

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)<sup>14,15,39</sup> (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)<sup>40</sup>, but failed to co-immunoprecipitate with KIR-deleted Flag–p62 (p62  $\Delta$ KIR) (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  $\beta$ -propeller structure with pseudo six-fold symmetry<sup>37,38</sup>. The KIR peptide binds to the bottom side of the  $\beta$ -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<sup>24–27</sup>. 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<sup>37,38</sup>.

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  $\beta$ -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 ( $K_{a,ETGE} = (1.90 \pm 0.40) \times 10^8 \text{ M}^{-1}$ , compared with  $K_{a,DLG} = (1.00 \pm 0.00) \times 10^9 \text{ M}^{-1}$ ) (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 ( $K_s = (5.4 \pm 0.3) \times 10^5 \text{ M}^{-1}$ ) 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*<sup>-/-</sup> mouse primary hepatocytes with adenovirus Flag-tagged wild-type p62 and p62 mutants ( $\Delta$ KIR, T352A and E354A) severely attenuated in their interactions with Keap1 (see Fig. 1d for  $\Delta$ KIR; 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

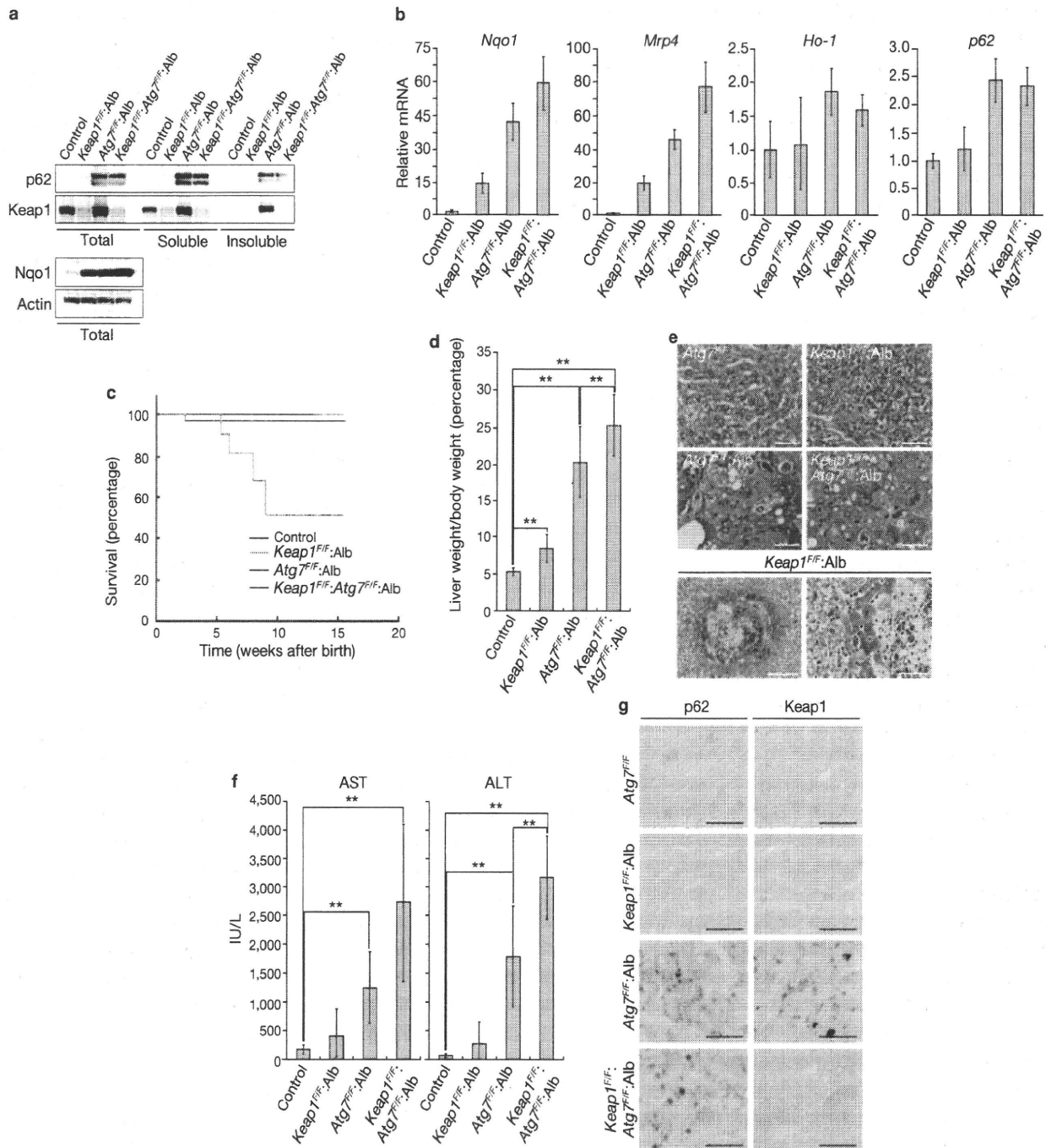
The next series of experiments served to clarify whether the Nrf2–Keap1 interaction is affected by the intracellular accumulation of p62 or p62-positive and ubiquitin-positive inclusion bodies, which is known to occur in autophagy-deficient liver<sup>16</sup>. These studies were conducted in *Atg7*<sup>+/+</sup>:Mx1 mice in which *Atg7* can be depleted in the liver by intraperitoneal injection of polyinosinic acid•polycytidylic acid (poly(I)•poly(C))<sup>2</sup>. Consistent with our previous work<sup>16</sup>, 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<sup>42</sup> (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*<sup>+/+</sup>:Mx1:p62<sup>-/-</sup> or *Atg7* p62 DKO)<sup>16</sup>. 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*<sup>+/+</sup>:Mx1 mice with *Nrf2*<sup>-/-</sup> mice<sup>31</sup> to produce *Atg7*:*Nrf2*-double-knockout mice (*Atg7*<sup>+/+</sup>:Mx1:*Nrf2*<sup>-/-</sup> 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<sup>43</sup>. 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<sup>2</sup>; 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*<sup>FF</sup>:Alb) and *Keap1* *Atg7*-deficient (*Keap1*<sup>FF</sup>:*Atg7*<sup>FF</sup>: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*<sup>FF</sup> liver (control). Data are means  $\pm$  s.d. for three experiments. (c) Kaplan-Meier curves of survival of *Atg7*<sup>FF</sup>:Alb and *Keap1*<sup>FF</sup>:*Atg7*<sup>FF</sup>:Alb mice. The survival analysis of control

(*n* = 33), *Keap1*<sup>FF</sup>:Alb (*n* = 15), *Atg7*<sup>FF</sup>:Alb (*n* = 12) and *Keap1*<sup>FF</sup>:*Atg7*<sup>FF</sup>: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  $\pm$  s.d. for *Atg7*<sup>FF</sup> (control) (*n* = 24), *Keap1*<sup>FF</sup>:Alb (*n* = 13), *Atg7*<sup>FF</sup>:Alb (*n* = 9) and *Keap1*<sup>FF</sup>:*Atg7*<sup>FF</sup>: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  $\mu$ m (left bottom panel); 20  $\mu$ m (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  $\pm$  s.d. for *Atg7*<sup>FF</sup> (control) (*n* = 24), *Keap1*<sup>FF</sup>:Alb (*n* = 13), *Atg7*<sup>FF</sup>:Alb (*n* = 9) and *Keap1*<sup>FF</sup>:*Atg7*<sup>FF</sup>: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  $\mu$ m.

### 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<sup>fl/fl</sup>;Atg7<sup>fl/fl</sup>;Alb* or *Keap1 Atg7* DKO) by crossing *Keap1<sup>fl/fl</sup>;Alb<sup>44</sup>* with *Atg7<sup>fl/fl</sup>* 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<sup>15</sup>. 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<sup>31</sup>. 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<sup>46</sup> 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  $7.69 \pm 1.25\%$  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<sup>47</sup>, 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  $\alpha_1$ -antitrypsin deficiency<sup>20</sup>. 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.

#### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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# PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy

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**P**arkinson's disease (PD) is a prevalent neurodegenerative disorder. Recent identification of genes linked to familial forms of PD such as *Parkin* and *PINK1* (*PTEN-induced putative kinase 1*) has revealed that ubiquitylation and mitochondrial integrity are key factors in disease pathogenesis. However, the exact mechanism underlying the functional interplay between Parkin-catalyzed ubiquitylation and PINK1-regulated mitochondrial quality control remains an enigma. In this study, we show that PINK1 is rapidly and constitutively degraded under steady-state conditions in a mitochondrial membrane potential-dependent manner and that a loss in

mitochondrial membrane potential stabilizes PINK1 mitochondrial accumulation. Furthermore, PINK1 recruits Parkin from the cytoplasm to mitochondria with low membrane potential to initiate the autophagic degradation of damaged mitochondria. Interestingly, the ubiquitin ligase activity of Parkin is repressed in the cytoplasm under steady-state conditions; however, PINK1-dependent mitochondrial localization liberates the latent enzymatic activity of Parkin. Some pathogenic mutations of PINK1 and Parkin interfere with the aforementioned events, suggesting an etiological importance. These results provide crucial insight into the pathogenic mechanisms of PD.

