mutant mice. Moreover, there were significant decreases in dopamine transporter and VMAT2 protein levels at these ages, consistent with the loss of dopaminergic nerve terminals (Fig. 3d and e).

No obvious behavioral defects in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice

Although observation of spontaneous, voluntary movements over 30 min in the open field test revealed that *parkin*-ko/PrP-*Pael-R*-tg mice tended to be more active at younger ages and less active at old ages compared with *parkin*-ko/*Pael-R*-non-tg mice, the difference failed to reach statistical significance (data not shown). In addition, these mice were similarly able to maintain their balance on the rotarod before falling off when young (data not shown). Although a tendency towards poor performance was observed in *parkin*-ko/PrP-*Pael-R*-tg mice at later stages, no significant difference was observed compared with *parkin*-ko/*Pael-R*-non-tg mice (data not shown).

Evidence for ER stress and activation of unfolded protein response in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice

We hypothesized that perturbation of ER homeostasis and triggering activation of unfolded protein response (UPR) underlies the progressive loss of dopaminergic neurons in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice. In the midbrain of *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice, significant increase in BiP mRNA levels was clearly detected at the age of 6 months and maintained higher levels of transcription throughout life (Fig. 4a). On the other hand,

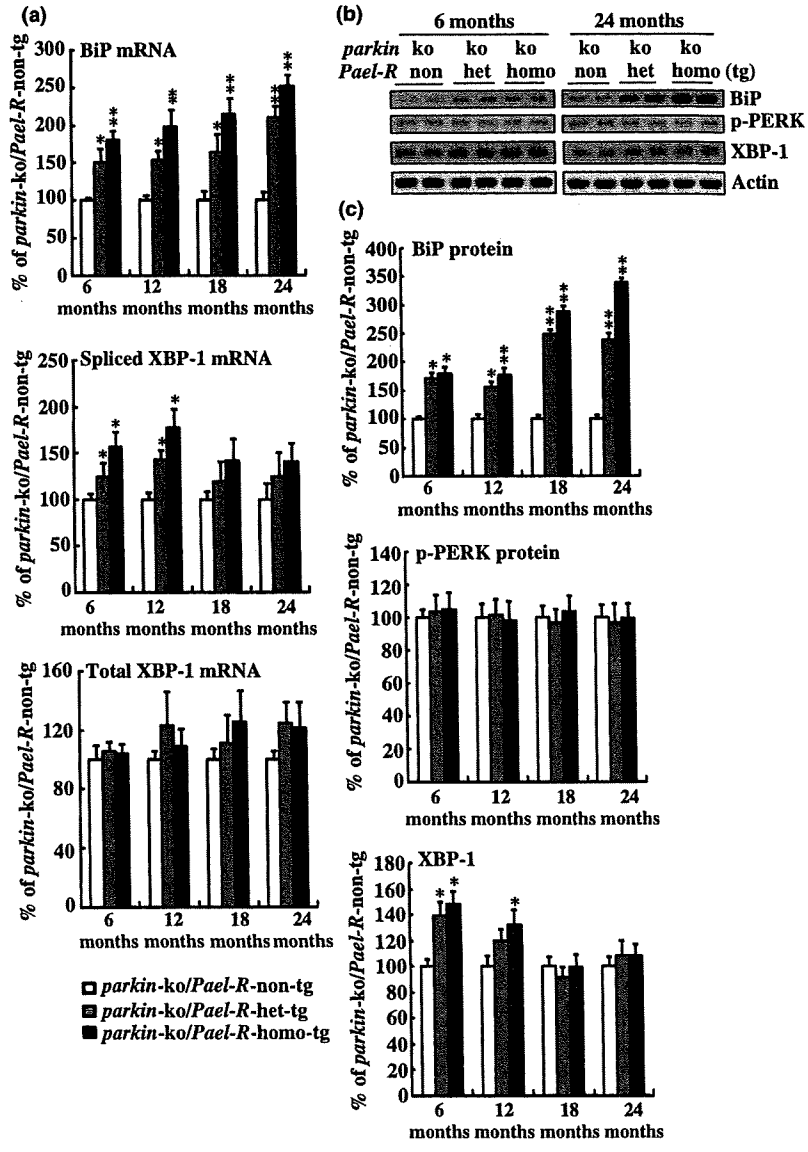

Fig. 4 Chronic and persistent activation of UPR in *parkin*-ko/*Pael-R*-tg double-mutant mice. (a) Real-time PCR was performed on mRNA extracted from the midbrain of *parkin*-ko/*Pael-R*-tg double-mutant mice. Data are expressed as mean ± SEM ($n = 6$). *, $p < 0.05$, **, $p < 0.01$ versus *parkin*-ko/*Pael-R*-non-tg. (b) Representative western blot images of BiP, phosphorylated form of PERK (p-PERK) and XBP-1 in the middle brain region of young (6 M) and aged (24 M) mice. Actin was used as a loading control. (c) The expression levels of BiP, p-PERK and XBP-1 protein (normalized to actin) were quantified using optical density. Data are expressed as mean ± SEM ($n = 6$). *, $p < 0.05$, **, $p < 0.01$ versus *parkin*-ko/*Pael-R*-non-tg.

the spliced form of XBP1 mRNA produced by 26-nucleotide splicing from primary XBP1 mRNA via the ribonuclease activity of IRE1 was increased only in early stages (Fig. 4a). Correspondingly, levels of BiP protein persistently increased in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice, whereas levels of XBP-1 increased only in the early stages (Fig. 4b and c). Collectively, these findings suggest that the IRE1 and ATF6 pathway of the UPR is activated in response to Pael-R accumulation *in vivo*.

On the other hand, PERK phosphorylation was unaltered at all time points examined (Fig. 4b and c), suggesting that global translational suppression induced by PERK phosphorylation may be transient in *parkin*-ko/*Pael-R*-tg double-mutant mice.

Evidence for ER-stress-mediated cell death: activation of JNK2, caspase-12 and CHOP/GADD153 in *parkin*-ko/*Pael*-R-tg double-mutant mice

Levels of CHOP were already substantially higher in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice at 6 months of age, followed by a nearly identical pattern of expression throughout life (Fig. 5a). The mRNA levels of caspase-12 and JNK2 were also increased at 12 months (Fig. 5a). The increase in levels of CHOP, JNK2 and caspase-12 mRNAs were accompanied by up-regulation of CHOP protein, phosphorylated JNK2 and cleaved form of caspase-12 (Fig. 5b and c).

Specific activation of UPR in dopaminergic neurons in *parkin*-ko/*Pael*-R-tg double-mutant mice

To confirm the specific occurrence of ER stress in dopaminergic neurons of the SNpc, double staining of TH and BiP or CHOP was performed. Up-regulation of BiP was restricted to TH-positive neurons and almost all TH-positive neurons exhibited intense expression of BiP in aged *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice (Fig. S4a). Intense expression of CHOP was also observed in dopaminergic neurons in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice (Fig. S4b).

Evidence for impairment of complex I in *parkin*-ko/*Pael*-R-tg double-mutant mice

An approximately 30% reduction in complex I activity was observed in mitochondria isolated from *parkin*-ko/PrP-*Pael-R*-tg double-mutant mouse midbrain as well as the whole brain obtained from 18- and 24-month-old mice (Fig. 6a and data not shown), whereas there was no significant differences in complex II-III or IV activities (data not shown).

