of liver dysfunction in autophagy-deficient mice by the additional loss of *Keap1*. (**a**) Immunoblotting of *Keap1*-deficient (*Keap1*$^{F/F}$:Alb) and *Keap1 Atg7*-deficient (*Keap1*$^{F/F}$:*Atg7*$^{F/F}$:Alb) livers. Liver homogenates from 8-week-old mice of these genotypes were separated into detergent-soluble and detergent-insoluble fractions. Total, soluble and insoluble fractions were subjected to SDS–PAGE and analysed by immunoblotting with the indicated antibodies (top section). Total lysates were subjected to SDS–PAGE and analysed by immunoblotting with antibodies against Nqo1 and actin (bottom section). Data were obtained from three independent experiments. Uncropped images of blots are shown in Supplementary Information, Fig. S11. (**b**) Quantitative real-time PCR analyses of Nqo1, Mrp4, p62 and Ho-1 in mouse livers. Total RNAs were prepared from livers of 9-week-old indicated genotypes. Values were normalized to the amount of mRNA in the *Atg7*$^{F/F}$ liver (control). Data are means ± s.d. for three experiments. (**c**) Kaplan–Meier curves of survival of *Atg7*$^{F/F}$:Alb and *Keap1*$^{F/F}$:*Atg7*$^{F/F}$:Alb mice. The survival analysis of control ($n = 33$), *Keap1*$^{F/F}$:Alb ($n = 15$), *Atg7*$^{F/F}$:Alb ($n = 12$) and *Keap1*$^{F/F}$:*Atg7*$^{F/F}$:Alb mice ($n = 14$) was based on a 16-week follow-up period. (**d**) Liver weight relative to body weight was measured for the different genotypes. Data are means ± s.d. for *Atg7*$^{F/F}$ (control) ($n = 24$), *Keap1*$^{F/F}$:Alb ($n = 13$), *Atg7*$^{F/F}$:Alb ($n = 9$) and *Keap1*$^{F/F}$:*Atg7*$^{F/F}$:Alb mice ($n = 7$). Two asterisks, $P < 0.01$ (Student's *t*-test). (**e**) Histological analysis of mouse liver of the indicated genotypes. The livers from 9-week-old mice were processed for haematoxylin/eosin staining. Scale bar, 50 μm (left bottom panel); 20 μm (other panels). (**f**) Liver function tests of the mice used in **d**. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured. Data are means ± s.d. for *Atg7*$^{F/F}$ (control) ($n = 24$), *Keap1*$^{F/F}$:Alb ($n = 13$), *Atg7*$^{F/F}$:Alb ($n = 9$) and *Keap1*$^{F/F}$:*Atg7*$^{F/F}$:Alb mice ($n = 7$). Two asterisks, $P < 0.01$. (**g**) Immunohistochemical analysis of cellular localization of p62 and Keap1. Paraffin sections of the liver from 9-week-old mice of the indicated genotypes were immunolabelled with anti-p62 (left) and anti-Keap1 (right) antibodies. Scale bars, 20 μm.

Liver injury in autophagy-deficient mice is exacerbated by loss of *Keap1*

To consolidate the notion that persistent activation of Nrf2 accompanied by defective autophagy causes liver injury, we generated hepatocyte-specific *Keap1*:*Atg7*-double-knockout mice (*Keap1*$^{F/F}$:*Atg7*$^{F/F}$:Alb or *Keap1 Atg7* DKO) by crossing *Keap1*$^{F/F}$:Alb[44] with *Atg7*$^{F/F}$ mice. *Keap1*-deficient mouse livers showed hyper-activation of Nrf2 (Fig. 6a, b), mild hepatomegaly (Fig. 6d) and occasional focal necrotic cell death (Fig. 6e, bottom panels), indicating that persistent activation of Nrf2 provokes cytotoxicity. Both the mRNA and protein levels of typical Nrf2 target genes in the *Keap1 Atg7*-DKO livers were significantly higher than those in single *Atg7*-knockout mouse livers (Fig. 6a, b), demonstrating that the loss of *Keap1* additively activates Nrf2 in autophagy-deficient livers. However, this additive effect was observed in only some Nrf2 target genes, such as *Nqo1* and *Mrp4*, but not in the other target genes, including those encoding p62 and haem oxygenase-1 (Ho-1) (Fig. 6a, b). Thus, transactivation by Nrf2 of certain Nrf2 target genes might be saturated in *Atg7*-deficient livers, so that levels of p62 and p62-positive aggregates in the *Keap1 Atg7*-DKO livers seemed to be similar to those in *Atg7*-deficient livers (Fig. 6a, g).

Although *Keap1 Atg7*-DKO mice were born at a Mendelian frequency, the survival rate of these mice diminished markedly 6 weeks after birth (Fig. 6c). We observed growth retardation at about 4 weeks (data not shown). Histological analysis showed pathological changes in DKO liver that were similar to those observed in *Atg7*-deficient liver: hepatocytic swellings and/or necrotic changes, appearance of acidophilic bodies, and cellular infiltration (Fig. 6e). The DKO mice showed increased liver weight (relative to body weight; Fig. 6d) and a high level of serum alanine aminotransferase (Fig. 6f) compared with *Atg7*-single-knockout mice. These results further support our conclusion that autophagy deficiency provokes deregulation of the Nrf2–Keap1 system and that constitutive activation of Nrf2 leads to hepatotoxicity.

DISCUSSION

In this study we have demonstrated that p62 serves as an endogenous protein inducer of Nrf2. We previously deciphered the unique association mechanism of Nrf2 and Keap1 in which two Keap1 molecules interact with one Nrf2 molecule through its DLG and ETGE motifs. This two-site binding facilitates the ubiquitylation and degradation of Nrf2 (refs 37, 38). This mechanism has been verified by the finding that, in cases of human lung cancer, somatic mutations in Nrf2 are located exclusively in the DLG and ETGE motifs[45]. We proposed the 'hinge and latch' model as the stress-sensing mechanism, by which the weak-affinity DLG motif acts as a latch for turning the ubiquitylation of Nrf2 on or off (refs 37, 41). Here we found that, on perturbation of autophagy, p62 accumulates and activates Nrf2 by competing with Nrf2 for its binding to Keap1. The following observations are significant: first, through its KIR motif, p62 interacts directly with Keap1; second, the binding mode of KIR is similar to that of Nrf2-ETGE or Nrf2-DLG, where it binds to the basic surface pocket at the bottom of Keap1; and third, p62-KIR binds to Keap1 with an affinity similar to that of Nrf2-DLG but much weaker than that of Nrf2-ETGE. It therefore seems most plausible that the KIR motif interferes directly with the binding of the DLG motif to Keap1. These results support our argument that the 'hinge and latch' mechanism actually operates *in vivo* (see the model in Supplementary Information, Fig. S10).

Our present study demonstrates that the pathological changes observed in autophagy-defective livers are due, at least in part, to persistent activation of Nrf2 by the excess accumulation of p62. This was an unexpected result because most Nrf2-dependent gene products retain cytoprotective function[31]. To verify this finding further, we performed a microarray analysis in *Atg7*-knockout and *Atg7 Nrf2*-DKO mouse livers. The analysis showed that more than 100 genes were activated in an Nrf2-dependent manner in autophagy-deficient livers, with a range of induction from 2-fold to as much as 100-fold (data not shown, and Fig. 5c). Furthermore, our comprehensive proteomics analysis reported previously[46] showed that the Nrf2 target gene products, such as Gst-m1 and Gst-p1, were prominent proteins in autophagy-deficient liver. Surprisingly, Gst-m1 constituted 7.69 ± 1.25% of the cytosolic proteins in *Atg7*-deficient livers (Supplementary Information, Fig. S8). Thus, the autophagy-deficient liver shows an abnormal accumulation of various Nrf2 target proteins. Because loss of *Keap1* in mouse hepatocytes resulted in only mild liver abnormality (Fig. 6d, e), the phenotypes detected in *Atg7*-deficient livers cannot be attributed solely to Nrf2 activation by loss of *Keap1*. It is therefore conceivable that the additional loss of autophagy greatly affects the development of liver abnormality. In other words, the collapse of the balance between the synthesis of cellular proteins (Nrf2-dependent robust protein synthesis) and their degradation (global turnover of cytoplasmic proteins through autophagy) could result in the appearance of destructive phenotypes (that is, hepatocytic hypertrophy due to the increased cellular protein volume followed by hepatomegaly and liver injury). In support of this conclusion, hepatocyte-specific *Keap1 Atg7*-DKO mice showing high-level Nrf2 activity exhibited more severe hepatomegaly and liver injury than the single *Atg7*-deficient mice (Fig. 6c–f).