## Introduction

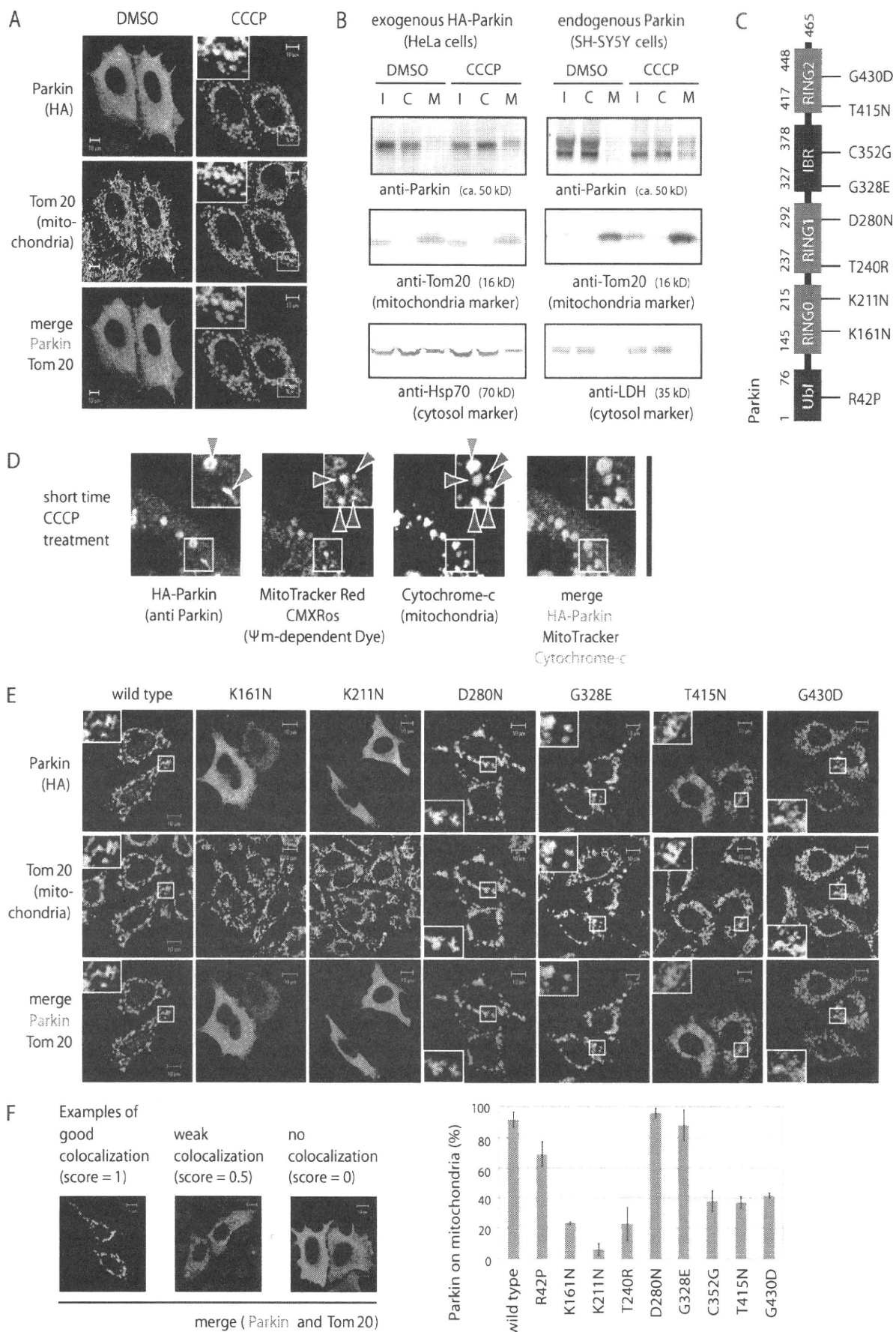
Parkinson's disease (PD) is a very common movement disorder characterized by dopaminergic neuronal loss. The majority of PD cases are sporadic; however, the discovery of genes linked to rare familial forms of this disease has provided important insight into the molecular mechanisms of disease pathogenesis (Moore et al., 2005; Hardy et al., 2006). In 2000, we and others found that dysfunction of an E3 ubiquitin ligase (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000) termed Parkin causes autosomal recessive juvenile Parkinsonism (Kitada et al., 1998). Since then, a multitude of papers have been published, but the mechanism by which dysfunction of Parkin causes autosomal recessive juvenile

Parkinsonism has largely remained obscure, and claims of pathogenicity remain controversial (Lim, 2007; Matsuda and Tanaka, 2010). In addition, *PINK1* (*PTEN-induced putative kinase 1*) was identified in 2004 as the gene responsible for another form of early-onset PD (Valente et al., 2004). PINK1 functions in mitochondrial maintenance, suggesting that mitochondrial integrity is another key factor in disease pathogenesis (Dodson and Guo, 2007; Schapira, 2008). Intriguingly, genetic studies using *Drosophila melanogaster* revealed that PINK1 and Parkin function in the same pathway, with PINK1 functioning upstream of Parkin (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). Little is known about how PINK1

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Abbreviations used in this paper: CCCP, carbonyl cyanide m-chlorophenylhydrazone; MEF, mouse embryonic fibroblast; Mt-GFP, mitochondria-targeting GFP; PD, Parkinson's disease.

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regulates Parkin, and our knowledge, especially in mammals, of their relationship is limited. In this study, we describe the mechanism underlying the functional interplay between ubiquitylation catalyzed by Parkin and mitochondrial quality control regulated by PINK1.

## Results and discussion

### Parkin localizes to and ubiquitylates mitochondria with low membrane potential

We initially sought to study the subcellular localization and E3 activity of Parkin using HeLa cells, which reportedly lack a functional *Parkin* gene (Denison et al., 2003). In support of that study, we found that endogenous Parkin was barely detectable in HeLa cells even when PRK8, the best-characterized specific anti-Parkin antibody (Pawlyk et al., 2003), was used (Fig. S1 A). Consequently, HA-Parkin was exogenously introduced into HeLa cells. Under steady-state conditions, HA-Parkin was diffusely localized throughout the cytosol and did not overlap with mitochondria, whereas Parkin was rapidly recruited to the mitochondria when HeLa cells were treated with the mitochondrial uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; Fig. 1 A), as reported by Narendra et al. (2008). Next we tried to confirm the redistribution of Parkin from the cytoplasm to the mitochondria using a biochemical approach. In fractionation experiments, detection of Parkin in the mitochondria-rich fraction was faint, probably because Parkin was weakly associated with the mitochondria and thus unstable during fractionation. Inclusion of the cross-linker DSP (dithiobis[succinimidyl] propionate) significantly strengthened the signal and further confirmed redistribution of exogenous (Fig. 1 B, left) and endogenous (Fig. 1 B, right) Parkin from the cytoplasm to a mitochondria-enriched fraction. (Note that endogenous Parkin in SH-SY5Y cells is detectable as a doublet, which is consistent with a previous study [Pawlyk et al., 2003].) To more convincingly demonstrate that Parkin is selectively recruited to depolarized mitochondria, we used MitoTracker red CMXRos, which accumulates in mitochondria with an intact membrane potential. Incomplete treatment with CCCP can generate cells in which healthy and damaged mitochondria coexist. Under these conditions, signals of Parkin and MitoTracker were mutually exclusive, and Parkin selectively localized on mitochondria with lower MitoTracker red staining (Fig. 1 D), indicating that Parkin was selectively targeted to mitochondria whose membrane potential had been lost.

Subsequently, we performed immunofluorescence staining using an antiubiquitin antibody. Under normal conditions, the ubiquitin signal was spread throughout the cell. In contrast,

when cells were treated with CCCP, the ubiquitin signal was concentrated in the mitochondria (Fig. 2, A and B). Mitochondrial ubiquitylation was only observed in Parkin-expressing cells (Fig. 2 A and Fig. S1 B) and disappeared when Parkin mutants deficient in E3 activity (T415N and G430D) were introduced (Fig. 2 A). Triple staining using mitochondria-targeting GFP (Mt-GFP), anti-Parkin, and anti-ubiquitin antibodies further confirmed the colocalization of Parkin, ubiquitin, and mitochondria after CCCP treatment (Fig. 2 C). Staining with single antibodies or Mt-GFP alone indicated that the aforementioned merged data were not derived from channel cross talk (Fig. S1, C and D). These results demonstrate that Parkin ubiquitylates mitochondria in response to a reduction in mitochondrial membrane potential.

### Disease-relevant mutations of Parkin impair mitochondrial localization

To confirm that translocation of Parkin to depolarized mitochondria is etiologically important, we selected nine pathogenic mutations and examined their subcellular localization (Fig. 1 C). In vitro experiments have previously shown that two of the mutations (T415N and G430D) in the RING2 domain abolish E3 activity of Parkin, whereas E3 activity is unaffected by the other mutations (Hampe et al., 2006; Matsuda et al., 2006). These mutants were serially introduced into HeLa cells, followed by CCCP treatment, and their subcellular localization was examined. Parkin with the D280N or G328E mutation in RING1 or the IBR (in between RING) domain, respectively, was recruited to the mitochondria in a manner similar to wild-type Parkin (Fig. 1, E and F). In contrast, the other pathogenic mutations altered to some degree the mitochondrial localization of Parkin; in particular, the K161N, K211N, and T240R mutations, which lie in or near the RING0 domain in the linker region (Hristova et al., 2009), severely compromised the mitochondrial localization of Parkin (Fig. 1, E and F). The aforementioned results suggest that mitochondrial localization of Parkin is pathologically significant and that the RING0 domain is important for the translocation of Parkin to the damaged mitochondria.