To investigate how Pael-R over-expression reduces complex I activity, we used a microarray approach to search for molecular markers and found that two transcripts, encoding Ndufs4 and Ndufa10, were significantly down-regulated. Consistent with the GeneChip data, semi-quantitative real time PCR confirmed decreased expression of Ndufs4 and Ndufa10 in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice when values were normalized to those for the house-keeping genes actin or 18S RNA (Fig. 6b). The reduction in expression of Ndufs4 (18 kDa) and Ndufa10 (42 kDa) proteins was confirmed by immunoblot analysis (Fig. 6c and d). We further used a mixture of monoclonal antibodies directed against various proteins in complexes of the electron transport chain to investigate the assembly of complex I in *parkin*-ko/PrP-*Pael-R*-tg mice. Quantitative band densitometry of western blot images revealed minor but significant reduction of protein from complex I, but not those from complexes II-V (Fig. 6c and d).

Abnormality of DA and its metabolites in *parkin*-ko/*Pael*-R-tg double-mutant mice

Striatal levels of DA were significantly increased in younger *parkin*-ko/PrP-*Pael-R*-het-tg and *parkin*-ko/PrP-*Pael-R*-homo-tg mice compared with age-matched *parkin*-ko/*Pael-R*-non-tg mice. Over time, however, the levels of DA in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice gradually decreased, and eventually significantly decreased at 24 months. Accordingly, the levels of 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the major metabolites of DA, were significantly increased in *parkin*-ko/PrP-*Pael-R*-homo-tg and *parkin*-ko/PrP-*Pael-R*-het-tg at younger stages (Fig. 7).

Evidence for oxidative damage in *parkin*-ko/*Pael*-R-tg double-mutant mice

It is widely believed that dopamine can induce neurotoxic effects via the formation of highly reactive oxygen species, quinones and semiquinones generated by dopamine auto-oxidation or via its enzymatic metabolism by MAO, leading to oxidative stress. The finding of increased levels of dopamine and its metabolites in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice therefore prompted us to investigate whether increased levels of oxidized proteins could be detected in these mice. Both *parkin*-ko/*Pael-R*-non-tg and *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice represented age-dependent increases in levels of protein carbonyls, a general marker of oxidative damage. Levels of protein carbonyls were significantly higher in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice compared with *parkin*-ko/*Pael-R*-non-tg controls in the midbrain region (Fig. 8a), but not in the cortex region (Fig. 8b), suggesting that dopamine and/or its metabolites play important roles in the production of protein carbonyls.

Discussion

Recently, we have published a mouse model of PD by infecting Pael-R encoding adenovirus in the substantia nigra and shown that Pael-R over-expression *in vivo* leads to ER stress-induced death of dopaminergic neurons over a couple of weeks (Kitao *et al.* 2007). To confirm the results in a

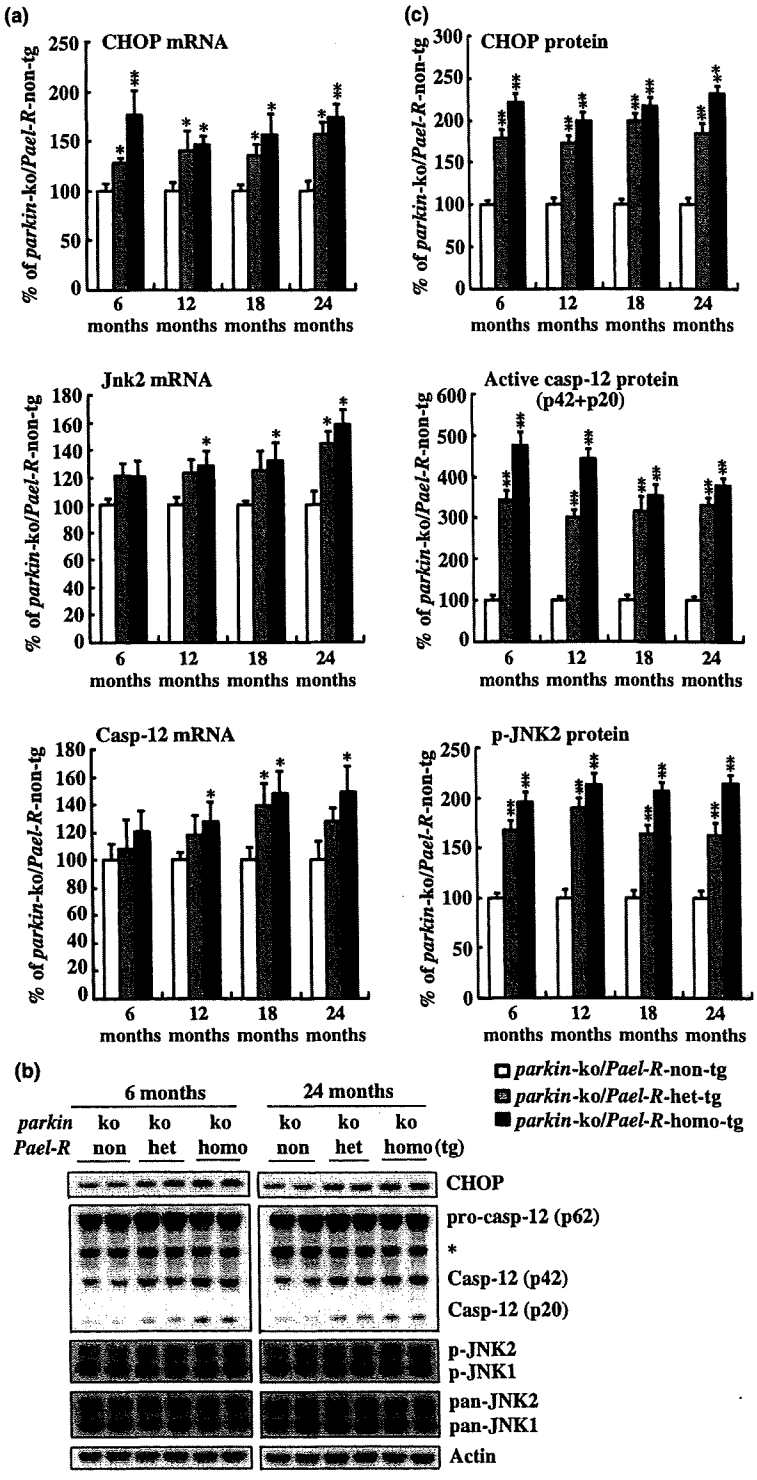

Fig. 5 Up-regulation of CHOP, JNK2 and caspase-12 in *parkin*-ko/PrP-*Pael-R*-het-tg and *parkin*-ko/PrP-*Pael-R*-homo-tg mice. (a) Real-time PCR was performed on the same samples as in Fig. 4a. Values were standardized to the level of actin mRNA and data are expressed as mean ± SEM ($n = 6$). *, $p < 0.05$, **, $p < 0.01$ versus *parkin*-ko/*Pael-R*-non-tg. (b) Representative western blot images of caspase-12 (Casp-12), CHOP, and phosphorylated form of JNKs (p-JNK1/2) in the midbrain region of young (6 M) and aged (24 M) mice. Actin was used as a loading control. The pan-JNK1/2 antibody was also used to assess the total amount of JNK proteins. The asterisk indicates a non-specific signal. (c) The expression levels of CHOP, p-JNK2 and active forms of caspase-12 (p42 and p20) (normalized to actin) were quantified using optical density. Data are expressed as mean ± SEM ($n = 6$). *, $p < 0.05$, **, $p < 0.01$ versus *parkin*-ko/*Pael-R*-non-tg.

genetic mouse model, we have generated *parkin*-ko/*Pael-R*-tg double-mutant mice, in which ER stress is evoked and selective and progressive catecholaminergic neuronal death without inclusion body formation occurs over 2 years, which recapitulates the main features of AR-JP. We obtained multiple lines of evidence indicating that chronic and persistent ER stress causes progressive loss of dopaminergic neurons over a long period of time in *parkin*-ko/*Pael-R*-tg