It has been reported that p62 accumulation in growing cell lines leads to a decrease in the ubiquitin–proteasomal (UPS) flux[47], implying that the cytotoxicity of p62 operates through inhibition of the UPS flux. However, overproduction of p62 did not induce inhibition of the UPS flux in primary culture hepatocytes (data not shown), suggesting that p62 accumulation is not cytotoxic towards quiescent cells. In fact, forced overexpression of p62 in primary culture hepatocytes lacking both *Atg7* and *p62* activated the expression of Nrf2 target genes but did not induce cell death (Supplementary Information, Fig. S9). We surmise that the hepatic injury caused by the *Atg7* deficiency is not attributable to cell-autonomous failure but rather to disintegration of the liver tissue as a result of hypertrophy of the hepatocytes. Indeed, both narrowing of sinusoidal capillaries and abnormal morphology of bile canaliculi were evident in *Atg7*-deficient liver (Figs 5e and 6e), suggesting that severe cholestasis and/or haemostasis might be the main causes of the liver injury.

Accumulation of p62, and/or p62-positive inclusions, has been reported in human liver diseases such as hepatocellular carcinoma, alcoholic hepatitis and α_1-antitrypsin deficiency[20]. We propose that in such pathological conditions, the high levels of p62 associated with the suppression of autophagy might result in activation of Nrf2. Further analysis is needed to clarify the regulation or dysregulation of the Nrf2–Keap1 pathway in these human diseases. □

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

M.K., K.T., I.U. and A.S. performed most of the experiments that characterized the knockout mice. I.U., Y.-S.S., Y.I. and A.K. performed the biochemical and cell biological experiments. Y.N. carried out microarray analyses. S.-i.I and T.N. performed mass spectrometric analyses. S.W. performed the histological and microscopic analyses. Structural and kinetics analyses were completed by H.K. and K.I.T. M.K., K.T. and M.Y. conceived the experiments. M.K., H.K., S.W., H.M., K.T. and M.Y. wrote the paper. E.K. and T.U. provided intellectual support. All authors discussed the results and commented on the manuscript.

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Review

The cellular pathways of neuronal autophagy and their implication in neurodegenerative diseases

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ABSTRACT

Autophagy is a tightly regulated cell self-eating process. It has been shown to be associated with various neuropathological conditions and therefore, traditionally known as a stress-induced process. Recent studies, however, reveal that autophagy is constitutively active in healthy neurons. Neurons are highly specialized, post-mitotic cells that are typically composed of a soma (cell body), a dendritic tree, and an axon. Despite the vast growth of our current knowledge of autophagy, the detailed process in such a highly differentiated cell type remains elusive. Current evidence strongly suggests that autophagy is uniquely regulated in neurons and is also highly adapted to local physiology in the axons. In addition, the molecular mechanism for basal autophagy in neurons may be significantly divergent from "classical" induced autophagy. A considerable number of studies have increasingly shown an important role for autophagy in neurodegenerative diseases and have explored autophagy as a potential drug target. Thus, understanding the neuronal autophagy process will ultimately aid in drug target identification and rational design of drug screening to combat neurodegenerative diseases.

1. Introduction

Autophagy is a conserved lysosomal degradation pathway. In mammals, three types of autophagy have been described: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). These three types of autophagy differ in their mode of delivery of their substrates to lysosomes for degradation [1]. While little is known about the microautophagy process, a large body of studies has contributed to the current understanding of the macroautophagy and CMA pathways. Macroautophagy is the prototype of autophagy involving formation, delivery, and degradation of autophagic vacuoles (also called autophagosomes) through lysosomes, and will be the only form discussed in this review (hereafter referred to as autophagy). Although autophagy occurs in virtually all cell types, and likely involves highly conserved molecular machinery, emerging evidence suggests cell type/tissue-specific regulation of autophagy.

Neurons were one of the few cell types that were used in the initial identification and characterization of autophagy. With early access to electron microscopy (EM) in the last century, Alex Novikoff, Christian De Duve, and colleagues discovered and described the cell "self-eating" process of autophagy in the form of distinct vacuoles through ultrastructural analysis [2,3]. The formation of the autophagosomes, which engulfed a portion of cytoplasm and occurred in a large number, especially following axotomy and excitotoxic insult to neurons, is associated with "chromatolysis", a phenomenon that describes the area in neuronal cytoplasm that is devoid of organelles and filled with various types of vesicles [4]. These initial observations of neuronal autophagic activity were followed by a series of EM studies that revealed the accumulation of autophagosomes in the neurons of several human neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). With little knowledge of autophagic process and regulation, especially in mammalian tissues, neuronal autophagy, marked by elevated levels of autophagosomes, was viewed traditionally as a cellular mechanism that was highly destructive, and therefore was suspected to be a driving force in cell death [5].

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The arrival of the molecular era of autophagy study provided important evidence showing that autophagy is a primary stress response for cell survival. For example, loss-of-function studies demonstrate the essential role for autophagy-associated genes in the removal of "obsolete" proteins and organelles, thus protecting cell or neuron survival [6,7]. Therefore, in injured neurons or neurons bearing disease-related genes, altered autophagy, associated with increased numbers of autophagosomes, can be viewed as a beneficial response of neurons in repairing or remodeling damaged cellular components necessary for sustaining normal neuronal function and survival. However, other studies also provide genetic and cellular evidence that otherwise argues for a role of autophagy in promoting neuronal death, especially in neurons with acute injury [8].

More recent studies have begun to dissect the autophagic process in neurons under various stress or pathological conditions. These studies suggest that, while accumulation of autophagosomes can arise from increased production in some cases [9], it can be caused by a mechanism that blocks fusion and degradation of autophagosomes through lysosomes in other scenarios [10]. Given the current studies exploring the potential of autophagy as a drug target for the treatment of neurodegenerative diseases, a thorough investigation of the detailed mechanism whereby autophagy participates in each disease condition, would be critical for designing therapeutic intervention and evaluating the efficacy of an "autophagy drug" [11].

Over the past several years, characterization of autophagy in mammalian cells and animal models has greatly advanced our knowledge of the autophagy process in mammals. However, despite the recent effort and exponential growth in autophagy research as a whole, the progress in understanding the basic process and regulation of neuronal autophagy remains relatively slow. Recent studies have revealed the connections between autophagy and major neurological disorders such as (AD), (PD), and (HD) [12–14]. A common theme emerging in those studies is the role of autophagy in degrading disease-related, aggregate-prone mutant proteins such as tau, huntingtin and alpha-synuclein [15]. In addition, specific pathogenic mechanisms of AD, PD, or HD may profoundly alter autophagic activity. For example, while "inappropriate" autophagic induction may contribute to the increased synthesis of β-amyloid (Aβ) [16], blocked autophagic clearance is also implicated in cytotoxicity in AD [10]. Recently, genetic animal models containing reduced autophagic activity were used to examine the role of autophagy in the pathogenesis of AD, HD, and PD. These studies provided important evidence linking dysfunctional autophagy to the specific disease process [17,18], Friedman and Yue, unpublished). Due to numerous recent reviews on the study of autophagy in PD, AD, or HD [12–14,19–21], this review will instead summarize recent research in understanding the basis of neuronal autophagy, especially in primary neuronal cultures and the nervous system of animal models, and autophagic activity associated with other types of neuropathological conditions. Although limited and sometimes conflicting in their current forms, these studies nonetheless begin to shed light on specific cellular pathways and the connection of the physiological function of autophagy to disease mechanism.

2. Biosynthesis of autophagosomes is conserved from yeast to human

Morphological evidence for autophagy was first reported in the 1960s [3], but the underlying molecular mechanisms were not elucidated for another three decades. In the early 1990s, genetic screens of yeast mutants identified a number of autophagy-related (*ATG*) genes essential for the autophagic molecular machinery [22,23]. Currently, 31 autophagy genes are known, many of which are required for autophagosome formation, at the nucleation, elongation, and/or fusion steps. Upon induction, an isolation membrane or phagophore forms and elongates, enveloping a portion of the cytosol, and encloses to form a double-membrane vacuole. Its outer membrane subsequently fuses with the lysosome, where its contents, together with the inner membrane, are digested by acidic hydrolases within the lysosomes (Fig. 1). Up to now, at least 14 mammalian homologues of yeast *ATG* genes have been identified, and characterization of their functions suggests that the autophagic machinery is highly conserved in mammals [24].

Studies in yeast revealed that, unlike endosomes and secretory vesicles, autophagosome formation does not require budding from existing organelles as its membrane source. Rather, autophagosomes may form from *de novo* membrane cisternae in the pre-autophagosomal structure (PAS), which contains several Atg protein complexes, and resides adjacent to the yeast vacuole [25–27]. It was shown that Atg9, the only known integral membrane protein associated with autophagic membranes, shuttles between a peripheral site in the cytoplasm and the PAS, therefore regulating the delivery of membrane to the PAS for expansion [27,28]. The Atg1 kinase complex, which contains regulatory subunit, Atg13, is involved in the retrieval of Atg9 from the PAS and is required for autophagosome formation [28,29]. Interestingly, the mammalian homologue of Atg9 (mAtg9) was shown to cycle between the trans-Golgi network (TGN) and late endosomes, which may serve as sources for membrane elongation. Starvation or rapamycin treatment induced redistribution of mAtg9 from TGN to peripheral late endosome membranes. Knock-down of Atg1 human homolog, ULK1, prevented this starvation-induced redistribution, suggesting that mAtg9 trafficking is ULK1-dependent [30].