### Parkin exerts E3 activity only when the mitochondrial membrane potential decreases

As shown in Fig. 2 C and Fig. S1 B, mitochondrial ubiquitylation was dependent on Parkin translocation to the mitochondria. Thus, we tried to determine whether the subcellular localization of Parkin modulates its E3 activity. To address this issue, we monitored the E3 activity of Parkin using an

**Figure 1. Mitochondrial localization of Parkin is etiologically important.** (A) HeLa cells expressing HA-Parkin were treated with CCCP or DMSO (control) and then immunostained with the indicated antibodies. (B) HeLa cells stably expressing HA-Parkin or intact SH-SY5Y cells were treated with CCCP or DMSO and subjected to fractionation experiments. I, C, and M indicate input, cytosol-rich supernatant, and mitochondria-rich membrane pellet, respectively. (C) Schematic diagram of disease-relevant mutants of Parkin used in this study. IBR, in between RING; Ubl, ubiquitin like. (D) Polarized mitochondria stained with MitoTracker red (red arrowheads) were not labeled by Parkin. In contrast, damaged mitochondria marked by Parkin (green arrowheads) were not stained with MitoTracker red. (E) HeLa cells expressing HA-Parkin with various pathogenic mutations were treated with CCCP, followed by immunocytochemistry. (A, D, and E) Higher magnification views of the boxed areas are shown in the insets. (F) Parkin colocalization with mitochondria was analyzed in >100 cells per mutation. Example figures indicative of robust colocalization (counted as 1), weak colocalization (counted as 0.5), and no colocalization (counted as 0) are shown. Error bars represent the mean  $\pm$  SD values of at least three experiments. Bars: (A, E, and F) 10  $\mu$ m; (D) 30  $\mu$ m.

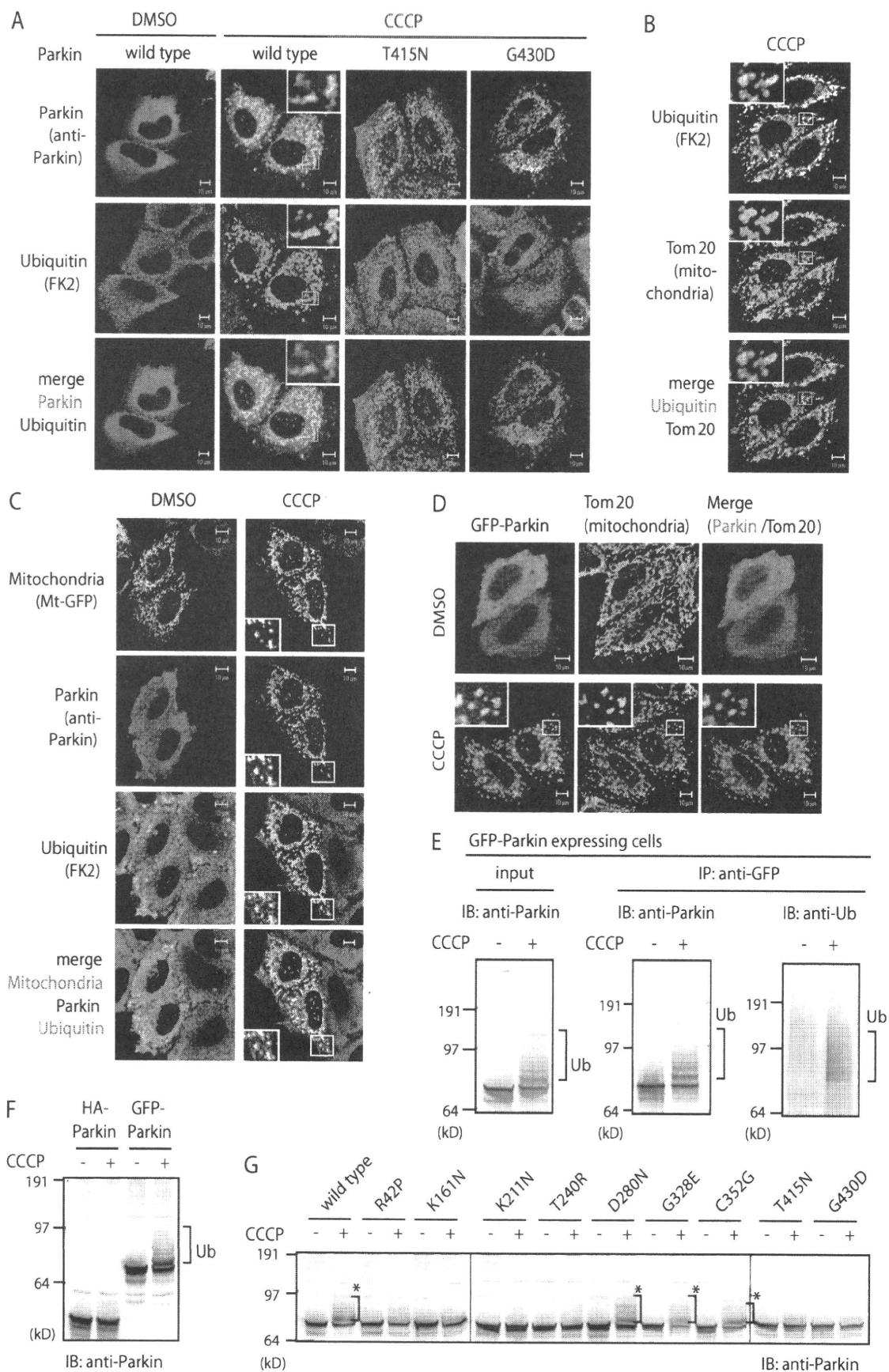


Figure 2. **Parkin exerts E3 activity only when the mitochondrial membrane potential is decreased.** (A) HeLa cells expressing wild-type Parkin or E3-inactivating mutations were treated with CCCP and then immunostained with the indicated antibodies. When E3-inactivating mutations were introduced into Parkin, the mitochondrial ubiquitylation signal disappeared. (B and C) HeLa cells expressing HA-Parkin (B) or expressing both Mt-GFP and HA-Parkin (C)

artificial substrate fused to Parkin. In vitro experiments have shown that Parkin can ubiquitylate an N-terminally fused lysine-rich protein as a pseudosubstrate (Matsuda et al., 2006). Similar ubiquitylation of pseudosubstrates even in the cytoplasm under normal conditions in vivo would be evidence that the E3 activity is constitutive; otherwise, E3 activity of Parkin is dependent on mitochondrial retrieval. A GFP tag is lysine rich and thus a good candidate for an in vivo pseudosubstrate, whereas an HA tag possesses no lysine residues and thus cannot function as a pseudosubstrate. GFP- and HA-Parkin expressed in HeLa cells treated with CCCP were both recruited to damaged mitochondria (Figs. 1 A and 2 D), but interestingly, a higher molecular mass population of only GFP-Parkin was observed (Fig. 2 F). Immunoprecipitation experiments demonstrated that GFP-Parkin was indeed ubiquitylated (Fig. 2 E). This was not based on autoubiquitylation of Parkin itself because mitochondria-associated HA-Parkin did not undergo ubiquitylation after CCCP treatment (Fig. 2 F and not depicted). Moreover, ubiquitylation of GFP-Parkin was absent in the T415N and G430D mutants, which lack E3 activities, suggesting that ubiquitylation of GFP-Parkin is not derived from other E3s (Fig. 2 G). The K161N and K211N mutants that impaired mitochondrial localization also inhibited ubiquitylation of GFP-Parkin (Fig. 2 G). Collectively, the aforementioned results indicate that Parkin ubiquitylates fused GFP only when it is retrieved to the mitochondria, suggesting that the latent E3 activity of Parkin is dependent on decreased mitochondrial membrane potential.

#### **PINK1 localization is stabilized by damaged mitochondria**

Recessive mutations in the human *PINK1* gene are also the cause of autosomal recessive early-onset PD (Valente et al., 2004). We next examined whether the subcellular localization of PINK1 was affected by mitochondrial membrane potential. As reported previously (Valente et al., 2004; Beilina et al., 2005; Takatori et al., 2008), N-terminal Myc- or N-terminal Flag-tagged PINK1 clearly localized to the mitochondria, whereas C-terminal Flag- or C-terminal V5-tagged PINK1 mainly localized to the cytoplasm (Fig. 3 A and not depicted). Exogenous nontagged PINK1 also localized to the cytoplasm under steady-state conditions (Fig. 3 B), suggesting that mitochondrial localization of PINK1 is an artifact of the N-terminal epitope. More importantly, similar to Parkin, untagged PINK1 and C-terminal Flag- or C-terminal V5-tagged PINK1 localized to the mitochondria after CCCP treatment (Fig. 3, A and B; and not depicted). These results suggest that the subcellular localization of PINK1 is also regulated by the mitochondrial membrane potential.