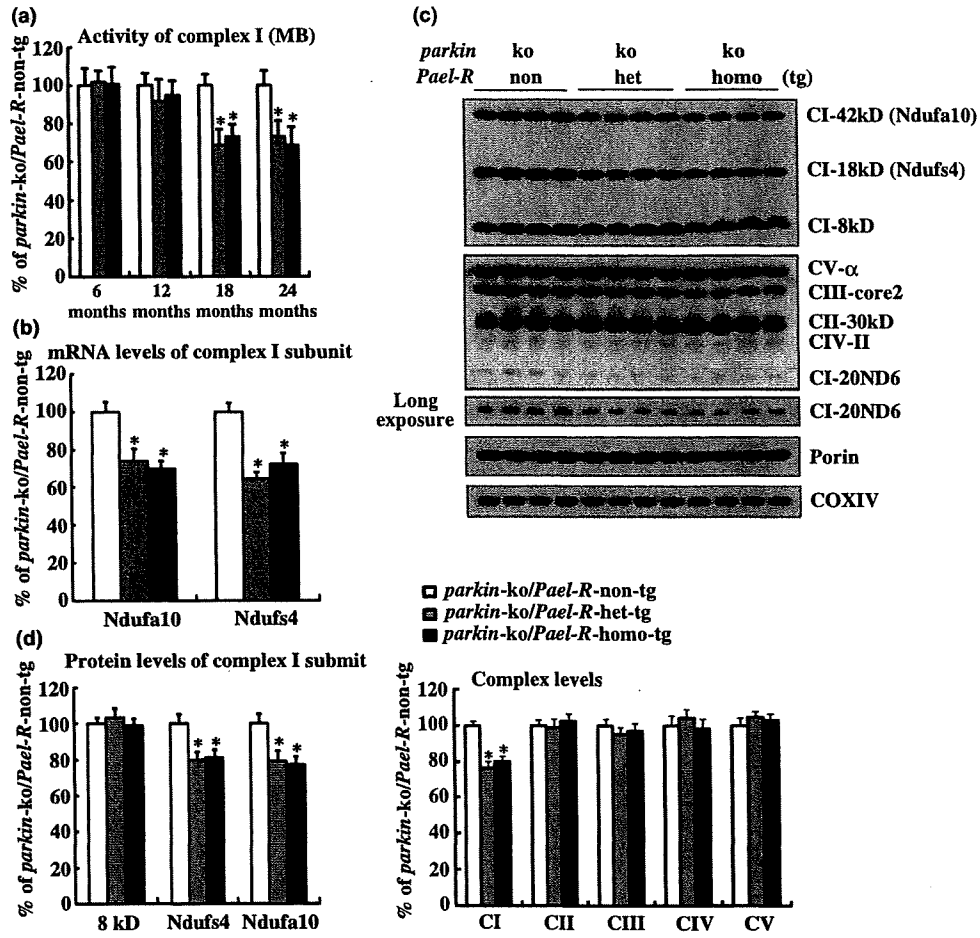

Fig. 6 Impaired mitochondrial complex I in *parkin*-ko/PrP-*Pael-R*-het-tg and *parkin*-ko/PrP-*Pael-R*-homo-tg mice. (a) Complex I activity analysis was performed on mitochondria isolated from *parkin*-ko/PrP-*Pael-R*-tg double-mutant mouse midbrains at later stages. Data are expressed as mean ± SEM ($n = 6$). *, $p < 0.05$ versus *parkin*-ko/*Pael-R*-non-tg. (b) Real-time PCR was performed on mRNA extracted from 18–24 months of *parkin*-ko/PrP-*Pael-R*-tg double-mutant mouse midbrains. The values were standardized to the level of actin mRNA and expressed as mean ± SEM ($n = 6$). *, $p < 0.05$ versus *parkin*-ko/*Pael-R*-non-tg. (c) Representative western blot images of subunits of complex I (Ndufa10, Ndufs4 and CI-8 kDa) and levels of complexes. Porin and COXIV were used as loading controls. (d) The expression levels of Ndufa10, Ndufs4, CI-8 kDa and complex I–V (normalized to COXIV) were quantified using optical density. Data are expressed as mean ± SEM ($n = 6$). *, $p < 0.05$ versus *parkin*-ko/*Pael-R*-non-tg.

double-mutant mice, providing a new genetic animal model to further explore the pathogenesis of PD.

In this study, parkin deletion promoted the accumulation of both soluble and insoluble Pael-R derived from the transgene, further supporting the idea that Pael-R is the substrate of parkin. Regarding the dopaminergic cell loss, it starts in PrP-*Pael-R*-het-tg mice at the age of 18 months, whereas it does in *Pael-R*-het -tg/*parkin*-ko double mutant mice at the age of 12 months (Figs 1a and 2e). Moreover, there were no difference in the dopaminergic cell number between 26-month-old PDGF-*Pael-R*-het-tg and wt mice, whereas 24-month-old PDGF-*Pael-R*-het-tg mice crossed with *parkin-ko* mice displayed reduced dopaminergic cell number compared with *parkin*-ko mice of the same age (Figs 1b, 2g and h). These data indicate that *parkin* deletion promotes neuronal loss by Pael-R accumulation.

Very recently, it was reported that around 35% TH-positive cell loss in the substantia nigra accompanied by motor behavioral abnormalities in foot print analysis occur in 24-month-old *parkin*-ko mice, raising the possibility that neurodegeneration phenotype of *parkin*-ko was simply added to that of Pael-R-tg mice(Rodriguez-Navarro *et al.* 2007). However, this is unlikely in our system, since 26-month-old *parkin*-ko mice displayed no dopaminergic neurodegeneration (Fig. 1c). The reasons for the discrepancies are unclear at this moment. Although we did not perform foot print

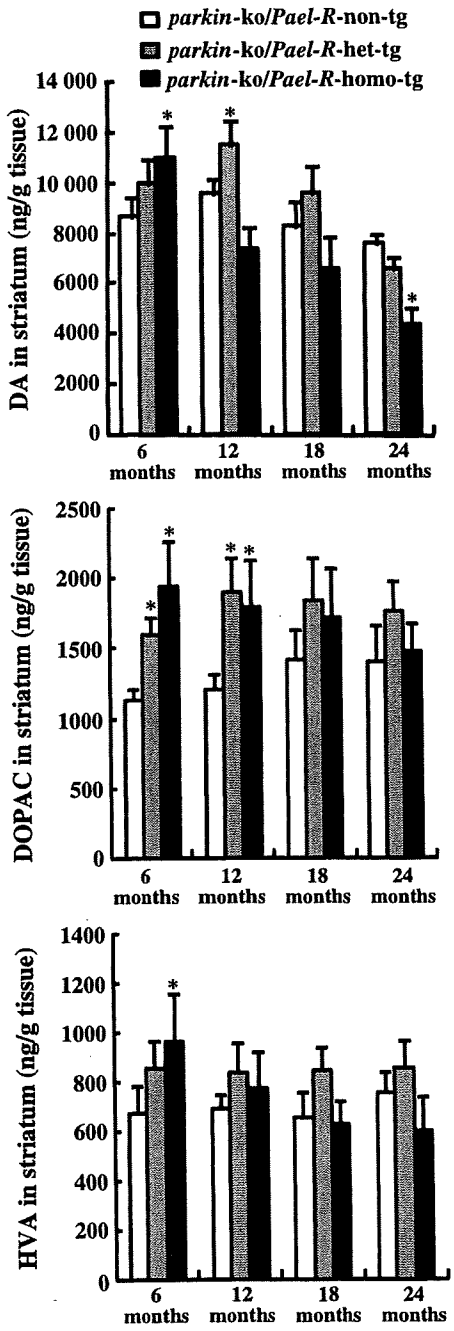

Fig. 7 HPLC analysis of DA and its metabolites in the striatum of *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice. Data are expressed as mean ± SEM ($n = 12$). *, $p < 0.05$, **, $p < 0.01$ versus *parkin*-ko/*Pael-R*-non-tg.