In addition to the recruitment of membranes, Atg9 may also play a role in assembling protein complexes at the PAS [31]. Vps34, a class III phosphatidylinositol (PtdIns) 3-kinase mediates vesicular trafficking through its interactions with Vps15 and Atg6, and forms two distinct complexes: one with Atg14, which regulates autophagy-specific function, and the other with Vps38, for endosome-to-Golgi trafficking [32]. The PtdIns 3-kinase complex produces phosphatidylinositol 3-phosphate (PtdIns(3)P) which may recruit effector proteins, such as the Atg18–Atg2 complex, and together, both complexes are essential for nucleation [25]. The mammalian homologues of Vps34, Vps15 and Atg6 are hVps34, p150 and Beclin 1, respectively. Recent studies have found additional proteins that interact with the hVps34–p150–Beclin 1 complex: UVRAG [33], Atg14L (putative yeast Atg14 homologue) [34] and Rubicon [35]. These studies suggest the existence of multiple hVps34–Beclin 1 kinase complexes, which are involved in specific membrane trafficking mechanisms, including autophagy [35].

Two ubiquitin-like conjugation systems mediate autophagic membrane elongation; one involving the conjugation of Atg5 with Atg12 [36] and the other involving the covalent linkage of Atg8 with phosphatidylethanolamine (PE) [37]. Both Atg12 and Atg8 modifications share a single E1-like activating enzyme, Atg7, but are processed by two separate E2-like conjugating enzymes; Atg10 and Atg3 respectively [38]. The two ubiquitin-like conjugation systems are highly conserved from yeast to mammals [36,39]. The majority of Atg5 and Atg12 exists in the conjugated form and interacts noncovalently with multimeric protein, Atg16, in yeast and its functional counterpart, Atg16L, in mammals [40]. In one of the first studies linking the small GTPase Rab family to Atg proteins, Atg16L was shown to directly interact with Golgi-resident Rab33 and modulates autophagosome formation [41]. The Atg5–Atg12·Atg16 complex is essential for autophagosome formation and facilitates Atg8 conjugation with PE [40] through E3-like activity of Atg5–Atg12 for Atg8 conjugation [42].

Microtubule-associated protein 1 light chain 3 (LC3) is a mammalian homolog of yeast Atg8 and is cleaved at its C-terminal region by cysteine protease Atg4. This processed form (LC3-I) resides in the cytoplasm until it undergoes two ubiquitin-like modifications to become covalently linked to PE. Both the lipidated form LC3 (termed LC3-II) and Atg12–Atg5·Atg16 are recruited to the isolation membrane [38]. Whereas Atg12–Atg5·Atg16 dissociates upon autophagosome completion, LC3-II remains coupled to the autophagic

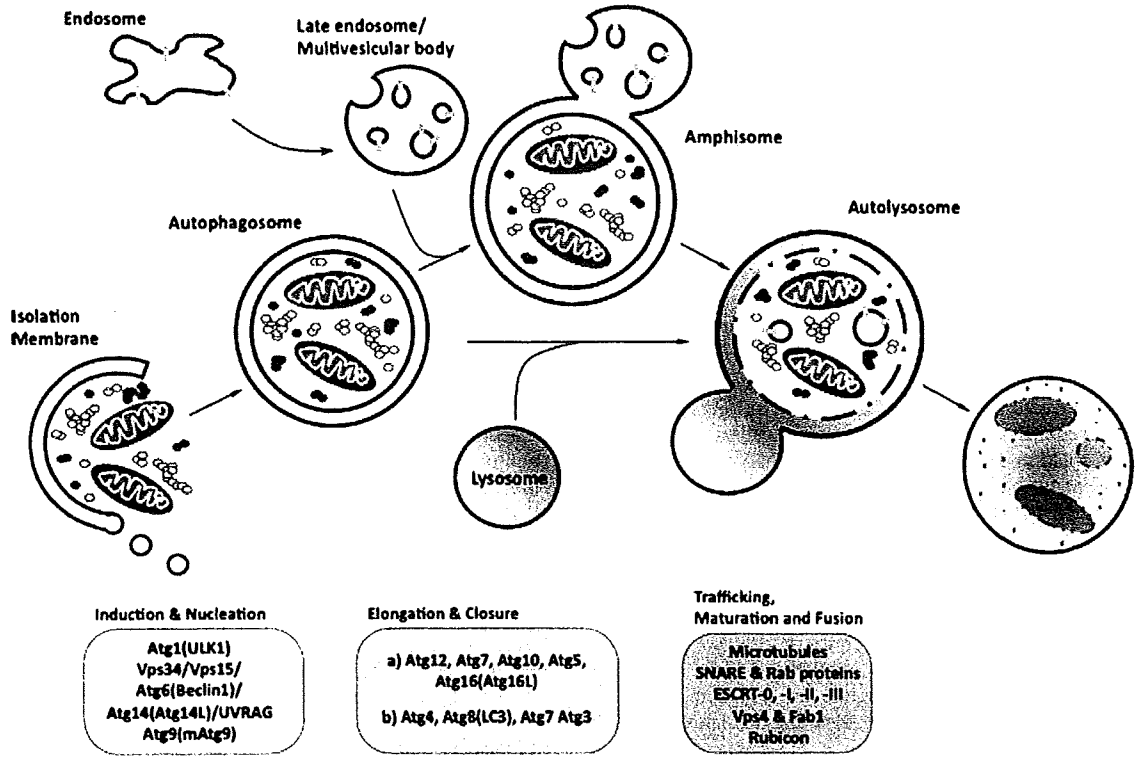

Fig. 1. A schematic representation of the macroautophagy process. Following induction, a phagophore or isolation membrane is formed. This process is regulated by Atg1 (Ulk1), Atg9, and the PtdIns 3-kinase complex, which includes Beclin 1, Atg14L, and UVRAG (nucleation). Two ubiquitin-like conjugation systems, which produce Atg8-PE (LC3II) and Atg5-Atg12, mediate the elongation of the isolation membrane, closure, and the formation a double-membrane vacuole known as the autophagosome. Autophagosomes can undergo maturation and fusion with early and late endosomes and MVBs, to generate the amphisome, followed by fusion with lysosomes, to form the autolysosome. Trafficking, maturation, and fusion events are mediated by microtubules and specific SNARE and Rab proteins. ESCRT proteins are also essential for MVB fusion with autophagosomes. Alternatively, immature autophagosomes can fuse directly with lysosomes.

membrane until fusion with the lysosome [39]. Prior to this fusion event, autophagosomes undergo a maturation process involving fusion with early and late endosomes, including multivesicular bodies (MVB), to form amphisomes [43]. Subsequently, these vacuolar bodies can then fuse with lysosomes to form the autolysosome (Fig. 1). These fusion events are mediated by SNARE proteins and Rab proteins, specifically Rab7, which is essential for maturation [44,45].

3. Neuronal autophagy: process and function in health and disease

3.1. Basal level autophagy

3.1.1. Unique feature of neuronal autophagy: scarcity of autophagosomes

Previous studies using mice expressing an autophagosome marker, green fluorescent protein-tagged LC3 (GFP-LC3), suggested that autophagy is distinctly regulated in different tissues [46]. For example, food limitation triggers a rapid upregulation of autophagy in liver and heart as indicated by the formation of a large number of GFP-LC3-associated autophagosomes, whereas it fails to induce GFP-LC3-labeled autophagosomes in the CNS, despite the strong expression of GFP-LC3 in many types of neurons. In addition, LC3 exists predominantly in a soluble form (LC3-I) in healthy neurons, and levels of the lipidated form (LC3-II) do not change after starvation [46]. Recently, we have investigated the localization of GFP-Atg5, a green fluorescent Atg5 fusion protein used for monitoring isolation membrane, in CNS neurons of transgenic mice [47], and the results show that like GFP-LC3, GFP-Atg5 is largely diffuse regardless of whether food is available or withdrawn (Yue, unpublished data). Indeed, ultrastructural analysis also confirmed scarcity of double-membrane vacuoles that are characteristic of autophagosomes in healthy neurons [48]. In contrast, many tissues, including liver and heart, have many GFP-LC3 puncta even when food is not limited, thus providing direct evidence for constitutively active autophagy under normal conditions.