We next sought to determine the subcellular localization of endogenous PINK1. Immunocytochemical experiments

showed, as reported previously (Zhou et al., 2008), that the endogenous PINK1 signal was barely detectable in HeLa cells under steady-state conditions. However, a decrease in mitochondrial membrane potential resulted in a mitochondria-associated PINK1 signal (Fig. 3, C and D). We found that CCCP treatment promoted the gradual accumulation of endogenous PINK1 in immunoblots as well (Fig. 3 E) and the presence of endogenous PINK1 in a mitochondria-enriched fraction (Fig. 3 F). More importantly, when CCCP was washed out, the accumulated endogenous PINK1 rapidly disappeared (within 30 min) both in the presence and absence of cycloheximide (Fig. 3 G and not depicted). Moreover, the N-terminal 34 aa of PINK1 sufficiently recruited GFP to the mitochondria even in the absence of CCCP (Fig. 3 H). These results support the hypothesis in which PINK1 is constantly transported to the mitochondria but is rapidly degraded in a membrane potential-dependent manner. We speculate that PINK1 is stabilized by a decrease in mitochondrial membrane potential and, as a result, accumulates in depolarized mitochondria.

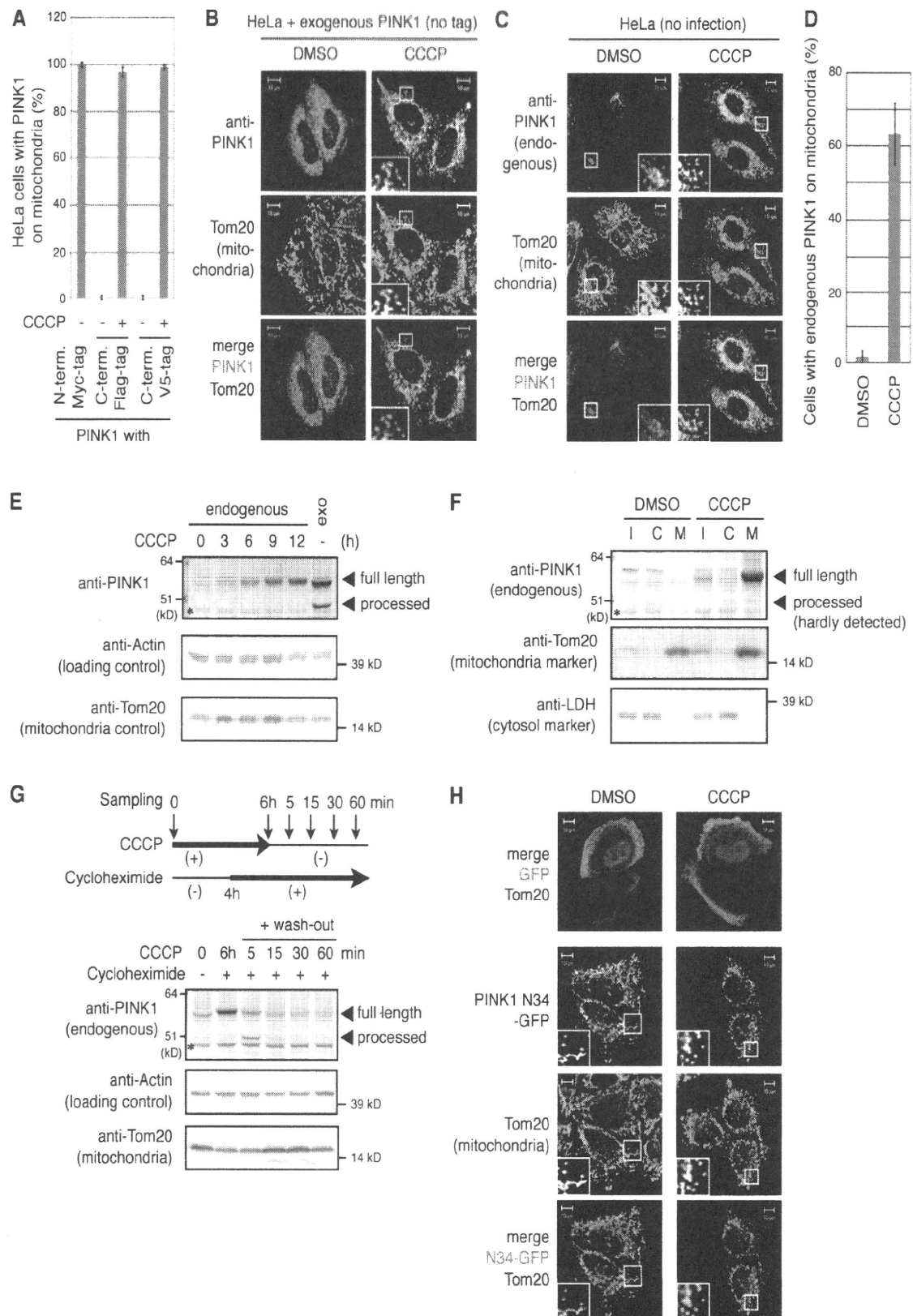
PINK1 normally exists as either a long (~60 kD) or a short (~50 kD) protein. Because the canonical mitochondria-targeting signal (matrix-targeting signal) is cleaved after import into the mitochondria, the long form has been designated as the precursor and the short form as the mature PINK1 (Beilina et al., 2005; Silvestri et al., 2005). The short (processed) form of PINK1 was clearly detected when untagged PINK1 was overexpressed (Fig. 3 E, sixth lane); however, this form of endogenous PINK1 was rarely detectable after CCCP treatment (Fig. 3 E, the first through the fifth lanes). Our subcellular localization study of endogenous PINK1 after CCCP treatment showed that the long form was recovered in the mitochondrial fraction (Fig. 3 F), suggesting that it is not the preimport precursor form. Moreover, by monitoring the degradation process of PINK1 after recovery of membrane potential, we realized that the short form of PINK1 transiently appeared soon after CCCP was washed out and then later disappeared (Fig. 3 G), suggesting that the processed form of PINK1 is an intermediate in membrane potential-dependent degradation. In conclusion, these results imply that PINK1 cleavage does not reflect a canonical maturation process accompanying mitochondrial import as initially thought.

#### **PINK1 retrieves Parkin from the cytoplasm to the mitochondria**

Because previous studies revealed that PINK1 functions upstream of Parkin (Clark et al., 2006; Park et al., 2006; Yang et al., 2006; Exner et al., 2007), we next examined the potential role of PINK1 in the mitochondrial recruitment of Parkin. To obtain clear-cut conclusions, we set up our experimental system using mouse embryonic fibroblasts (MEFs) derived from control

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were treated with CCCP or DMSO (control) and then immunostained with the indicated antibodies. (D) Localization of GFP-Parkin to the mitochondria after CCCP treatment. (A–D) Higher magnification views of the boxed areas are shown in the insets. (E) HeLa cell lysates expressing GFP-Parkin were immunoprecipitated by anti-GFP antibody, followed by immunoblotting with the indicated antibodies. (F) Straight immunoblotting of HA- and GFP-Parkin in the absence or presence of CCCP. Note the slower migrating ladders derived from ubiquitylation (Ub) in only the GFP-Parkin with CCCP lane. (G) GFP-Parkin-expressing HeLa cells with various pathogenic mutations (Fig. 1 C) were treated with CCCP and subjected to immunoblotting. Asterisks show ubiquitylation of GFP-Parkin. Vertical black lines indicate that intervening lanes have been spliced out. IB, immunoblot; IP, immunoprecipitation. Bars, 10  $\mu$ m.



**Figure 3. PINK1 is constitutively degraded in a mitochondrial membrane potential-dependent manner and localizes to depolarized mitochondria.** (A) The number of HeLa cells with N-terminal- or C-terminal-tagged PINK1 localized to the mitochondria was counted in >100 cells. (B and C) Exogenous non-tagged PINK1 (B) or endogenous PINK1 (C) in HeLa cells was immunostained with the indicated antibodies. (D) The number of HeLa cells with endogenous PINK1 localized to the mitochondria was counted as in A. (A and D) Error bars represent the mean  $\pm$  SD values of least three experiments. (E) Endogenous PINK1 gradually accumulated after CCCP treatment. The first through the fifth lanes show endogenous PINK1, and the sixth lane shows overexpressed untagged PINK1. Note that the asterisk indicates a cross-reacting band because it was not affected by overproduction of untagged PINK1. (F) Subcellular fractionation of endogenous PINK1. Intact SH-SY5Y cells were treated with CCCP or DMSO and subjected to fractionation experiments (same sample as