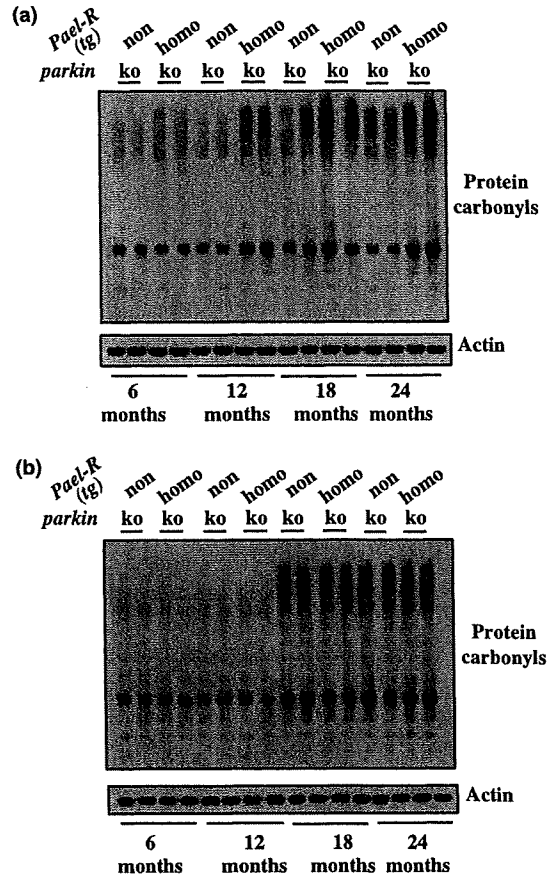

Fig. 8 Increased oxidative damage in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice. Carbonyl proteins were evaluated using lysates isolated from midbrain (a) and cortex (b). Incubation of the same blots with anti-actin antibody confirmed equivalent loading of proteins in each lane.

analyses, they might have detected abnormalities in *parkin*-ko/*Pael-R*-homo-tg double mutant mice which display 40% TH-positive cell loss at the age of 24 months.

Speculation regarding the involvement of ER stress in neuronal death has grown recently, due in part to reports of activation of the UPR in *in vitro* and *in vivo* models of neurodegenerative diseases (Nakagawa *et al.* 2000; Imai *et al.* 2001; Southwood *et al.* 2002; Takahashi and Imai 2003; Rao and Bredesen 2004; Tessitore *et al.* 2004). BiP has been shown to accompany ER stress and to be anti-apoptotic, while the failure of cells to counteract ER stress initiates activation of multiple pathways that lead to apoptosis (Breckenridge *et al.* 2003). CHOP is a member of CCAAT/enhancer-binding protein family that is induced by ER stress and participates in ER stress-mediated apoptosis (Oyadomari and Mori 2004). Excessive ER stress can also activate caspase-12, which resides on the outside of ER membrane (Nakagawa *et al.* 2000). Moreover, it has been shown that the ER transmembrane kinase/nuclease IRE1 can activate the c-Jun N-terminal kinase (JNK) by recruiting TRAF2 in response to ER stress (Urano *et al.* 2000). The early and consistent up-regulation of BiP, accompanied by activation of caspase-12, CHOP and JNK2 in *parkin*-ko/PrP-

Pael-R-tg double-mutant mice might represent cellular efforts to relieve ER stress. Over time, however, the cellular mechanisms fail to correct the continuous protein-folding defects, eventually leading to activation of multiple ER stress-mediated apoptotic processes. The finding of lack of alteration of phosphorylation of PERK is interesting, and suggests that persistent UPR induced in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice is not identical to conventional acute UPR.

Chronic and mild ER stress is known to induce UPR which allows for adaptation, instead of apoptosis, although UPR is designed to facilitate both adaptation to stress and apoptosis. Kaufmann and his colleagues have recently reported that survival is favored during chronic stress as a result of the intrinsic instabilities of mRNAs and proteins that promote apoptosis (Rutkowski *et al.* 2006; Rutkowski and Kaufman 2007). Consistent with their data, the scale of BiP up-regulation of double mutant mice at 24 months of age was greater than that of CHOP (Figs 4b and 5b).

It has been reported by several groups that complex I is decreased in the substantia nigra, skeletal muscle and platelets of patients with PD. Moreover, complex I inhibitors such as 1-methyl-4-phenyl-1,2,2,6-tetrahydropyridine and rotenone have been shown to cause dopaminergic cell death (Mizuno *et al.* 1998; Betarbet *et al.* 2000; Schapira 2001; Dauer and Przedborski 2003). More recently, phosphatase and tensin homologue-induced kinase 1 (PINK1), a mitochondrial protein, and DJ-1, a protein involved in oxidative stress partly located at mitochondria, turned out to be the genes responsible for familial PD termed PARK6 and PARK7, respectively (Dawson and Dawson 2003; Miller *et al.* 2003; Shen and Cookson 2004; Valente *et al.* 2004). These findings strongly support the idea that mitochondrial dysfunction, especially complex I deficiency plays a crucial role in the pathogenesis of PD. In this study, we found that complex I activity was decreased in Pael-R over-expressing mice, suggesting an important link between ER stress and mitochondrial dysfunction, both of which are thought to be involved in the pathogenetic mechanisms underlying PD.

In *parkin*-ko/*Pael-R*-tg double-mutant mice, the decrease in complex I activity was ascribable to transcriptional down-regulation of Ndufs4 and Ndufa10 subunits. Although we examined whether tunicamycin, thapsigargin or Pael-R over-expression-induced UPR is responsible for the down-regulation of Ndufs4 and Ndufa10 in cultured cells, only negative results were obtained (data not shown). The mechanism underlying down-regulation of Ndufs4 and Ndufa10 subunits of complex I in Pael-R tg mice is thus still unknown.

It is worth noting that down-regulated subunits Ndufs4 and Ndufa10 in PrP-*Pael-R*-tg mice are two exclusively phosphorylated proteins among complex I subunits (Schulenberg *et al.* 2003, 2004; Smeitink *et al.* 2004). Phosphorylation of mitochondrial proteins is pivotal to the regulation of respiratory activity in cells, and to signaling pathways leading to apoptosis, as well as for other vital mitochondrial processes. Ndufs4 has been suggested to be involved in assembly of functional complex I (Scacco *et al.* 2003). In addition, cyclic AMP-dependent intracellular signal transduction via phosphorylation of Ndufs4 has been reported to regulate the activity of complex I (Papa *et al.* 2001; Smeitink *et al.* 2001). The functional consequences of Ndufa10 phosphorylation await further investigation but might affect the binding affinity to NADH and in turn regulate the amount of fully active complex I (Schulenberg *et al.* 2003). Ndufa10 has appeared late in mitochondrial evolution and has been referred to as a "mammalian-specific" subunit of complex I (Cardol *et al.* 2004), consistent with a more regulatory role for this phosphorylated protein. It should also be noted that the phosphorylation sequence of Ndufa10 is likely to be a casein kinase I-like consensus motif, giving rise to the possibility that PINK1 is responsible for its phosphorylation (Schilling *et al.* 2005). Moreover, recent studies indicated that PINK1 and Parkin function, at least in part, in the same pathway, with PINK1 functioning upstream of Parkin, based on the observations that PINK1 and Parkin deficient *Drosophila* exhibit the identical phenotype with male sterility, apoptotic muscle degeneration and defects in mitochondrial morphology (Clark *et al.* 2006; Park *et al.* 2006; Yang *et al.* 2006). In this regard, whether Ndufs4 and Ndufa10 are substrates of PINK1 is an important issue to be clarified.