The above evidence suggests that neurons normally maintain low levels of autophagosomes, and perhaps a low rate of autophagosome biosynthesis, even with the fluctuation of available nutrients. The current hypothesis is that neurons are prohibited from large-scale autophagy induction in response to starvation due to their ability to utilize multiple energy sources to maintain normal neuronal function. For example, glial cells provide nutrient and neurotrophin support to neurons and peripheral organs supply necessary nutrients to the CNS under hypothalamic regulation [49]. Thus, it is possible that even after 48 h of fasting in mice, it is insufficient to cause nutrient or trophic factor depletion in the CNS neurons due to compensatory mechanisms of nutrient supply. Interestingly, without showing the availability of nutrient or trophic factors, a recent study reported that mTOR activity (an important regulator of autophagy) was significantly reduced, at least in hypothalamic neurons, after 48 h of starvation in mice [50]. Despite the reduced mTOR activity, there was no report of the formation of GFP-LC3 or GFP-Atg5 puncta in hypothalamic neurons (Yue, unpublished data), [46]. While the mTOR activity in hypothalamic neurons of GFP-LC3 or GFP-Atg5 transgenic mice after 48 hour-starvation remains to be determined, other studies observed mTOR-independent regulation of autophagy in neurons [51,52]. Importantly, Young et al., showed that insulin plays a critical role in suppressing the induction of autophagy in primary neuron cultures. Through screening culture medium components, they found that insulin (but not other components, including amino acids) was the key factor and that

the absence of insulin induced autophagy in primary neurons, in an mTOR-dependent manner. Surprisingly, a highly potent Akt inhibitor also efficiently induces autophagy, however, via an mTOR-independent pathway [51]. This result suggests that the active insulin signaling is responsible for the low level of autophagosome formation in healthy neurons, and therefore, the insulin pathway may provide a critical mechanism for controlling basal autophagy in neurons. Although the autophagy control mediated by insulin signaling is not restricted to neurons [53], it can be adapted to the neuronal physiology that is related to the high consumption of energy via distinct sources. In addition, multiple parallel signaling pathways, including the mTOR pathway, exist in neurons to regulate autophagy.

Apart from the insulin pathway, specific proteins or protein modifications expressed in neurons may also contribute to additional regulation of basal autophagy in neurons. Previously, we showed that expression of a microtubule associated protein 1B (MAP1B), which is enriched in neurons and binds LC3 with high affinity, remarkably affects the formation of GFP-LC3 puncta (autophagosomes), and this effect is dependent on the status of certain modifications of MAP1B [54], (see section 4.2 of this review).

Taken together, we propose that the regulation of basal autophagy in CNS neurons at least involves the following mechanisms: (1) a non-cell-autonomous mechanism whereby nutrients, hormones (insulin), or neurotrophic factors are supplied by peripheral organs or glial cells, (2) a cell-autonomous control mechanism by intrinsic nutrient-mediated signaling or specific factors expressed in neurons. Furthermore, current evidence also raises the question as to what context or extent does mTOR-mediated signaling regulates autophagy in neurons.

While the hypothesis for the low level of basal autophagy (in the form of "classical" autophagosomes) and its regulation remains to be tested further, a recent study provides evidence suggesting an alternative hypothesis that autophagosomes, once formed, are rapidly cleared by fusion with lysosomes in primary neuronal culture. It further proposes that the clearance of autophagosomes by basal autophagy is highly efficient in neurons and accounts for the low levels of autophagosomes [10]. Apparently, future experiments focused on directly measuring or imaging the dynamics of autophagy flux will be needed to solve this issue.

3.1.2. Essential role of basal autophagy in cellular homeostasis and neuroprotection

Current evidence suggests that neuronal autophagy is tightly regulated and autophagosomes are maintained at minimal levels. However, recent studies in mutant mice with targeted deletion of *Atg5* or *Atg7* specifically in brain, unequivocally demonstrate the importance of basal autophagy in CNS neurons [6,7]. Unlike *Atg5*- or *Atg7*-deficient yeast or mammalian MEF cells that survive and grow normally with regular culture conditions, CNS neurons lacking *Atg5* or *Atg7* in animal models undergo progressive degeneration, even when mice are housed under normal conditions. Importantly, *Atg5* or *Atg7* deficient neurons develop a massive number of inclusion bodies that are labeled by ubiquitin, suggesting that continuous autophagy is required to prevent the build-up of intracytoplasmic protein aggregation. These studies provide undeniable proof for the existence and critical function of basal autophagy in neurons. Paradoxically, the evidence that autophagy is in continuous action through autophagosome–lysosome degradation is at odds with the scarcity of autophagosomes or low level of basal autophagy in the healthy neurons. Although it appears to be consistent with the hypothesis of highly efficient basal autophagy [10], an alternative hypothesis is that basal autophagy in neurons proceeds via a distinctive mechanism (e.g. in the absence of "classical" autophagosomes formation). Even though autophagy genes (ATG system) are required, they participate in the lysosomal degradation pathway that differs from canonical autophagy (in the presence of well-documented autophagosome formation) [55]. One well-known example for such an autophagy-related cellular process, is the yeast Cvt (cytoplasm-to-vacuole targeting) pathway, which shares most of its protein components with autophagy, but is biosynthetic and functions to specifically deliver certain enzymes to vacuoles [56]. Future studies should investigate the possibility of non-canonical or adapted autophagy in neurons.

Although CNS neurons are generally vulnerable to the loss of the autophagy pathway, the degree of vulnerability and the formation of intracellular inclusions vary significantly among different neuron types as observed in the mutant brains deficient in *Atg5* or *Atg7*. The ablation of the *Atg5* or *Atg7* gene may trigger a cell-type specific cellular response to autophagy deficiency and/or a cell type-dependent mechanism contributing to the neurotoxicity. For example, Purkinje cells deficient in *Atg5* or *Atg7* display very few ubiquitin-associated inclusions, whereas these cells are among the most vulnerable neuron types [57,58]. In contrast, a large number of ubiquitin-associated inclusions are seen in the brain region where neuronal loss was hardly detected when autophagy was genetically inhibited (Waguri S, Komatsu M, unpublished data). While it is possible that the compensatory pathways to the loss of autophagy vary in different neuronal types and account for the difference in their vulnerability, it may also reflect the disparity in their intrinsic demands for autophagy, as well as relative levels of basal autophagy. Neurons exposed to high levels of various stresses may contain high levels of basal autophagy and are therefore, far more vulnerable, whereas the neurons that require low levels of basal autophagy through their lives are relatively resistant to autophagy deficiency.

3.2. Induced autophagy in neurons: signaling and distinctive stress response

The primary function of autophagy is to provide cellular response to nutrient limitation by mobilization of autophagosome–lysosome degradation pathway. A large body of evidence shows that nutrient-related signaling pathways regulate autophagic activity in mammals. These pathways include insulin and amino acid pathways, mTOR kinase complex [59–61], AMP-activated protein kinase (AMPK) [62] and Beclin 1/Vps34 (class III PI-3 kinase, PtdIns3K) lipid kinase complex [63]. Despite the growing evidence linking these signaling pathways to autophagy regulation, the details for how they control the complex process of autophagy is lacking. Furthermore, little is known about tissue/cell type-dependent regulation. A recent study in primary cortical neuronal culture shows that insulin plays a critical role in controlling the induction of autophagy [51].

A unique feature of neurons, with respect to their fuel supply, is that they use glucose (or ketones) almost exclusively as a blood-borne energy substrate to provide energy and carbon chains for protein synthesis. Therefore, compared to other tissues/cell types, CNS neurons may depend less on autophagy to provide free amino acids or energy under physiological conditions. It can be further speculated that the primary function of neuronal autophagy at the basal level is different than as a primary nutrient-starvation response [49]. Accordingly, neurons are likely to use a distinctive mechanism for autophagy regulation. Adding to the complexity of autophagy regulation in neurons, a recent study shows that cultured neurons from male rats more readily undergo autophagy in response to 24 h of nutrient deprivation compared to female neurons [64], indicating gender differences in autophagic capacity of neurons.

While nutrient-related pathways are likely the conserved mechanism for the control of neuronal autophagy [10,51], the direct *in vivo* evidence that these pathways contribute to the induction of autophagy (e.g. synthesis of a large number of autophagosomes) in CNS neurons remains to be shown. Despite the lack of *in vivo* evidence of neuronal autophagy mediated by nutrient signaling, recent studies in animal models indicate that, in contrast to nutrient starvation which rarely induces autophagy, a variety of stress-related signals,

neuron injuries, and neuropathogenic pathways cause rapid formation and accumulation of autophagosomes in neurons. As a result, the intracytoplasmic area occupied by autophagic activity is sharply increased and the capacity of autophagic degradation could be maximally expanded. During the process of autophagy induction, neurons may undergo a significant change in autophagy regulation, involving a deregulation process that allows neurons to transition from basal level (neuron-specific process) to the activated state (well-conserved and known as induced autophagy) involving large-scale biosynthesis of autophagosomes. Here we summarize some examples of induced autophagy in neurons.