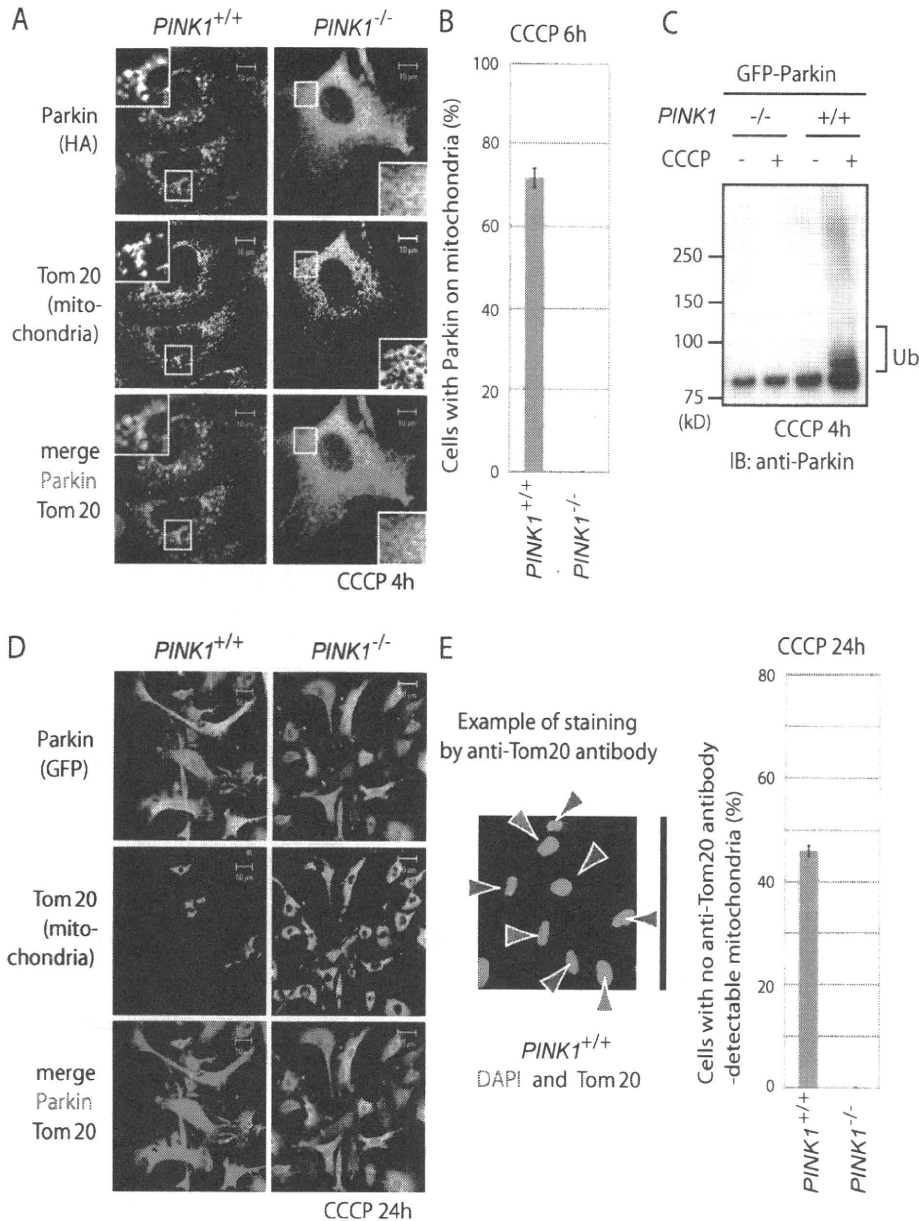


Figure 4. **PINK1 recruits cytoplasmic Parkin to damaged mitochondria.** (A) *PINK1* knockout (*PINK1*<sup>-/-</sup>) or control [*PINK1*<sup>+/+</sup>] MEFs were transfected with HA-Parkin, treated with CCCP, and subjected to immunocytochemistry with the indicated antibodies. Higher magnification views of the boxed areas are shown in the insets. (B) The number of MEFs with Parkin localized to the mitochondria was counted as in Fig. 3 A. (C and D) Neither activation of Parkin nor mitochondrial degradation was observed in *PINK1*<sup>-/-</sup> MEFs. MEFs stably expressing GFP-Parkin were treated with CCCP for 4 h and subjected to immunoblotting (C) or for 24 h, followed by immunocytochemistry (D). IB, immunoblot; Ub, ubiquitylation. (E) The number of MEFs without anti-Tom20 antibody-detectable mitochondria was counted as in Fig. 3 A. In the example figure (left), blue arrowheads indicate cells without anti-Tom20 antibody-detectable mitochondria, and red arrowheads indicate cells harboring anti-Tom20 antibody-detectable mitochondria. (B and E) Error bars represent the mean ± SD values of least three experiments. Bars: (A) 10 μm; (D) 50 μm; (E) 150 μm.

(*PINK1*<sup>+/+</sup>) or *PINK1* knockout (*PINK1*<sup>-/-</sup>) mouse (Gautier et al., 2008). Endogenous Parkin is undetectable in MEFs (Fig. S1 A); consequently, HA- or GFP-Parkin was introduced into these cells by retroviral transfection (Kitamura et al., 2003). In control MEFs (*PINK1*<sup>+/+</sup>), Parkin was selectively recruited to the mitochondria after CCCP treatment (Fig. 4 A) and subsequently

resulted in the disappearance of the mitochondria (Fig. 4, D and E). This mitochondrial clearance was considerably impeded by *Atg7* (essential gene for autophagy) knockout (Fig. S2; Komatsu et al., 2005), suggesting that Parkin degrades mitochondria by selective autophagy as reported previously (Narendra et al., 2008). In sharp contrast, Parkin was not

Fig. 1 B). I, C, and M indicate input, cytosol-rich supernatant, and the mitochondria-rich membrane pellet, respectively. (G) HeLa cells were treated with CCCP and cycloheximide as depicted, followed by immunoblotting with the indicated antibodies. LDH, lactate dehydrogenase. (F and G) Asterisks indicate a cross-reacting band. (H) N-terminal 34 aa of PINK1 recruited GFP to the mitochondria both in the absence and presence of CCCP. The top panel shows control HeLa cells expressing only GFP. (B, C, and H) Higher magnification views of the boxed areas are shown in the insets. Bars, 10 μm.

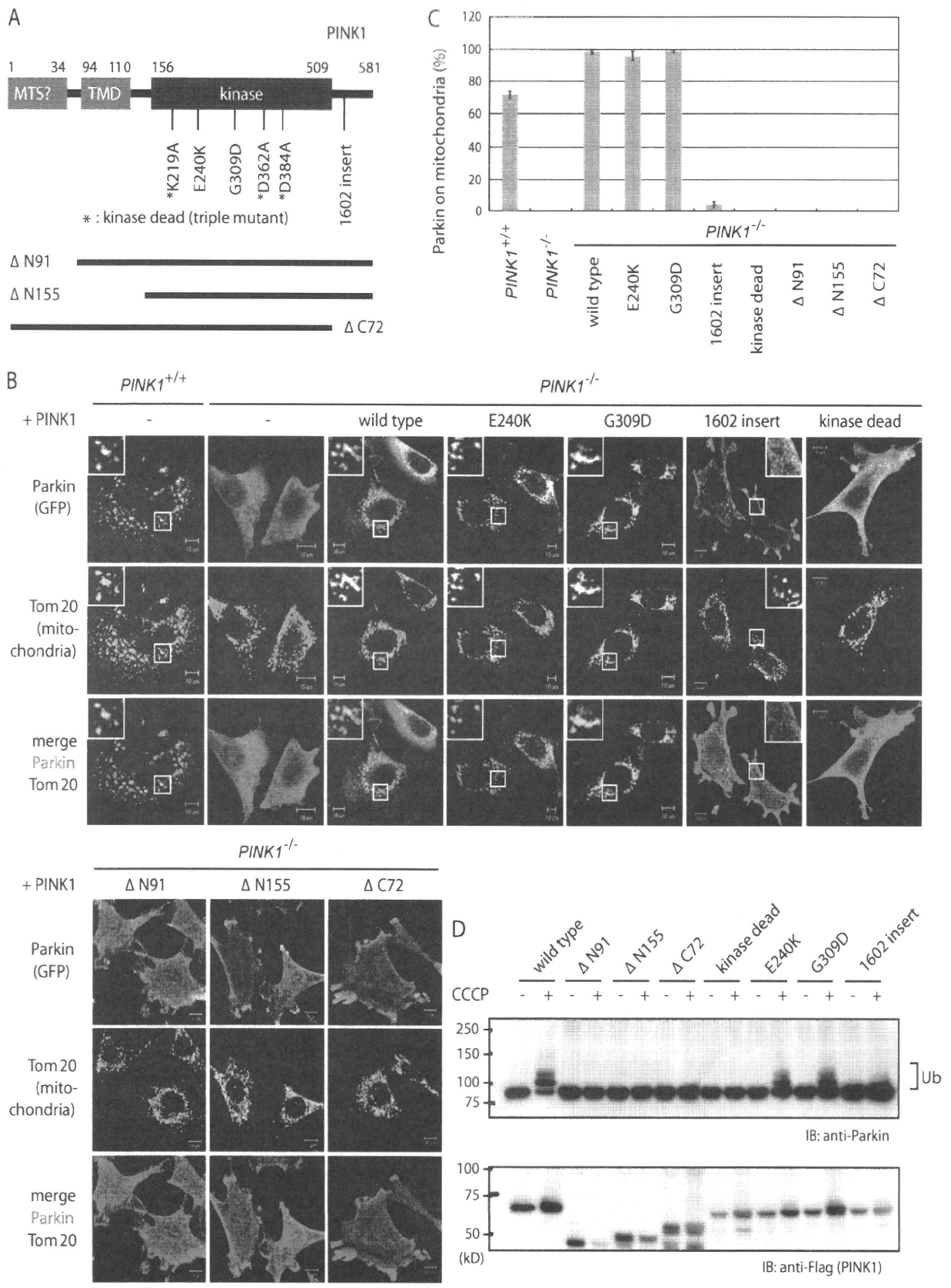


Figure 5. Kinase activity and mitochondrial targeting of PINK1 is imperative for mitochondrial localization of Parkin. (A) Schematic depiction of pathogenic and deletion mutants of PINK1 used in this study. MTS, mitochondria-targeting sequence; TMD, transmembrane domain. (B) Subcellular localization of Parkin in *PINK1*<sup>-/-</sup> cells complemented by various pathogenic and deletion mutants of PINK1-Flag. Cells were treated with CCCP. Higher magnification views of the boxed areas are shown in the insets. (C) The number of cells with Parkin-positive mitochondria was counted as in Fig. 3 A. Error bars represent the mean ± SD values of least three experiments. (D) *PINK1*<sup>-/-</sup> MEFs complemented by various PINK1 mutants were treated with CCCP and subjected to immunoblotting using anti-Parkin or anti-Flag (tag of PINK1) antibodies. IB, immunoblot; Ub, ubiquitylation. Bars, 10 μm.

translocated to the mitochondria in *PINK1* knockout (*PINK1*<sup>-/-</sup>) MEFs after CCCP treatment (Fig. 4, A and B). Subsequent activation of Parkin and mitochondrial degradation were also completely impeded (Fig. 4, C–E). To exclude the possible role of retroviral integration of Parkin in the aforementioned phenotype, we checked whether reintroduction of PINK1 rescued this phenotype. Untagged or C-terminal Flag-tagged PINK1 complemented the mislocalization of Parkin in *PINK1*<sup>-/-</sup> MEFs (Fig. 5, B and C; and not depicted), confirming that the aforementioned defects were caused by the loss of PINK1.