One feature common to dopaminergic neurons is the constitutive synthesis of dopamine within their cytoplasm. This is potentially important, given that metabolism of dopamine gives rise to various molecules that can act as endogenous toxins. If not properly handled, cytoplasmic dopamine might provoke neuronal damage through the generation of reactive oxygen species and, therefore, through mechanisms of oxidative stress (Shen and Cookson 2004). It has been shown that dopamine facilitates the transition of non-toxic α-synuclein protofibrils to toxic fibrils present in Lewy bodies (Lee *et al.* 2001; Sulzer 2001). It has also been reported that covalent modification of Parkin by dopamine lead to substantial inhibition of its E3 activity (LaVoie *et al.* 2005). Moreover, reduced level of VMAT2, leading to increase of cytoplasmic dopamine, is shown to result in progressive nigrostriatal neurodegeneration in mice (Caudle *et al.* 2007). This suggests the possibility that inappropriate metabolism of dopamine or its signaling or both might contribute to the selective degeneration of dopaminergic neurons. Notably, panneuronal expression of Pael-R in *Drosophila* causes age-dependent selective degeneration of dopaminergic neurons, and knockdown of *parkin* exacerbates this phenotype (Yang *et al.* 2003). Moreover, we have recently found that Alpha-methyl-p-tyrosine (AMPT), a TH inhibitor, ameliorates dopaminergic cell death induced by infection of adenovirus encoding Pael-R, implicating the pathological role of dopamine and its metabolites (Kitao

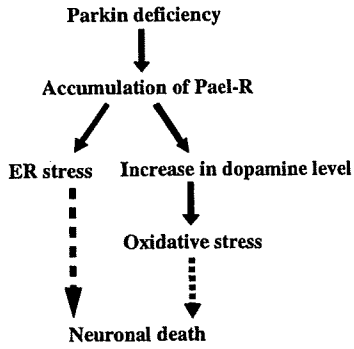

Fig. 9 The mechanisms underlying neuronal death in *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice.

et al. 2007). *parkin*-ko/PrP-*Pael-R*-tg double-mutant mice demonstrate higher levels of DA, DOPAC and HVA early in the disease process and maintain higher levels of DOPAC and HVA throughout the lifetime. Correspondingly, these mice show higher levels of protein carbonyls, a well-known marker of oxidative damage specifically in the midbrain (Fig. 9). It is recently reported that oxidative stress compensatory mechanisms are impaired in 24-month-old *parkin* ko mice, suggesting that they may contribute to the increase of oxidative stress at the end-stage of double mutant mice(Rodriguez-Navarro *et al.* 2007).

Based on these observations, both chronic ER stress and excessive dopamine-mediated oxidative stress are likely to contribute to dopaminergic neuron-specific degeneration (Fig. 9). It is an intriguing question whether Pael-R is involved in the pathogenesis of sporadic PD, since Pael-R is localized to Lewy bodies(Murakami *et al.* 2004). The examination of Pael-R level accumulated in post-mortem brain of patients with sporadic PD will provide important clues to this question in the future. Taken together, *parkin*-ko/*Pael-R*-tg double-mutant mice provide an excellent opportunity to dissect the molecular mechanisms underlying AR-JP as well as other degenerative diseases caused by chronic ER stress.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Expression of Pael-R in distinct regions of brain.

Fig. S2 Loss of TH-positive neurons in *parkin-ko/PrP-Pael-R-tg* double-mutant mice.

Fig. S3 No reduction of Nissl-positive neurons in 24 months *parkin-ko/PrP-Pael-R-tg* double-mutant mice in the dentate gyrus region of hippocampus.

Fig. S4 Specific activation of UPR in *parkin*-ko/PrP-*Pael-R*-tg mice.

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ORIGINAL ARTICLE

Parkin as a tumor suppressor gene for hepatocellular carcinoma

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The *parkin* was first identified as a gene implicated in autosomal recessive juvenile Parkinsonism. Deregulation of the *parkin* gene, however, has been observed in various human cancers, suggesting that the *parkin* gene may be important in tumorigenesis. To gain insight into the physiologic role of parkin, we generated *parkin*−/− mice lacking exon 3 of the *parkin* gene. We demonstrated here that *parkin*−/− mice had enhanced hepatocyte proliferation and developed macroscopic hepatic tumors with the characteristics of hepatocellular carcinoma. Microarray analyses revealed that *parkin* deficiency caused the alteration of gene expression profiles in the liver. Among them, endogenous *follistatin* is commonly upregulated in both nontumorous and tumorous liver tissues of *parkin*-deficient mice. *Parkin* deficiency resulted in suppression of caspase activation and rendered hepatocytes resistant to apoptosis in a follistatin-dependent manner. These results suggested that *parkin* deficiency caused enhanced hepatocyte proliferation and resistance to apoptosis, resulting in hepatic tumor development, partially through the upregulation of endogenous *follistatin*. The finding that *parkin*-deficient mice are susceptible to hepatocarcinogenesis provided the first evidence showing that *parkin* is indeed a tumor suppressor gene.
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Keywords: *parkin*; hepatocellular carcinoma; follistatin

Introduction

The *parkin* was first identified as a gene implicated in autosomal recessive juvenile Parkinsonism (ARJP), the most frequent form of familial Parkinson disease (Farrer, 2006). Mutations in the *parkin* gene have been found among ARJP families worldwide (Kitada *et al.*, 1998). Parkin protein is characterized by a ubiquitin-like domain at the N terminus and two RING-finger motifs and in between RING-finger (IBR) motif at the C terminus (Kahle and Haass, 2004). There are an increasing number of studies, including ours, showing that parkin is an E3 ubiquitin ligase that targets a variety of candidate substrate proteins, resulting in proteosomal degradation (Jackson *et al.*, 2000; Shimura *et al.*, 2000; Takahashi *et al.*, 2003). Possible substrates for parkin include: *o*-glycosylated α-synphilin; α-synuclein interacting protein, synphilin-1 and Pael-R (Dawson and Dawson, 2003). These substrates suggest crucial roles for parkin in several cellular processes. However, the physiological role of parkin, especially in organs other than the brain, has not been clarified.

Human cancer develops through a multistep process involving the accumulation of genetic alterations that drive the progression of normal cells into malignant derivatives (Lengauer *et al.*, 1998). It has been shown that the development of human cancers can be triggered by various allelic deletions, which could theoretically contain tumor suppressor genes. Indeed, many tumors are associated with deletion of chromosomal regions containing the tumor suppressors *p53* and *Rb* (Hahn and Weinberg, 2002). One thing to be noted is that the loss of heterozygosity (LOH) within chromosomal region 6q25–q27 is frequently associated with various types of solid tumor, including carcinomas of the ovary (Saito *et al.*, 1996; Tibiletti *et al.*, 1996), breast (Rodriguez *et al.*, 2000), kidney (Morita *et al.*, 1991), lung (Kong *et al.*, 2000) and melanomas (Millikin *et al.*, 1991). On the other hand, the transfer of human chromosome 6 to melanoma cells resulted in the loss of their ability to form tumors in nude mice (Trent *et al.*, 1990). Moreover, normal chromosome 6 altered tumor growth properties *in vitro* and suppressed the tumorigenicity of breast cancer cells (Negrini *et al.*, 1994). These findings suggest that undefined genes present in chromosome 6 may play roles as tumor suppressor genes. Recently, physical mapping combined with LOH analysis identified *parkin* as a possible tumor suppressor gene, as the chromosomal region containing the highly

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unstable FRA6E common fragile site region at 6q25–q27 is frequently deleted in breast and ovarian tumors (Cesari *et al.*, 2003; Denison *et al.*, 2003). In addition, another study reported that 4 of 11 hepatocellular carcinoma (HCC) cell lines had heterozygous deletion of *parkin* exon, and that parkin protein expression was significantly decreased or absent in all 11 HCC cells (Wang *et al.*, 2004). These findings suggest that *parkin* is important as a tumor suppressor and is involved in the development of human cancers. To gain insight into the physiological and pathological role of parkin, we generated *parkin*−/− mice and analysed their phenotypes.