3.2.1. Hypoxic-ischemia

A combined hypoxic-ischemia (H/I) procedure in rodents has been widely used as a model for studying human ischemia or stroke. H/I causes extensive CNS neuron damage or injury and consequent neuronal death. Two recent studies of mouse models, one in neonatal brains [8] and the other in adult brains [65], showed that H/I induced robust formation of autophagosomes within a very short period of time (hours), concomitant with increased production of LC3-II levels. This induced autophagy occurred in different types of neurons in the hippocampus and striatum, suggesting that it is not cell type-specific. The rapid appearance of autophagic hallmarks in these two *in vivo* models demonstrated that H/I is a powerful stimulus for autophagy induction in CNS neurons. At the cellular level, H/I produces pro-inflammatory cytokines and simultaneous activation of both pro-survival (e.g., upregulation of Hsp70, phosphorylation of ERK and AKT) and pro-apoptotic signaling pathways (e.g., release of cytochrome *c* and AIF from mitochondria, cleavage of caspase-9 and -8) [65]. Therefore, at present, it is unknown which signals of autophagy induction are triggered by the H/I procedure. Interestingly, Koike et al. further demonstrates that mice deficient in essential autophagy gene, *Atg7*, show nearly complete protection from H/I-induced neuron death, arguing strongly for the involvement of autophagy in a pro-death pathway in this specific setting [8]. Although the significant role of autophagy in triggering cell death pathways has yet to be clarified, this study suggests that H/I-induced autophagy, rather than an epiphenomenon of neuronal death, is an active process that controls cell fate. More importantly, inhibition of autophagy represents an attractive strategy for drug design in alleviating neuronal damage associated with H/I injury.

3.2.2. Excitotoxic stimuli

Many excitotoxic stimuli provoke neurodegeneration through poorly defined cell mechanisms. The Clarke group has previously used NMDA (glutamate mimetic)-treated organotypic hippocampal slices to investigate this mechanism [66]. They found that, within 2 h following the drug administration, numerous autophagosomes appeared in CA1 and CA3 pyramidal neurons, concomitant with signs of neurodegeneration. Moreover, this induction of autophagosome formation was inhibited by treatment of a JNK-signal blocking peptide, which also prevented neuronal death [66]. In another excitotoxicity-related model with focal injection of kainic acid, hippocampal extracts contain a significant increase in LC3-II levels as early as 4 h following the injection [67]. Consistent with autophagic activity elicited in hippocampus, local administration of kainic acid caused a rapid increase in autophagosomes and autolysosome formation in the cytoplasm of striatal cells. In addition, it also augmented the ratio of LC3-II/LC3-I, LAMP2, cathepsin B, release of cytochrome *c*, and activation of caspase-3, suggesting stimulation of both apoptosis and autophagy [68]. Pre-treatment of autophagy inhibitor 3-methyladenine (3-MA) reversed the change in LC3-II levels, autophagosome formation, and loss of striatal cells [68]. In *Lurcher* mice, a genetic mouse model of excitotoxicity associated with Purkinje cell-specific degeneration, constitutive activation of glutamate δ2 receptor results in autophagy induction, preceding Purkinje cell degeneration. Moreover, the mutant glutamate δ2 receptor-mediated cell toxicity in transfected cells was blocked with autophagy inhibitor 3-MA [69].

Current knowledge indicates that excitotoxicity occurs when excitatory glutamate receptors, such as those for NMDA or kainic acid, are overexcited by high dosage of these ligands. The activated receptors allow the receptor channels to open, resulting in Ca^{2+} influx and consequent activation of a number of enzymes, including phospholipases, endonucleases, and proteases, such as calpain. Recent studies show that Ca^{2+} triggers strong stimulation of autophagic activity [70]. Therefore, it is possible that excitotoxic-related signals induce elevated levels of autophagy by a common Ca^{2+}-mediated pathway. Furthermore, the above studies suggest that manipulation of autophagy, by blocking its activation, may be beneficial in the treatment of excitotoxic-associated neuronal death.

3.2.3. Methamphetamine and MPP^+

Administration of methamphetamine (METH) in animals generally produces selective degeneration of dopamine (DA) neuron terminals without significant cell body loss [71]. This model is currently used to study specific aspects of neuron terminal dystrophy and degeneration in PD. To investigate the mechanism underlying the METH-mediated toxicity, the Sulzer group found that METH treatment promoted the rapid formation of autophagosomes, particularly in neuronal varicosities and, ultimately, within cell bodies of DA neurons from midbrain neuronal cultures [72]. A recent study in PC12 cells showed a similar response to METH administration, and provided additional evidence that suppression of autophagy, by blocking PI-3K class III activity, precipitates neuronal death. This study implicates a beneficial role of METH-induced autophagy in neural protection, perhaps through repairing or remodeling injured neurons [73].

A related PD cell model is the treatment of the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), which produces mitochondria-targeted injury and contributes to parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine in mammals. Administration of MPP^+ in primary DA neuronal culture elicited an increased profile of intracellular autophagosomes, which was suppressed by extracellular signal-regulated protein kinase (ERK) kinase, a MEK inhibitor [74]. However, it is unclear why 3-MA, an inhibitor for autophagy, did not impede autophagosome formation induced by MPP^+ in this setting. Interestingly, RNAi knock-down of autophagy gene LC3 or block of MEK with a specific inhibitor in MPP^+-treated human neuroblastoma SH-SY5Y cells reduced formation of autophagosomes and cell death. One plausible explanation for MPP^+-induced autophagy is through injured mitochondria, which elicited signals involving MEK for increased autophagic activity. Although the result shows that reduced autophagy is correlated with delay of cell death, whether or not autophagy acts as an executor of cell death is unclear [74].

3.2.4. Proteasome inhibition: a compensatory induction

Emerging evidence implicates inhibition of the ubiquitin–proteasome system (UPS) in neurodegenerative diseases, such as AD and PD. Autophagy-lysosomal degradation and UPS are the two major proteolytic pathways, but they differ in various aspects including their substrates, capacity, and molecular and cellular machinery. Previous study of the effects of chronic low-level proteasome inhibition on neural homeostasis indicated that inhibition of UPS causes increased autophagic activity in clonal SH-SY5Y cells [75]. The view that increased autophagic activity is a compensatory response of impaired UPS system is supported by a few other studies. Pandey et al. used the fly genetic system to provide *in vivo* evidence that significantly strengthened and expanded this view. They showed that histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase that interacts with polyubiquitinated proteins, plays a critical role in this compensated induction of autophagy for impaired UPS function. Moreover, they found that forced expression of HDAC6 is able

to rescue degeneration associated with UPS dysfunction in an autophagy-dependent fashion [76]. This study raises an important question regarding the cross-talk between the two main cellular degradation routes – autophagy and UPS – and whether or not HDAC6 is positioned at the intersection of these two pathways. Interestingly, a more recent report shows that genetic deletion of 26S proteasomes in mouse brain causes neurodegeneration and Lewy-like inclusions in midbrain DA neurons, which is accompanied by increased expression of autophagy genes and formation of numerous vacuoles resembling autophagosomes [77]. Although the mechanism of interaction between the two pathways is unclear, the compensatory up-regulation of autophagy for degradation of protein aggregates, which normally exceed the capacity of UPS degradation, should be explored as drug target for the treatment of neurodegenerative diseases.

3.2.5. *Lysosomal enzyme/lipid storage or suppression: autophagy "vicious circle"*

A number of studies exploring autophagy in lysosome-related neurological diseases provide a new understanding of the pathogenic mechanisms of "old" diseases. A subset of these diseases, also known as lysosomal storage disease, is caused by deficiencies of lysosomal hydrolases, resulting in accumulation of undigested materials in the lysosomal or related compartment. These disorders include Niemann–Pick type C (NPC) [78,79], mucolipidosis type IV with mutations in Mucolipin 1 (MCOLN1), multiple sulfatase deficiency and mucopolysaccharidosis type IIIA [80]. A common cellular pathologic feature in these diseases and their animal models is the manifestation of aberrant autophagic activity as evidenced by the accumulation of autophagosomes and increased levels of LC3-II, accompanied by neurodegeneration. Direct deletion of the genes encoding lysosomal enzymes such as cathepsin D (CD−/−) or combination of cathepsin B and L (CB−/− and CL−/−) in mouse brain, resulted in a similar phenotype [81]. The current view is that lipid trafficking molecules or lysosomal enzymes are required for the delivery, fusion or clearance of autophagosomes; deficiencies in these molecules causes disruption of this process, resulting in build-up of autophagosomes/autolysosomes unable to complete the digestion. This view supports a hypothesis that impaired autophagic activity is part of the disease mechanism for lysosomal storage diseases due to the inability of neurons to clear autophagosomes. However, these studies also provide evidence that otherwise amends this hypothesis. Pacheco et al. showed that, in addition to impaired autophagy flux, there is also induction of autophagy in NPC by signaling through a complex of the class III phosphoinositide 3-kinase/beclin-1 [79]. A significant number of nascent autophagosomes (immature or before fusion with lysosomes) are found in CD−/− or CB−/− and CL−/− brains, suggesting that they are newly synthesized autophagosomes [81]. In a study of fibroblasts obtained from patients carrying mucolipidosis type IV mutation, Vergarajauregui et al. suggest that the autophagosome accumulation is due to increased *de novo* autophagosome formation and delayed fusion of autophagosomes with late endosomes/lysosomes [80]. Therefore, the available evidence indicates that there is induced synthesis of autophagosomes in addition to the compromised autophagosome fusion or clearance in lysosomal storage diseases.