To examine whether pathogenic mutations of PINK1 affect its mitochondrial localization, we expressed PINK1 mutants harboring the missense mutations E240K and G309D, or a CAA nucleotide insertion behind C1602 (referred to hereafter as 1602-insert) in *PINK1*<sup>-/-</sup> MEFs. Similar to wild-type PINK1, these PINK1 mutants colocalized with mitochondria after CCCP treatment (Fig. S3). Next, GFP-Parkin was introduced into these cells to examine whether pathogenic mutations of PINK1 affect the mitochondrial localization and activation of Parkin. The E240K and G309D mutants restored the mitochondrial localization and activation of Parkin as well as wild-type PINK1, whereas recruitment of Parkin to the mitochondria by the 1602-insert mutant was abolished (Fig. 5, B–D), suggesting that the pathology of this PINK1 mutation is mislocalization and consequent inactivation of Parkin.

#### Mitochondrial localization and kinase activity of PINK1 are essential for translocation of Parkin to damaged mitochondria

Finally, we investigated the role of various PINK1 domains (Fig. 5 A) in the mitochondrial recruitment of Parkin. PINK1 is composed of an atypical N-terminal mitochondrial localization signal and transmembrane domain, a kinase domain in the middle, and a conserved C-terminal domain (Zhou et al., 2008). Deletion of the N-terminal 91 aa abolished the mitochondrial localization of PINK1 (Zhou et al., 2008). We also confirmed that the  $\Delta$ N91 and  $\Delta$ N155 mutants did not target to the mitochondria even after CCCP treatment (Fig. S3). We also generated a mutant containing the triple K219A, D362A, and D384A mutations that abolish kinase activity (Beilina et al., 2005) and a C-terminal domain deletion mutant associated with PINK1 dysfunction (Sim et al., 2006; Yang et al., 2006). The kinase-dead and  $\Delta$ C72 mutants of PINK1 colocalized with damaged mitochondria similar to wild type (Fig. S3). When introduced into *PINK1*<sup>-/-</sup> cells harboring GFP-Parkin, the mutants were unable to complement the mislocalization and inactivation of Parkin (Fig. 5, B–D), even though the mutant PINK1 proteins were expressed (Fig. 5 D and Fig. S3). These results indicate that the kinase activity and mitochondrial targeting of PINK1 are essential for the mitochondrial recruitment of Parkin.

#### Conclusion

In summary, we have shown that (a) PINK1 is a Parkin-recruitment factor that recruits Parkin from the cytoplasm to damaged mitochondria in a membrane potential-dependent manner for mitochondrial degradation, (b) endogenous PINK1 is constitutively degraded at the mitochondria, but its localization

is specifically linked to a decrease in membrane potential, (c) under steady-state conditions, the E3 activity of Parkin is repressed in the cytoplasm but is liberated by PINK1-dependent mitochondrial localization, and (d) the aforementioned phenomena are presumably etiologically important in part because they were impeded for the most part by disease-linked mutations of PINK1 or Parkin. We believe that these results provide solid insight into the molecular mechanisms of PD pathogenesis not only for familial forms caused by *Parkin* and *PINK1* mutations but also major sporadic forms of PD.

## Materials and methods

#### Cell culture and transfection

MEFs derived from embryonic day 12.5 embryos of PINK1 knockout mice (provided by J. Shen, Harvard Medical School, Boston, MA) were mechanically dispersed by repeated passage through a P1000 pipette tip and plated with MEF media containing DME, 10% FCS,  $\beta$ -mercaptoethanol (Sigma-Aldrich), 1 $\times$  nonessential amino acids, and 1 mM L-glutamine. Various stable transformants of MEFs were established by infecting MEFs with recombinant retroviruses. HA-Parkin, GFP-Parkin, PINK1 (provided by Y. Nakamura, T. Iwatsubo, and S. Takatori, University of Tokyo, Bunkyo-ku, Tokyo, Japan), or various PINK1 mutants were cloned into a pMXs-puro vector. Retrovirus packaging cells, PLAT-E (provided by T. Kitamura, University of Tokyo; Kitamura et al., 2003), were transfected with the aforementioned vectors and were cultured at 37°C for 24 h. After changing the medium, cells were further incubated at 37°C for 24 h, and the viral supernatant was collected and used for infection. MEFs were plated on 35-mm dishes at 24 h before infection, and the medium was replaced with the aforementioned undiluted viral supernatant with 8  $\mu$ g/ml polybrene (Sigma-Aldrich). 2 d later, transformants were selected by the medium containing 10  $\mu$ g/ml puromycin.

#### Cell fractionation and immunoprecipitation

To depolarize the mitochondria, HeLa and SH-SY5Y cells were treated with 10  $\mu$ M CCCP, and MEFs were treated with 30  $\mu$ M CCCP. For fractionation experiments, HeLa and SH-SY5Y cells were treated with CCCP for 1–5 h and subsequently treated with 1 mM DSP (Thermo Fisher Scientific) in PBS for 1 h on ice, inactivated by 10 mM glycine in PBS three times, and suspended in chappell-perry buffer (0.15 M KCl, 20 mM Hepes-NaOH, pH 8.1, 5 mM MgCl<sub>2</sub>, and protease and phosphatase inhibitor [Roche]). Cells were disrupted by five passages through a 25-gauge needle (with 1-ml syringe), debris was removed by centrifugation at 1,000 g for 7 min, and the supernatant was subjected to 10,000 g for 10 min to separate the mitochondria-rich fraction from the cytosol-rich fraction. Immunoblotting and immunoprecipitation were performed by conventional methods. To detect the ubiquitylation of GFP-Parkin, the cell lysate of HeLa cells (10  $\mu$ M CCCP for 1 h) or MEFs (30  $\mu$ M CCCP for 3 h) was collected in the presence of 10 mM N-ethylmaleimide to protect ubiquitylated Parkin from deubiquitylation enzymes. To monitor the degradation of endogenous PINK1, HeLa cells were treated with 10  $\mu$ M CCCP and 50  $\mu$ g/ml cycloheximide as depicted in Fig. 3 G and were subjected to immunoblotting.

#### Immunocytochemistry

To depolarize the mitochondria, HeLa cells (provided by A. Tanaka and R. Yocle, National Institutes of Health, Bethesda, MD) were treated with 10  $\mu$ M CCCP for 1 h (exogenous Parkin and PINK1) or 5 h (endogenous PINK1), and MEFs were treated with 30  $\mu$ M CCCP for 3–4 h (Figs. 4 A and 5 B; and Fig. S3 B) or 24 h (Fig. 4 D and Fig. S2 A). For immunofluorescence experiments, cells were fixed with 4% paraformaldehyde, permeabilized with 50  $\mu$ g/ml digitonin, and stained with primary antibodies described in the next section and with the following secondary antibodies: mouse and/or rabbit Alexa Fluor 488, 568, and 647 (Invitrogen). N-terminal 34 aa of PINK1 were fused to GFP to stain mitochondria in the triple staining experiments. To monitor the mitochondrial membrane potential, MEFs were treated with 50 nM MitoTracker red CMXRos (Invitrogen) for 15 min, washed three times, and incubated for an additional 10 min before fixation, as reported previously (Narendra et al., 2008). Cells were imaged using a laserscanning microscope (LSM510 META; Carl Zeiss, Inc.) with a Plan ApoChromat 63 $\times$  NA 1.4 oil differential interference contrast objective lens. Image contrast and brightness were adjusted in Photoshop (Adobe).

## Antibodies

Antibodies used in this study are as follows: antiactin [AC-40; Sigma-Aldrich], anti-cytochrome c (6H2.B4; BD), anti-Flag [M2; Sigma-Aldrich], anti-GFP [3E6 [Wako Chemicals USA, Inc.]; or A6455 [Invitrogen]], anti-HA [12CA5; Roche], anti-Hsp70 [MBL], anti-lactate dehydrogenase [Abcam], anti-Parkin (#2132 [Cell Signaling Technology] for immunocytochemistry; or PRK8 [Sigma-Aldrich] for immunoblotting), anti-PINK1 [Novus], anti-Tom20 [FL-145 and F-10; Santa Cruz Biotechnology, Inc.], antiubiquitin [P4D1 [Santa Cruz Biotechnology, Inc.]; or FK2 [MBL]], and anti-V5 [Invitrogen].