Results

Development of hepatocellular carcinoma in parkin−/− mice

The mutant *parkin* allele lacking exon 3 was generated by homologous recombination in murine AK18.1 ES cells (129S4/SvJaeSor), and the *parkin* mutation was maintained in a mixed 129/C57BL6SJL (75/25) genetic background (Kitao *et al.*, 2007). *Parkin*−/− mice were born alive and appeared healthy, but body weight was significantly reduced in both male and female *parkin*−/− mice compared to wild-type mice (31.77 ± 0.72 g for *parkin*−/− male mice vs 36.23 ± 0.58 g for wild-type male mice at 48 weeks of age; $n=42$ for each; Figure 1a). *Parkin*−/− mice were neurologically normal with no obvious behavioral abnormalities, and no neuropathologic changes were observed in aged *parkin*−/− mice, which is consistent with recent reports (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Palacino *et al.*, 2004; Von Coelln *et al.*, 2004; Perez and Palmiter, 2005). In contrast to the lack of neuropathologic abnormalities, a striking phenotypic change occurred in the livers of *parkin*−/− mice. Despite the low body weight of *parkin*−/− mice, their livers were enlarged: at 48 weeks of age, their liver weight was 1.5 times greater than that of wild-type mice (1.55 ± 0.24 vs 1.09 ± 0.34 g; Figure 1b). Thus, the ratio between liver weight and body weight of *parkin*−/− mice was about twofold greater than that of wild-type mice at 48 weeks of age.

The livers of aged *parkin*−/− mice had a distinct phenotype. In addition to hepatomegaly, macroscopic hepatic tumors developed in 33% (12/36) and 45% (19/42) of *parkin*−/− mice at 72 and 96 weeks of age, respectively. In contrast to the parkin−/− mice, no hepatic tumors were admitted in the wild-type and *parkin*+/− mice examined at the same age. Histological examination revealed that these tumors were trabecular-type liver cancers, which were similar to human HCC (Figure 2a). It is noteworthy that all HCC tissues examined expressed α-fetoprotein (AFP), the best known tumor marker for human HCC, whereas no AFP expression was observed in the nontumorous regions of either *parkin*−/− livers or wild-type livers (Figure 2b). Moreover, strong immunoreactivity for β-catenin was observed in HCC tissues of the *parkin*−/− mice, whereas staining for β-catenin was absent in the nontumorous region of the *parkin*-deficient liver (Figure 2c).

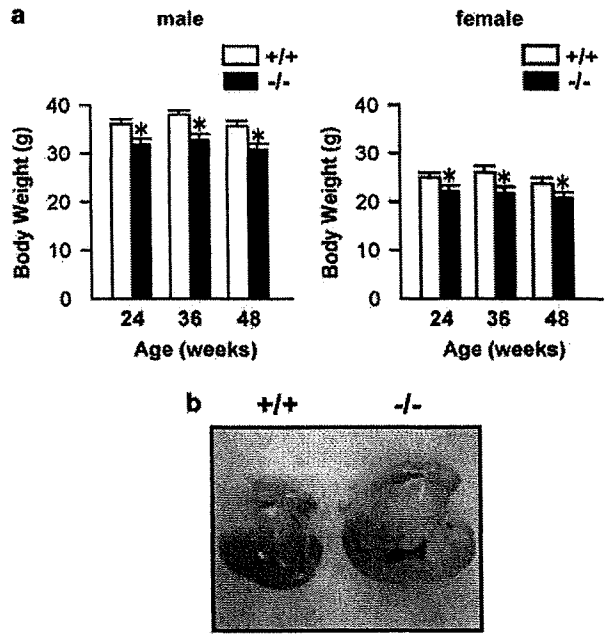

Figure 1 Enhanced proliferation of hepatocytes in the *parkin*-deficient liver. (**a**) Evolution of body weight for wild-type (+/+, open bars) and *parkin*-deficient (−/−, black bars) mice. **P*-value of <0.05 as determined by Student's *t*-test. (**b**) Liver anomalies in *parkin*−/− mice. Macroscopic hepatomegaly was observed in 48-week-old *parkin*−/− mice.

Gene expression profiles in nontumorous and tumorous liver tissues of parkin−/− mice

Consistent with previously reported *parkin*−/− mice models (Goldberg *et al.*, 2003), immunoblot analyses revealed no significant changes in the expression levels of several parkin substrate proteins (data not shown). On the other hand, it has been demonstrated that parkin has a RING-IBR-RING motif, which has been predicted to be involved in the regulation of gene expression (Morett and Bork, 1999). To provide an insight into the molecular mechanism by which *parkin* deficiency contributes to hepatocarcinogenesis, we analysed gene expression profiles of nontumorous and tumorous liver tissues from *parkin*−/− mice using cDNA microarray analyses. Out of 43 800 genes analysed, we identified a number of genes that were upregulated or downregulated in the liver tissues of *parkin*−/− mice. Among them, the expression ratios of 302 genes (including 59 genes of unknown function) were greater than fivefold in the tumorous liver tissues compared to those in normal livers of wild-type mice (Supplementary Table 1). Similarly, we identified 96 genes (including 31 genes of unknown function) that were upregulated in nontumorous liver tissues of *parkin*−/− mice compared to liver tissues of wild-type

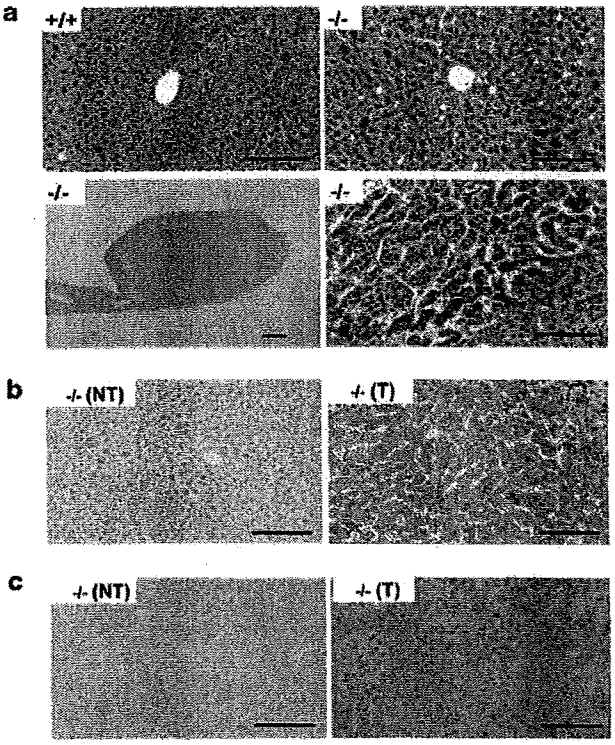

Figure 2 Development of hepatocellular carcinoma in *parkin*-deficient mice. (**a**) Histologic analyses of hepatic tumor specimens from *parkin*−/− mice obtained at 72 weeks of age. The upper panels show ×200 magnification of hematoxylin and eosin (HE)-stained liver sections of nontransgenic controls (+/+, left) and the nontumorous region of *parkin*-deficient mice (−/−, right). Scale bar = 200 μm. The lower panels show liver tumors of *parkin*−/− mice. Macroscopic view of representative hepatic tumor (left) and HE-stained section of the liver tumor (right; ×200) are shown. Scale bar = (left) 1 mm, (right) 200 μm. (**b**, **c**) Immunostaining for α-fetoprotein (AFP) and β-catenin in the hepatic tumor developed in *parkin*−/− mice. Immunohistochemical study for AFP (**b**) and β-catenin (**c**) protein was carried out in the nontumorous region (NT) and the tumor (T) of *parkin*−/− mice (magnification: ×200). Scale bar = 200 μm.

mice (Supplementary Table 2). To identify the key molecule responsible for enhanced hepatocyte proliferation, and thus hepatocarcinogenesis, in *parkin*−/− mice, we searched for genes that were commonly upregulated in both nontumorous and tumorous liver tissues of the *parkin*−/− mice. This ensured that the genes selected were not confined to nontumorous or to tumorous tissues. Using this filter, we identified 13 genes that were commonly upregulated in livers of *parkin*−/− mice (Table 1). These 13 upregulated genes included those expressing enzymes (cytochrome p450 (4a10), abhydrolase domain containing 1, hydroxysteroid dehydrogenase-4 and asparagine synthetase), molecules involved in metabolism, chemokine ligand 1 and the activin-antagonist, *follistatin*. The filter also identified genes that were downregulated in nontumorous (104 genes) and tumorous liver tissues (138 genes) of *parkin*−/− mice whose expression ratio was less than fivefold than those of corresponding genes in normal liver. Among them, 17 genes were commonly downregulated in both nontumorous and tumorous liver tissues of the *parkin*−/− mice (Supplementary Table 3), however, the role of these genes in tumorigenesis is unclear at present. The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at National Center for Biotechnology Information (NCBI) under the accession number GSE9651.