The deficiency in lysosomal degradation is expected to affect the net outcome of all forms of autophagy, including macroautophagy, microautophagy and chaperon-mediated autophagy. In line with the concept that blocking one type of degradation (e.g., proteasome or CMA) stimulates compensatory pathways (e.g. autophagy), intracellular accumulation of non-degraded materials resulting from lysosomal enzyme deficiency is expected to feed back to the upstream signaling which triggers the biosynthesis of autophagosomes. Since autophagosome degradation through lysosomes is impaired, it creates a "dilemma" for neurons to dispose autophagosomes, amassing a large number of autophagosomes of various stages. Thus, we propose a hypothesis that explains the sequence of the events in the disease, considering lack of detectable autophagosomes in healthy neuron. The accumulation of undigested materials due to lysosomal deficiency triggers *de novo* autophagosome biosynthesis in lysosomal storage disease. Impaired lysosomal degradation will trap these autophagosomes, causing build-up of the induced autophagosomes, and more undigested materials. This can continue in circles, and as a result of this rampant autophagy "vicious circle", neurons may exhaust their energy and generate various toxic species that are harmful.

3.3. *ESCRT: autophagy meets endocytosis*

Previous studies from the Seglen group showed that autophagosomes can fuse with endosomes and form a hybrid product, termed amphisomes, providing early evidence of interaction between autophagy and endocytosis [82]. Although this observation suggests the convergence of autophagosomes and endosomes, perhaps under specific settings, the significance of this process has not been fully appreciated. Recently, several studies showed that mutations in CHMP2B, a subunit of the endosome sorting complex required for transport (ESCRT)-III, are linked to frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) and prevents autophagic degradation [83]. Along with these studies, genetic and ultrastructural analysis in *Drosophila melanogaster* showed that subunits of the ESCRT- I, -II and -III, as well as their regulatory ATPase Vps4 and the endosomal PtdIns(3)P 5-kinase Fab1, are all required for normal autophagy function [84,85]. These studies are important in several aspects. First, they reveal a novel mechanism involving dysfunctional autophagy in the pathogenesis of specific types of FTD and ALS. Second, since the ESCRT proteins are known to function in the sorting of transmembrane proteins into the inner vesicles of the multivesicular body (MVB) during endocytosis [86], these studies suggest that MVB or endocytic pathways are critical routes for the trafficking of autophagosomes to lysosomes, and defective ESCRT function prevents fusion or maturation of autophagosomes. Third, they suggest that neurons are particularly vulnerable to abnormal function in the autophagosome-MVB (endosomes) pathway, and this may be associated with dendritic maintenance [83].

Several outstanding questions, however, arise from these studies. The ESCRT machinery is evolutionarily conserved [86], and its function in MVB biogenesis has been shown in many species including yeast and human. Curiously, although ESCRT/MVB, as well as autophagy, are highly conserved and well-characterized in yeast, the interaction between ESCRT/MVB and autophagy has not been demonstrated. Therefore, it raises a question whether the ESCRT-MVB pathway is only important for autophagy in higher eukaryotes, including fly and mammals. Furthermore, amphisomes are not detected in yeast, and autophagosomes fuse directly with the vacuole, which is mediated by SNARE proteins [87]. A related question is whether ESCRT-MVB represents the primary, if not the only, route for delivery of autophagosomes to lysosomal degradation, and whether this route occurs only in specific cell/tissue context. Previous studies also suggest that a considerable proportion of endocytosed cargo merges with autophagic pathway prior to being degraded by lysosomes in neurons [20].

It is also conceivable that the ESCRT-MVB pathway is actually part of the yet-to-be-defined basal autophagy process in neurons. Formation of ubiquitin- or p62/SQSTM1-labeled inclusions and autophagosomes accumulation are two important features associated with cells or neurons deficient in ESCRT components. Interestingly, time-course studies show that the formation of ubiquitinated inclusions occurs prior to the appearance of autophagosomes in cortical neuronal culture expressing mSnf7-2 siRNA or CHMP-2B^{intron5}. The early formation of ubiquitinated protein inclusions in these neurons defective in ESCRT function phenocopies results from *Atg5* or *Atg7* deficient neurons [6,7], suggesting that the ESCRT-MVB pathway is likely related to the *Atg5* or *Atg7*-regulated function, which is required

for the maintenance of protein homeostasis or basal autophagy function. Since impaired ESCRT pathway does not affect biosynthesis of autophagosomes (unlike *Atg5* or *Atg7*), autophagosome biosynthesis is induced as a response to the increasing levels of ubiquitinated proteins. Thus, an alternative hypothesis is that formation of ubiquitin- or p62-labeled protein inclusions is the early response of neurons to the deficiency in basal autophagy caused by dysfunctional ESCRT, whereas the accumulation of autophagosomes is the secondary response to the increased levels of undigested protein and/or a result of impaired ESCRT function, which may be related to autophagosome fusion or maturation. The relationship of ESCRT with basal autophagy should be explored in the future.

4. Specialized neuronal autophagy in the axons

4.1. Role of neuronal autophagy in axonal homeostasis

The axon is a highly specialized neuronal compartment that performs many functions independently from the soma. Accumulating evidence has revealed that, not only is autophagy uniquely regulated in the neuron, but it may also have a distinct function and regulation within the axonal compartment, independent from the dendrites and soma. Early morphological evidence showed that, following axotomy [88] or excitotoxic injury [4], double-membrane vacuoles resembling autophagosomes started accumulating and localized in dilated axon terminals. In later studies, Hollenbeck described the sequestering of autophagic substrates in autophagosomes at the most distal region of the axon, which were retrogradely transported to the soma [89]. Mammalian autophagosomes form in various regions throughout the cytoplasm. Upon maturation, they move toward lysosomes, which are primarily located in the juxtanuclear cytoplasm of the cell body [90,91]. More recently, autophagosome formation in response to an excitotoxic insult was monitored in *Lurcher* mice expressing GFP-LC3. The constitutive activation of the GluRδ2 receptor resulted in an excitotoxic injury that triggered a rapid and robust accumulation of GFP-LC3-labeled autophagosomes in axonal dystrophic swellings [54]. The link between increased autophagy activity and axonal dystrophy would suggest local biosynthesis of autophagosomes at the distal axon terminals.

To elucidate the physiological role of neuronal autophagy, mutant mice containing a neural cell type-specific deletion of *Atg7* were generated. Establishment of these mutant mice allowed the study of cell-autonomous events in cerebellar Purkinje cells deficient in autophagy. Characterization of the mutant Purkinje cells revealed the accumulation of aberrant organelles and membrane structures in dystrophic axon terminals. This result suggests a specific role for neuronal autophagy in the maintenance of membrane homeostasis at the axon terminal. We hypothesize that this highly specialized neuronal autophagy is required for keeping the balance of the membrane network, which normally involves cycling of membranous structures or vesicles, at the axon terminals to support synaptic activity. This particular function of autophagy is no surprise considering that the typical autophagic process involves dynamic membrane rearrangement and turnover. Future study should investigate in detail how this "self-eating" process participates in axonal membrane turnover and what membrane substrates axonal autophagy removes under physiological conditions. The answers to these questions are expected to advance our current understanding of autophagy in the neuron, as well as the disease processes that are associated with dysfunctional autophagy in the neuron and the axon.

4.2. MAP1B-LC3 interaction regulates autophagosome formation and possibly axonal transport

LC3 is best known as an autophagosome-associated protein. Despite the abundance of LC3 in neurons, the vast majority of LC3 is in soluble form and is not associated with autophagosomes. To understand neuron-specific autophagosome formation, Wang et al. identified and analyzed the interaction between LC3 and microtubule-associated protein 1B (MAP1B), which is an abundant protein in the axons and plays an important role in regulating microtubule stability. They found that MAP1B binds to LC3 with high affinity, and overexpression of MAP1B in non-neuronal cell culture significantly reduces the number of LC3-associated autophagosomes, presumably through the MAP1B–LC3 interaction [54]. Since MAP1B (or MAP1A) is highly expressed in neurons, this result may potentially explain the scarcity of the autophagosomes in healthy neurons and the lack of GFP-LC3 puncta in GFP-LC3 transgenic brain. In contrast to MAP1B, phosphorylated MAP1B (MAP1B-P) was found to be associated with LC3-autophagosomes. It was further shown that LC3 was associated with increased levels of MAP1B-P in dystrophic axon terminals, coincident with the presence of a large number of autophagosomes [54]. Moreover, during development, MAP1B-P is highly expressed and is most concentrated in the distal axons of growing neurons [92]. The conserved role of MAP1B-P in axonal growth or repair during development or injury implicates autophagy in the remodeling of axon terminal structures for regeneration. Thus, we propose that interactions of LC3 with MAP1B or MAP1B-P provide a mechanism that regulates autophagosome formation in neurons or the axons.