## Online supplemental material

Fig. S1 shows various control experiments for immunoblotting and immunocytochemistry. Fig. S2 shows that Parkin promoted degradation of depolarized mitochondria via autophagy. Fig. S3 shows the subcellular localization of pathogenic and deletion mutants of PINK1 after CCCP treatment. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200910140/DC1>.

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**Note added in review.** While our manuscript was under review, Geisler et al. (2010), Narendra et al. (2010), and Vives-Bauza et al. (2010) independently published results that are consistent with those described herein.

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# Phosphorylated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis

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**Objective:** TAR DNA-binding protein of 43kDa (TDP-43) is deposited as cytoplasmic and intranuclear inclusions in brains of patients with frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS). Previous studies reported that abnormal phosphorylation takes place in deposited TDP-43. The aim of this study was to identify the phosphorylation sites and responsible kinases, and to clarify the pathological significance of phosphorylation of TDP-43.

**Methods:** We generated multiple antibodies specific to phosphorylated TDP-43 by immunizing phosphopeptides of TDP-43, and analyzed FTLD-U and ALS brains by immunohistochemistry, immunoelectron microscopy, and immunoblots. In addition, we performed investigations aimed at identifying the responsible kinases, and we assessed the effects of phosphorylation on TDP-43 oligomerization and fibrillization.

**Results:** We identified multiple phosphorylation sites in carboxyl-terminal regions of deposited TDP-43. Phosphorylation-specific antibodies stained more inclusions than antibodies to ubiquitin and, unlike existing commercially available anti-TDP-43 antibodies, did not stain normal nuclei. Ultrastructurally, these antibodies labeled abnormal fibers of 15nm diameter and on immunoblots recognized hyperphosphorylated TDP-43 at 45kDa, with additional 18 to 26kDa fragments in sarkosyl-insoluble fractions from FTLD-U and ALS brains. The phosphorylated epitopes were generated by casein kinase-1 and -2, and phosphorylation led to increased oligomerization and fibrillization of TDP-43.

**Interpretation:** These results suggest that phosphorylated TDP-43 is a major component of the inclusions, and that abnormal phosphorylation of TDP-43 is a critical step in the pathogenesis of FTLD-U and ALS. Phosphorylation-specific antibodies will be powerful tools for the investigation of these disorders.

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Tau-negative and ubiquitin-positive inclusions (UPIs) that include neuronal cytoplasmic inclusions (NCIs), neuronal intranuclear inclusions (NIIs), and dystrophic neurites (DNs) are the pathological hallmarks of frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) with or without clinical features of motor neuron disease (MND).<sup>1</sup> Recently, several genes and chromosomal loci, including the progranulin (*PGRN*) gene,<sup>2,3</sup> valosin-containing protein (*VCP*) gene,<sup>4</sup> and an unidentified site at chromosome 9p,<sup>5,6</sup>

have been reported to be associated with familial FTLD-U. Ubiquitin-positive, tau-negative NCIs have also been recognized in patients with the classic type of MND, amyotrophic lateral sclerosis (ALS),<sup>7</sup> in which skein-like cytoplasmic inclusions are found in the lower motor neurons of the hypoglossal nucleus and spinal cord.<sup>8,9</sup> In both FTLD-U and ALS, understanding why these inclusions form may provide critical clues to the neurodegenerative process.

Recently, TAR DNA-binding protein of 43kDa

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(TDP-43), which functions in regulating transcription and alternative splicing,<sup>10,11</sup> was identified as a component of these UPIs.<sup>12-14</sup> TDP-43 appears to belong to the group of two RNA-binding domain-Glycine RNA-binding proteins, which include the heterogeneous nuclear ribonucleoprotein (hnRNP) family and factors involved in RNA splicing and transport.<sup>15</sup> TDP-43 binds hnRNP A/B and hnRNP A1 through its C-terminal region, inhibiting premessenger RNA splicing.<sup>16</sup> Several disorders, including FTL-D-U, FTL-D-MND, and ALS are now referred to as TDP-43 proteinopathies.<sup>12-14</sup> Immunoblot analysis of the sarkosyl-insoluble fraction extracted from brains of patients afflicted with these disorders shows an abnormal TDP-43-immunoreactive band at 45kDa. The electric mobility of this band changes after dephosphorylation, suggesting that abnormal phosphorylation takes place in accumulated TDP-43.<sup>12,13</sup> However, the phosphorylation sites, responsible kinases, and pathological significance of phosphorylation are still unknown.

In this report, we demonstrate that multiple antibodies raised against TDP-43 phosphopeptides label UPIs in histological sections from FTL-D-U and ALS brains. These antibodies may offer advantages over previous antibodies used to identify these structures because they appear to be more sensitive than anti-ubiquitin antibodies and, unlike commercially available anti-TDP-43 antibodies, do not stain normal neuronal nuclei. In addition, these antibodies specifically recog-

nize abnormal TDP-43 species on immunoblots of sarkosyl-insoluble fractions extracted from FTL-D-U and ALS brains. Furthermore, we show that the multiple phosphorylation epitopes identified in aggregated TDP-43 are generated by casein kinase-1 (CK1), and that oligomerization or fibril formation of TDP-43 is promoted by phosphorylation with CK1 in vitro. These results suggest that phosphorylated TDP-43 is a critical component of UPIs in FTL-D-U and ALS, and that phosphorylation of TDP-43 by CK1 may be involved in the accumulation of the protein.

## Subjects and Methods

### Materials

Human brain tissue was obtained from the Brain Donation Program at Sun Health Research Institute (Sun City, AZ), Aichi Medical University (Japan), Jichi Medical University (Japan), National Shimofusa Mental Hospital (Japan), and Tokyo Metropolitan Matsuzawa Hospital (Japan). Small blocks of brain tissue were dissected at autopsy and frozen rapidly at -70 to 80°C or fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 2 days. Brain tissue from sporadic FTL-D-U, familial FTL-D-U with PGRN mutations (mPGRN), sporadic ALS, and sporadic FTL-D-MND was compared with brain tissue from Alzheimer's disease (AD) and neurologically normal control subjects. The age, sex, brain regions examined, diagnosis, and histopathological subtyping for these cases are given in Table 1. Neuropathological diagnoses of FTL-D-U, FTL-D-MND, ALS, and AD were made in accordance with published guidelines.<sup>1,9,17-19</sup>

**Table 1. Subjects, Brain Regions, Pathological Diagnosis, and Subtypes Examined**

Case No.	Age (yr)	Sex	Region	Diagnosis	Subtype
1	67	M	Hip, T, F	FTLD-U (sporadic)	1
2	59	M	Hip, T	FTLD-U (sporadic)	1
3	68	F	Hip, T	FTLD-U (sporadic)	1
4	49	F	T	FTLD-MND	2
5	76	M	Prec	FTLD-MND	2
6	66	M	F, SC	ALS	2
7	70	M	Prec, SC	ALS	2
8	69	M	Prec	ALS	2
9	53	M	Hip, T, F	FTLD-U (mRGRN)	3
10	56	F	Hip, T, F	FTLD-U (mRGRN)	3
11	54	M	Hip, T, F	FTLD-U (mRGRN)	3
12	68	F	Hip, T	AD	—
13	83	F	Hip, T	AD	—
14	65	M	Hip, T	Control	—
15	72	M	Hip, T	Control	—

Hip = hippocampus; T = temporal; F = frontal; FTL-D-U = frontotemporal lobar degeneration with ubiquitinated inclusions; MND = motor neuron disease; Prec = precentral; SC = spinal cord; ALS = amyotrophic lateral sclerosis; mPGRN = mutations of progranulin gene; AD = Alzheimer's disease.

Although none of the three ALS cases had a documented history of dementia, all had immunohistochemical evidence of pathology in the neocortex.

#### *Preparation of Antibodies*

Immunogens consisted of 39 synthetic phosphopeptides representing 36 of the 64 Ser/Thr/Tyr sites in the human TDP-43 molecule. All peptides were conjugated at the amino or carboxyl terminal by a cysteine linkage to synthetic thyroglobulin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as a coupling reagent.<sup>20</sup> The rabbit antisera were purified by obtaining flow-through fractions from a Toyopearl AF-Tresyl-650M (TOSOH, Tokyo, Japan) or SulfoLink Coupling Gel (Pierce Biotechnology, Rockford, IL) precoated with the nonphosphorylated synthetic peptide. The specificities of the antibodies were verified by enzyme-linked immunosorbent assay and immunoblot. A phosphorylation-independent rabbit polyclonal antibody to TDP-43 was also produced using a C-terminal peptide of TDP-43 (405-414) as immunogen.

#### *Immunohistochemistry*

After cryoprotection in 15% sucrose in 0.01M phosphate-buffered saline (pH 7.4), paraformaldehyde-fixed tissue blocks were cut on a freezing microtome at 30 $\mu$ m thickness. The free-floating sections were immunostained with an anti-ubiquitin antibody (DF2, a gift from Dr Mori, 1:200),<sup>21</sup> a commercially obtained phosphorylation-independent anti-TDP-43 antibody (10782-1-AP; ProteinTech Group, Chicago, IL; 1:2,000), and a panel of phosphorylation-dependent anti-TDP-43 antibodies including pS409/410, using methods previously described.<sup>13</sup> Double-labeled immunofluorescence was performed using fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-conjugated secondary antibodies; sections were examined with a confocal laser microscope (LSM5 PASCAL; Carl Zeiss MicroImaging GmbH, Jena, Germany).