Upregulation of endogenous follistatin in parkin−/− *mice*

Follistatin is an endogenous antagonist of activin and controls proliferation, differentiation and apoptosis of numerous cell types in an autocrine and paracrine manner (Chen *et al.*, 2002; Harrison *et al.*, 2005). Recent studies demonstrated that overexpression of follistatin is associated with enhanced hepatocyte proliferation, resulting in enlargement of the liver *in vivo* (Kogure *et al.*, 2000; Takabe *et al.*, 2003). As *follistatin* expression is commonly upregulated in the livers of *parkin*−/− mice, we focused on the hepatic expression of follistatin to clarify the mechanism by which *parkin* deficiency causes HCC. To confirm the reliability of microarray data, we first examined the expression of *follistatin* using semiquantitative and quantitative real-time RT–PCR assays with primers specific for mouse *follistatin*. Expression of β-*actin* and *18S rRNA* transcripts served as internal controls. Although transcription of *follistatin* was low in the normal livers of wild-type mice, expression of *follistatin* was substantially upregulated in both tumorous and nontumorous liver tissues of *parkin*−/− mice (Figure 3a). Transcripts of the transforming growth factor-β superfamily genes in liver tissues, including the genes for *inhibin-A*, *inhibin-B*, *BMP2* and *BMP4*, were also examined, but there were no differences in their expression between wild-type and *parkin*−/− mice (Figure 3b). To further confirm the upregulation of *follistatin* expression in *parkin*−/− mice, we performed immunoblot analyses using antibodies specific for follistatin. The follistatin protein was not detected in lysates from liver tissues of wild-type mice. In contrast, follistatin protein was clearly detectable in the tumorous liver tissues of *parkin*−/− mice (Figure 3c), consistent with the data obtained from the quantitative real-time RT–PCR analyses. To examine the tissue-specific expression of *follistatin*, total RNA samples extracted from wild-type and *parkin*−/− mice tissues (liver, kidney, lung, small intestine and brain) were analysed using quantitative real-time RT–PCR. We found that *follistatin* mRNA was highly expressed in the livers of *parkin*−/− mice, whereas substantially lower levels of *follistatin* expression were observed in various tissues of the wild-type mice and in the liver of the *parkin*+/− mice (Figure 3d).

To determine whether parkin is involved in transcriptional regulation of *follistatin*, we examined the effect of *parkin* on expression of endogenous follistatin using cultured hepatoma-derived cells. First, we confirmed that follistatin protein expression was markedly decreased in Hep3B cells with parkin expression

Table 1 List of commonly upregulated genes in both nontumorous and tumorous liver tissues of *parkin*–/– mice

Gene name	*Tumor/nontumor*[a]	*Ref seq ID*	*Function*
Cell growth			
Chemokine ligand 1 (Cxcl1)	21.5/11.7	NM_008176	Angiogenesis
Follistatin (Fst)	10.7/8.0	NM_008046	Antagonist of activin
Biological process			
Asparagine synthetase (Asns)	92.4/39.2	NM_012055	Unknown
Apolipoprotein A-4 (Apoa4)	58.8/5.3	NM_007468	Lipid binding
Camello-like 4 (Cml4)	15.7/27.1	NM_023455	Unknown
Cytochrome p450 (4a10)	14.7/18.5	NM_177406	Monooxygenase
Sulfotransferase family 1E, member 1	6.36/14.4	NM_023135	Metabolism
Heat-shock protein 1A (Hspa1a)	5.63/5.07	NM_010479	Response to heat
Metabolism			
Hydroxysteroid dehydrogenase-4 (Hsd3b4)	8.29/29.1	NM_008249	Biosynthesis of steroids
Hydroxysteroid dehydrogenase-5 (Hsd3b5)	7.69/24.9	NM_008295	Biosynthesis of steroids
Cellular component			
WAP four-disulfide core domain 15	60.7/5.04	NM_138685	Extracellular space
Abhydrolase domain containing 1	8.65/14.7	NM_021304	Unknown
Serum amyloid P component	6.49/7.54	NM_011318	Precursor of amyloid

Genes that were upregulated in both nontumorous and tumorous liver tissues of *parkin*–/– mice whose expression ratio was greater than fivefold compared to normal livers of wild-type mice are shown.
[a]Values are fold increases in nontumorous and tumorous liver tissues of *parkin*–/– mice relative to normal livers of wild-type mice.

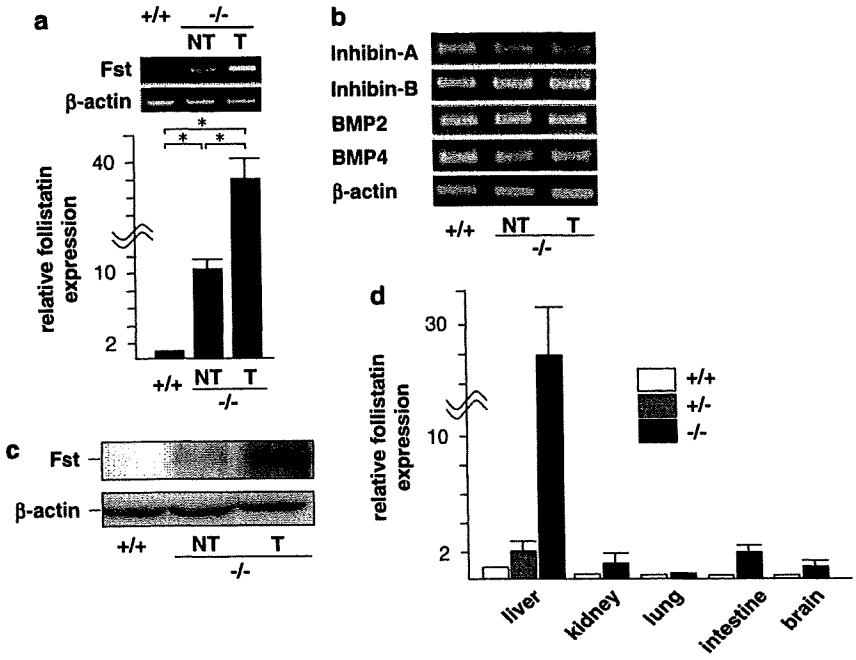

Figure 3 Upregulation of *follistatin* expression in the *parkin*-deficient liver. (**a**) Expression of endogenous *follistatin* (Fst, upper panel) or β-actin (lower panel) in the livers of wild-type (+/+), nontumorous (NT) and tumorous (T) tissues of *parkin*-deficient mice. The lower graphs show the relative mRNA levels of *follistatin* measured by quantitative real-time RT–PCR using *18S rRNA* as an internal control (mean ± s.d.; $n=3$). *P-value of <0.05 as determined by Student's t-test. (**b**) Total RNA was isolated from the liver tissues of wild-type (+/+), NT and T liver tissues of *parkin*–/– mice. RT–PCR was performed using 0.5 μg of each RNA as a template and oligonucleotide primer sets specific for mice *inhibin-A*, *inhibin-B*, *BMP2*, *BMP4* and *β-actin*. (**c**) Total protein was isolated, and immunoblot analyses were carried out using anti-Fst (upper panel) or anti-β-actin (lower panel) in the livers of wild-type (+/+), NT and T liver tissues of *parkin*–/– mice. (**d**) Expression levels of endogenous *follistatin* in various organs. Quantitative real-time RT–PCR was carried out using the total RNA isolated from the various organs of wild-type (+/+, open bars) and *parkin*–/– mice (black bars). The expression levels of Fst transcripts in the liver of the *parkin*+/– are also shown as a gray bar.