The hypothesis that neuronal autophagy maintains axonal homeostasis is also in line with the idea that autophagy substrates are removed from the axons through axonal transport back to the soma where lysosomes perform degradation [89,93]. Interestingly, recent studies have revealed a role of MAP1B in retrograde axonal transport [54]. It is possible that the MAP1B-P–LC3 interaction may regulate axonal transport of "autophagic cargo". In support of this idea, microtubules and the dynein motor complex were previously shown to be required for autophagy process [90]. Dynein, the minus-end directed motor protein, mediates retrograde transport and interacts with the dynactin protein complex, which is implicated in cargo binding [94]. Inhibition of dynein impeded rapid movement of autophagosomes in mouse embryonic fibroblasts (MEF) cells and inhibited clearance of mutated alpha-synuclein or huntingtin by preventing fusion with the lysosome [90,95]. Furthermore, microtubule networks may facilitate the transport of LC3-positive autophagosomes [96], but are not required for fusion with lysosomes [54,97,98].

Although limited, recent studies involving live imaging showed that GFP-LC3 "dots" undergo retrograde transport in primary cerebellar granule cells [93]. In addition, both anterograde and retrograde transport of GFP-LC3-labeled autophagosomes were detected in neurites of differentiated PC12 and normal rat kidney cells [91,99]. Although bidirectional movement was observed, Jahreiss et al. propose an overall bias for microtubule-dependent movement toward the nucleus, which is mediated by dynein [91]. In summary, we anticipate that future study, using live-imaging, should elucidate the dynamic process of autophagy in neuron or axon.

4.3. Ulk1 and axonal autophagy

Additional evidence supporting the hypothesis that autophagy is important in axonal transport and function comes from the study of yeast Atg1 and its homologue, Unc-51 (*C. elegans uncoordinated-51*). Atg1/Unc-51 is a highly conserved serine/threonine kinase. In initial *C. elegans* screenings, *unc-51* mutants displayed disruptions in axon ultrastructure, including the presence of abnormal membranous structures in the axon and large swellings in the terminal [100]. The murine homologue, Unc51.1, is required for neurite extension in primary cerebellar granule neurons and governs vesicular membrane organization during outgrowth [101]. Thus, Unc-51 may provide a potential link between local membrane dynamics and autophagy at the axon terminal. Interestingly, the human homolog, Unc-51-like

kinase 1 (ULK1), interacts with two MAP1B-LC3 related proteins: Golgi-associated ATPase enhancer (GATE-16) and $GABA_A$ receptor associated protein (GABARAP), and have recently been shown to associate with p62/SQSTM1 [102,103]. Nerve growth factor (NGF) binding with TrkA receptors facilitates K-63 polyubiquitination of ULK1, promoting its association with the UBA domain of p62/SQSTM1 and recruiting ULK1 into the active TrkA complex. ULK1 interacts with syntenin and SynGAP (which regulate endocytosis) and results in the trafficking of NGF-bound TrkA receptors into endocytic vesicles [104]. These results provide a possible mechanism for the cross-talk between two important membrane trafficking pathways – autophagy and endocytosis – and their role in axonal physiology. One possibility is that, by fusion with autophagosomes, some types of membrane compartments, including endosomes, are removed from the axons and delivered to lysosomes for degradation. This process may be essential to maintain the homeostasis of the axonal membrane network [58].

4.4. Axonal autophagy and axonopathy

Axonal dystrophy, a hallmark of axonopathy, can be triggered by neuronal injuries, excitotoxicity, and various neurodegenerative conditions. Despite the prevalence of this pathology, the molecular mechanisms underlying axonopathy, as well as the connection between axonopathy and neurodegeneration, remain poorly understood. Dysfunctional autophagy has recently been implicated in axonal dystrophy. Severe axonal swellings were observed when autophagy was suppressed in the mouse brain [6,7]. Genetic ablation of *Atg7*, specifically in Purkinje cells of mutant mice, however, resulted in cell-autonomous axonal dystrophy and degeneration, implying an essential role for autophagy in membrane trafficking and turnover in axons. In contrast to *Lurcher* mice, axon terminals of *Atg7* deficient mice lacked autophagosome-like vesicles, but amassed abnormal organelles and membranous structures [58]. Moreover, mice with Purkinje-cell specific deletion of either *Atg5* or *Atg7*, exhibited axonal dystrophy much earlier than dendritic tree atrophy and cell death [57,58]. Thus, overactive or insufficient autophagy may contribute to axonopathy, which is a prominent feature of human neuropathology.

Accumulating evidence indicates that axonal degeneration precedes neuronal cell body death and undergoes a self-destruct mechanism that is distinct from apoptotic death in the soma [105,106]. A classic example of axonal degeneration is Wallerian degeneration, in which transected axons or neurites undergo complete fragmentation distal to the site of injury [106]. In mice carrying the spontaneous-occurring slow Wallerian degeneration (*Wld*^{s}) mutation, Wallerian degeneration is markedly delayed following transection. However, the "dying back" model, a progressive retrograde degeneration of the distal axon, may better reflect the chronic injury associated with neurodegenerative diseases [106]. NGF deprivation induced accumulation of autophagosomes in the distal tips of PC12 neurites supporting the hypothesis that autophagy induction contributes to dying back degeneration in which distal neurites are more fragile than proximal segments. Indeed, knocking down levels of *Atg7* or *Beclin1* caused a significant delay of neurite degeneration after NGF deprivation in sympathetic neurons [107]. These findings suggest that autophagy activation contributes to neurite degeneration, thus providing a valuable clue about the possible involvement of autophagy in the mechanism of axonal degeneration.

In summary, based on current evidence, we propose that basal autophagy plays an important role in the maintenance of axonal homeostasis by the removal of "autophagic cargo" from distal axons or terminals through retrograde axonal transport. Although the "autophagic cargo" can be associated with LC3, it is largely unclear whether they are in the form of typical autophagosomes [55]. Future study should investigate the nature of "autophagic cargo" in healthy neurons or axons. In contrast, pathological conditions can induce autophagy involving stimulated local synthesis of autophagosomes in axons. Degradation by the autophagic process may occur in the axons, which may require the accessibility of lysosomes or late endosomes in the axons. Or degradation may take place in the soma, which then requires retrograde transport of autophagosomes. Nonetheless, the inability of axons to either degrade or transport a large number of autophagosomes produced locally would have deleterious consequences to the axons, causing axonal dystrophy and degeneration (Fig. 2).

5. Specific function of p62/SQSTM1 in autophagy-mediated protein degradation

5.1. Role of neuronal autophagy in protein homeostasis

One of the critical findings in the study of *Atg5* or *Atg7*-deficient neurons is the recognition that autophagy is required to

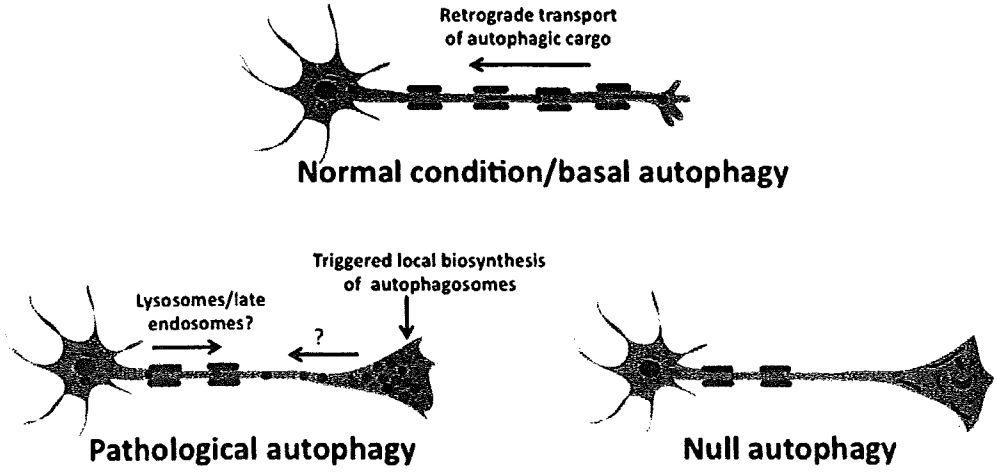

Fig. 2. Proposed model for neuronal autophagy in the axons. Under normal conditions, basal autophagy maintains axonal homeostasis by removal of "autophagic cargo" (top). The "autophagic cargo" undergoes retrograde axonal transport to the soma and fuses with lysosomes for degradation. Stress or injury can induce local biosynthesis of autophagosomes, resulting in their accumulation in axons and axon terminals. It has been suggested that, under pathological conditions, precursors of degradative vesicles or lysosomes may be anterogradely transported to the axon terminal and contribute to the degradation of autophagosomes (bottom left). Neurons deficient in autophagy amass proteins, organelles, and aberrant membrane structures at axon terminals, resulting in gross axonal swellings or dystrophy (bottom right).

suppress spontaneous protein aggregation. Loss of these autophagy genes causes accumulation of polyubiquitinated proteins. These appear as inclusion bodies, which increase in size and number with aging. However, proteasome function, which is generally known to cause abnormal ubiquitin-mediated proteolysis when impaired, is not compromised. Thus, blocking neuronal autophagy results in delayed global turnover of cytoplasmic components, accumulation of misfolded and/or unfolded proteins, followed by ubiquitination and the formation of inclusion bodies. Furthermore, these findings reveal an important role for basal autophagy in the protein clearance or protein homeostasis in neurons. While autophagy is absolutely required to degrade proteins for the maintenance of homeostasis in healthy neurons, its function becomes even more prominent in neurons expressing disease-related proteins. Recent studies show that autophagy contributes to the degradation of aggregate-prone proteins such as polyglutamine containing proteins and mutant alpha-synuclein [15]. Although it remains largely unclear how autophagy may achieve selective degradation of these disease-related proteins, expression of disease-related proteins in cells or neurons stimulates autophagy activation, and elevated autophagy is correlated with reduced levels of protein aggregates, concomitant with a decrease in neurotoxicity [61,108].