#### *Immunoelectron Microscopy*

Tissue blocks of ALS lumbar spinal cord were fixed in paraformaldehyde and embedded in LR White Resin (London Resin, Reading, United Kingdom). Ultrathin sections were incubated with pS409/410 (1:1,000), and the immunoreaction products were probed using colloidal gold particles (1:10 dilution; BBInternational, Cardiff, United Kingdom) according to a standard immunogold-based postembedding electron microscopic procedure.<sup>22</sup>

#### *Immunoblotting*

Sarkosyl-insoluble, urea-soluble fractions were extracted from the frontal and the temporal regions of control, FTLU, and ALS brains as previously described.<sup>23</sup> The samples before (-) and after (+) the treatment with lambda protein phosphatase ( $\lambda$ PPase) were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins in the gel were then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 3% gelatin in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (50mM Tris-HCl, pH 7.5, 150mM NaCl), membranes were incubated overnight with the primary antibody

ies. After incubation with an appropriate biotinylated secondary antibody, labeling was detected as described previously.<sup>13,23</sup>

#### *In Vitro Phosphorylation and Fibrillization of TDP-43*

Human TDP-43 complementary DNA were subcloned into pRK172 expression vectors and transformed into *Escherichia coli* BL21(DE3). For in vitro phosphorylation, crude extracts from *E. coli* that expressed human TDP-43 were used to prepare partially purified TDP-43 using heparin-Toyopearl column chromatography and elution with 0.5M NaCl. The elutes were phosphorylated with CK1 (10,000U/ml; New England Biolabs, Beverly, MA), casein kinase-2 (CK2) (10,000U/ml; New England Biolabs), or glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) (10,000U/ml; New England Biolabs) at 30°C for 14 hours. To study fibrillization, we incubated partially purified TDP-43 aliquots in 30mM Tris-HCl at pH 7.5 containing 4mM magnesium chloride, 2mM ATP, with or without CK1 (10,000U/ml) at 30°C for 3 days. A few drops of reaction solution was then applied to a carbon-coated copper grid and allowed to air-dry. The grid was placed on a drop of blocking solution (10mg/ml bovine serum albumin in phosphate-buffered saline) for 10 minutes and then placed on a drop of primary antibody (pS409/410, 1:200) for 2 hours at room temperature. After washing with 10mg/ml bovine serum albumin in phosphate-buffered saline, the grid was placed on a drop of the secondary antibody conjugated to 10nm colloidal gold particles (1:50; Sigma, St. Louis, MO) for 1 hour at room temperature. Finally, after another round of washing, the grid was negatively stained with 2% sodium phosphotungstate and examined with the electron microscopy (JEM-1230; JEOL, Akishime, Japan).

## **Results**

### *Multiple Sites within TDP-43 Are Abnormally Phosphorylated in Frontotemporal Lobar Degeneration with Ubiquitinated Inclusions and Amyotrophic Lateral Sclerosis*

There are multiple potential phosphorylation sites within human TDP-43, including 41 serine (Ser), 15 threonine (Thr), and 8 tyrosine (Tyr) residues. To identify the critical phosphorylation sites of TDP-43, we raised antibodies against 39 different synthetic phosphopeptides, representing 36 of 64 candidate phosphorylation sites (Table 2). The major strategy was to choose Ser and Thr residues that cover known protein kinase consensus phosphorylation motifs, including R-X-pSer/Thr for protein kinase A, pSer/Thr-X-X-Ser/Thr for CK1, pSer/Thr-X-X-E/D for CK2, and pSer/Thr-X-X-X-Ser for GSK3 and CK1. In addition, Ser/Thr residues in C-terminal region of TDP-43 were chosen because they are analogous to abnormal phosphorylation sites found in tau or  $\alpha$ -synuclein.

Of the generated antibodies, pS379, pS403/404, pS409, pS410, and pS409/410 intensely immunostained the UPIs in FTLU and ALS, and demonstrated, on immunoblots of sarkosyl-insoluble fractions

**Table 2. Antigen Peptides for Immunization of Rabbits**

	Site	Antigen Peptide
1	pT8	EYIRVT(p)EDENDEC
2	pS20	PIEIPS(p)EDDGTC
3	pS29	GTVLLS(p)TVTAC
4	pT88	CNYPKDNKRKMDDET(p)D
5	pS91	CDETDAS(p)SAVKVKR
6	pS92	CDETDASS(p)AVKVKR
7	pS91/92	DETDAS(p)S(p)AVKVC
8	pT103	CKRAVQKT(p)SDLIVLG
9	pS104	CKRAVQKTS(p)DLIVLG
10	pT116	PWKTT(p)EQDLKEC
11	pT141	KKDLKT(p)GHSKGC
12	pT153	CGFVRFT(p)EYETQVK
13	pS180	CKLPNS(p)KQSQDE
14	pS183	CPNSKQS(p)QDEPLR
15	pS190	CKQSQDEPLRS(p)RK
16	pT199	CT(p)EDMTEDLE
17	pT203	RCTEDMT(p)EDELK
18	pT233	CRAFADVT(p)FADDQ
19	pS254	IIKGIS(p)VKISC
20	pS258	CISVHIS(p)NAEPK
21	pS266	CPKHNS(p)NRQLER
22	pS273	CRQLERS(p)GRFGGN
23	pS292	CGFGNS(p)RGGGA
24	pS305	CNNQGS(p)NMGGG
25	pS317	CFGAFS(p)INPAM
26	pS332	CAALQS(p)SWGMM
27	pS350	CQSGPS(p)GNNQN
28	pS375	GNNSYS(p)GSNSGC
29	pS379	CSGSNS(p)GAAIG
30	pS389	CGWGSAS(p)NAGS
31	pS393	CASNAGS(p)GSGF
32	pS395	CAGSGS(p)GFNGG
33	pS403	CGFNGGFGS(p)SMD
34	pS404	CNGGFGSS(p)MDSK
35	pS403/ 404	CNGGFGS(p)S(p)MDSK
36	pS407	CGSSMDS(p)KSSGW
37	pS409	CMDSKS(p)SGWGM
38	pS410	CMDSKSS(p)GWGM
39	pS409/ 410	CMDSKS(p)S(p)GWGM

extracted from brains of the FTLD-U and ALS cases, an abnormal band of 45kDa but not the 43kDa band corresponding to normal TDP-43. The results suggest that these sites are phosphorylated in the abnormal aggregates of TDP-43 present in FTLD-U and ALS.

#### *Immunohistochemical Characterization of Phosphorylated TDP-43*

Immunohistochemical staining showed that five of the phosphorylation-specific anti-TDP-43 antibodies identified UPIs in both FTLD-U and ALS brains. Of the phosphorylation-specific antibodies, the immunoreactivity of pS409/410 was particularly robust (Fig 1). In comparison, the commercially obtained, phosphorylation-independent, anti-TDP-43 antibody labeled NCIs, DNs, and neuronal nuclei in the dentate gyrus (see Fig 1A) and the temporal cortex (see Fig 1B) of the sporadic FTLD-U cases, and skein-like inclusions and neuronal nuclei in the spinal cord of ALS cases (see Fig 1C). It was particularly difficult to distinguish the staining of NCIs from that of neuronal nuclei in the dentate gyrus of the sporadic FTLD-U cases (see Fig 1A). By contrast, NCIs and DNs were unambiguously identified by the phosphorylation-specific antibody pS409/410, with no nuclear staining (see Figs 1D, E). Similarly, pS409/410 clearly labeled skein-like (see Fig 1F) and glial inclusions (see Fig 1G) in the spinal cord, and NCIs in the frontal and precentral cortices of the FTLD-MND and ALS cases (see Fig 1H). Glial inclusions in the frontal and precentral regions of the FTLD-MND and ALS cases were also immunopositive (data not shown). In the cases of familial FTLD-U with PGRN mutations, pS409/410 intensely stained NCIs, DNs, and NIIs in the cerebral cortex (see Figs 1I–K) and abundant positive structures in the cerebral white matter (see Fig 1L). The results of double-immunofluorescence staining showed that pS409/410 identified more NCIs than the ubiquitin antibody (see Figs 1M–O).

Based on morphological aspects, TDP-43 proteinopathies have been classified into four subtypes.<sup>24</sup> Type 1 is characterized by DNs with few NCIs and no NIIs, type 2 has numerous NCIs with few DNs and no NIIs, type 3 has numerous NCIs and DNs and an occasional NII, and type 4 has numerous NIIs and DNs with few NCIs. Type 4 is specific for familial FTLD-U with mutations of valosin-containing protein (VCP) gene. In this study, using the commercial anti-TDP-43 antibody, all of the sporadic FTLD-U cases showed type 1 pathology, all FTLD-MND and ALS cases showed type 2 pathology, and all FTLD-U with mPGRN showed type 3 pathology, in agreement with previous reports.<sup>24,25</sup>

The staining patterns obtained with our phosphorylation-dependent antibodies were similar to those seen