5.2. Link of p62/SQSTM1 to autophagic degradation

Emerging evidence suggests that p62/SQSTM1, originally identified as an ubiquitin-associated protein, provides a link between autophagy and selective protein degradation. Recent studies show that p62/SQSTM1 is an LC3-interacting protein [103,109]. Because p62/SQSTM1 can bind a large number of proteins through its multiple protein–protein interaction motifs, it may mediate diverse signaling pathways including cell stress, survival, and inflammation. Structural analysis reveals that p62/SQSTM1 N-terminal *P*hox and *Bem1* (PB1) domain exhibits self-oligomerization, and the C-terminal *ub*iquitin-*a*ssociated (UBA) domain can bind ubiquitinated proteins, suggesting a link between p62/SQSTM1 and disease-related protein inclusion formation. Consistent with the notion that p62/SQSTM1 is likely more related to autophagy-lysosome degradation, lysosomal inhibition, but not proteasomal inhibition, resulted in marked accumulation of p62 as well as LC3-II. Moreover, ablation of autophagy in neurons leads to a rapid and robust increase in p62 protein levels [54,109]. These observations suggest that p62 is selectively degraded by autophagy via the LC3-interaction. In addition, since autophagosome formation and long-lived protein degradation are intact in *p62*-knockout mice, p62 is considered a specific substrate of autophagy rather than a molecule involved in autophagosome formation.

Recent studies have identified the *L*C3 *r*ecognition *s*equence (LRS) in uncharacterized linker regions between the zinc finger and UBA domains in murine p62. The LRS is comprised of 11 amino acids (Ser334–Ser344), which include an acidic cluster and hydrophobic residues (DDD or DEE and WXXL or WXXV). Interestingly, this sequence is almost identical to a previously reported *L*C3-*i*nteracting *r*egion (LIR) of human p62. The crystal structure of the LC3-LRS complex, solved at 1.56 Å resolution, reveals that the acidic cluster of Asp337–Asp339 in LRS interacts with basic residues in the N terminus of LC3, and that the Trp-340 and Leu-343 residues are inserted into two hydrophobic pockets, exposed on the ubiquitin domain of LC3. LC3 has basic residues at its N-terminal α-helix surface, and these residues are involved in the interaction with the acidic cluster of LRS, whereas the other two Atg8 mammalian homologues, GATE-16 and GABARAP, have acidic residues in their respective N-terminal α-helical surfaces [110]. It is thus conceivable that p62 is a more favorable target for LC3 than GATE-16 or GABARAP.

5.3. Role of p62 in inclusion body formation

Characterization of protein inclusions in autophagy-deficient cells or neurons reveals that nearly all inclusions are positive for ubiquitin and p62 [109]. This raises an important question regarding the role of p62 in the formation of protein inclusions. To answer this, Komatsu et al. crossed p62−/− mice to mutant mice with autophagy deficiency in specific tissues. They found that loss of *p62* greatly reduces the formation of ubiquitinated protein inclusions resulting from impaired autophagy in mice. Similar studies were also performed in fruit flies and again the results indicate that the presence of p62 is necessary for the occurrence of protein inclusions in neurons [111]. To understand the molecular basis of this function of p62, it was shown that p62 proteins harboring mutations in LRS, escape efficient degradation by autophagy, leading to inclusion formation despite normal autophagy in cells. In addition, degradation of the PB1 mutant of p62, which is defective in oligomerization, is markedly attenuated, but no protein inclusions occur. These results suggest that increased levels and oligomerization of p62 are necessary for the formation of inclusion bodies, and furthermore, oligomerization of p62 via PB1 is a critical event for facilitating their degradation by autophagy.

Considerable evidence has shown that p62 is a component of protein inclusion bodies found in human disorders such as in liver injuries (e.g., alcoholic hepatitis, steatohepatitis, and α1-antitrypsin deficiency) and neurodegenerative diseases (e.g., AD, PD, and ALS). Although autophagy is beneficial in the clearance of toxic protein aggregates and prevents cellular toxicity, whether or not p62 is indeed essential for the formation of disease-related inclusions and the exact role of p62 in the disease process remains unknown at present. Intriguingly, the formation of ubiquitin-positive aggregates induced by proteasome inhibition is also suppressed in *p62*-deficient cells, suggesting that p62 is a general mediator of inclusion formation. Thus, we hypothesize that p62 normally functions as an adaptor that links certain proteins to autophagy machinery for degradation. When cellular degradation systems (proteasomes or autophagy-lysosomes) are compromised or overwhelmed, p62 levels are sharply increased (especially with impaired autophagy) and it simply behaves as "glue" that bonds many autophagic protein substrates together, manifesting as protein aggregates and eventually large protein inclusions.

5.4. Potential mechanism of neurodegeneration caused by autophagy-deficiency

Emerging evidence indicates that soluble proto-fibrils or small oligomers of disease-related proteins are the cytotoxic species, whereas large inclusion bodies derived from the sequestration of toxic protein oligomers are protective. Therefore, we can hypothesize that the presence of p62 is protective in suppressing the toxicity of proto-fibrils or protein oligomers, given the role of p62 in facilitating the formation of relatively less toxic inclusion bodies. Accordingly, loss of p62 in autophagy-deficient neurons would exacerbate the neurodegenerative process because toxic proteins would accumulate intracellularly and no inclusion bodies would form. Surprisingly, in *Atg7/p62* double knockout mice, loss of p62 was found to be associated with reduced liver dysfunction or little change in the neurodegenerative process caused by autophagy-deficiency, despite the absence of any inclusion bodies [109]. Apart from the specific function of p62 in liver, these results seem to argue against a critical role of protein inclusions in the context of cytotoxicity, especially in the absence of disease-related proteins. Alternatively, they may suggest that the proteins accumulated upon impairment of autophagy are largely neutral or at least neurons are tolerant to the build-up of those proteins. In this regard, the degradative function of autophagy in protein homeostasis control is likely more significant towards disease-related, aggregate-prone proteins than normal long-lived protein substrates of autophagy.

So what could be the primary cause that leads to neuronal death when neuronal autophagy is impaired? As discussed above, analyses of knock-out mice with Purkinje cell-specific *Atg7* deletion (*Atg7*$^{flox/flox}$; Pcp2-Cre) demonstrated that loss of Atg7 initially causes cell-autonomous, progressive dystrophy (manifested by axonal swelling) and degeneration of the axon terminals followed by cell-autonomous Purkinje cell death and mouse behavioural deficits. Furthermore, the mutant Purkinje cells developed aberrant organelles in the swelling axons, suggesting the important role of autophagy in the regulation of local axonal membrane trafficking and turnover [58]. The study also implicates impairment of axonal autophagy as a mechanism for axonopathy associated with neurodegeneration. Interestingly, such axonopathy associated with *Atg7*-deficient Purkinje cells and hypothalamic neurons is still observed in *Atg7*/*p62*-double knockout neurons, indicating that the development of axonopathy in *Atg7*-deficient neurons is *p62*-independent [109]. Therefore, we hypothesize that axonal dystrophy and degeneration provide an important mechanism for the neurodegeneration caused by autophagy-deficiency.

6. Conclusion and perspectives

The past several years have witnessed a rapid growth of autophagy research. With emerging evidence that links the aberrant autophagic activity to various neurodegenerative diseases, it is of upmost importance to understand the basic process of neuronal autophagy at molecular and cellular levels. This would aid in revealing the exact role of autophagy in the neuropathogenesis associated with different diseases. Currently, the particular challenge facing us is to establish robust and physiological cellular and animal models that allow us to dissect neuronal autophagy, and to identify specific factors that contribute to the regulation of neuronal autophagy process. We anticipate that, with the development of new experimental approaches, particularly in the area of cell biology, mouse genetics, live-imaging and proteomics, we will make significant progress in this field and provide valuable knowledge to the design of drugs targeted at autophagy in the treatment of neurodegenerative diseases.

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