(Supplementary Figure 1a). Quantitative real-time RT–PCR analyses revealed that overexpression of *parkin* in cultured hepatoma-derived cells resulted in the substantial downregulation of *follistatin* expression, whereas mutant *parkin* lacking exons 3–4 had no significant effects on the expression of *follistatin*

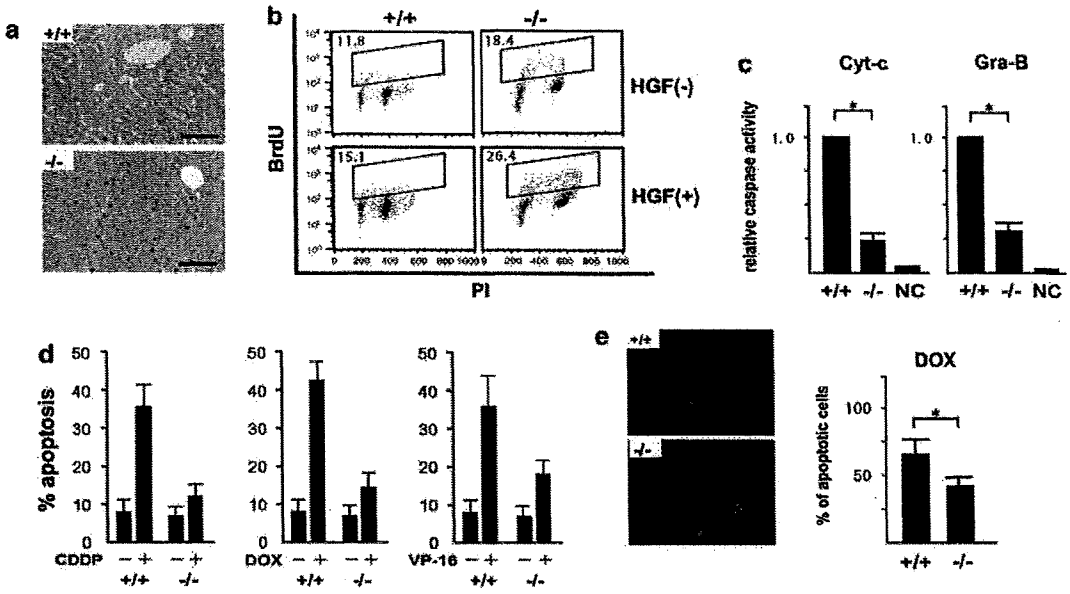

Figure 4 Increased cell proliferation and reduced apoptosis of hepatocytes of *parkin*−/− mice. (**a**) Increased proliferation of hepatocytes derived from the nontumorous region of *parkin*−/− mice. Hepatocyte proliferation in wild-type (+/+) and *parkin*-deficient (−/−) mice was evaluated by proliferating cell nuclear antigen (PCNA) staining. Scale bar = 200 μm. (**b**) 5-Bromodeoxyuridine (BrdU) labeling on the primary hepatocytes before and after the treatment with hepatocyte growth factor (HGF). Mouse primary hepatocytes were established from *parkin*−/− or wild-type (+/+) mice and cultured in fresh medium for 24 h, followed by the treatment with recombinant HGF (10 ng/ml) for 48 h. The levels of BrdU labeling were determined by flow cytometric analyses. (**c**) *Parkin* deficiency caused an overall inhibition of caspase activation induced by cytochrome *c* (Cyt-c) and granzyme B (Gra-B). Lysates were prepared from liver specimens of wild-type (+/+), *parkin*−/− mice and normalized for total protein content. Caspase activity was measured using the caspase-3 substrate, Ac-DEVD-pNA (10 mM), after the addition of Cyt-c and dATP (left graph) or Gra-B (right graph). Results are expressed relative to caspase activity generated in the normal liver of wild-type mice (mean ± s.d.; $n = 3$). Reaction mixture without Cyt-c or Gra-B was included as a negative control (NC). **P*-value of <0.05 as determined by Student's *t*-test. (**d**, **e**) *Parkin*−/− hepatocytes show resistance to apoptosis induced by various anticancer drugs. Primary hepatocytes were established from wild-type (+/+) or *parkin*−/− mice. Then, cells were treated for 48 h with 200 μM cisplatin (CDDP), 10 μM doxorubicin (DOX) or 400 nM etoposide (VP-16). Cell viability was determined by a trypan blue dye exclusion assay (**d**) and Annexin-V staining (**e**) (mean ± s.d.; $n = 3$). The percentage of apoptotic cells was determined from >200 cells, manually counted in triplicate. **P*-value of <0.05 as determined by Student's *t*-test.

(Supplementary Figure 1b). Moreover, expression of *parkin* in Huh7 cells resulted in dose-dependent reductions in the relative amounts of *follistatin* transcripts (Supplementary Figure 1c). Use of reporter plasmids encoding the promoter region of the *follistatin* gene revealed that parkin expression resulted in a substantial decrease in luciferase activity in the transfected cells (Supplementary Figure 1d). Finally, we investigated the expression profiles of *follistatin* as well as the *parkin* gene in clinical specimens of human HCC tissues. We found that most of the liver cancer specimens that lacked *parkin* expression showed upregulation of *follistatin* compared to the nontumorous region of the liver (Supplementary Figure 2a). In contrast, human HCC specimens that had a *parkin* expression level similar to the nontumorous regions did not show such enhanced expression of *follistatin* transcripts (Supplementary Figure 2b). Taken together, these findings indicate that the expression of *follistatin* was specifically elevated in the *parkin*-deficient livers and suggest that *parkin* is involved in the transcriptional regulation of *follistatin* expression in hepatocytes.

Parkin deficiency comprising follistatin upregulation renders hepatocytes more resistant to apoptosis

To investigate the molecular basis of hepatomegaly in *parkin*−/− mice, hepatocyte proliferation rate was determined using PCNA (proliferating cell nuclear antigen) labeling. Consistent with the development of hepatomegaly, enhanced hepatocyte proliferation was observed in the livers of 48-week-old *parkin*−/− mice (Figure 4a). Increased proliferation of *parkin*−/− hepatocytes was further supported by 5-bromodeoxyuridine (BrdU) labeling of primary hepatocytes, which showed that hepatocyte growth factor (HGF) stimulation resulted in a higher level of BrdU labeling in *parkin*−/− hepatocytes than in those of wild-type mice (Figure 4b). To clarify the functional significance of *follistatin* upregulation, we investigated whether *parkin*-deficient hepatocytes are resistant to apoptosis because of upregulation of *follistatin*. To determine whether caspase activation is suppressed in *parkin*-deficient hepatocytes, cytosolic extracts from the livers of *parkin*−/− and wild-type mice were prepared, normalized for total protein content and then treated with either cytochrome *c* or granzyme B. In control extracts

from wild-type mice, cytochrome *c* or granzyme B induced the activation of caspases, as measured by cleavage of the caspase substrate, Ac-DEVD-pNA. By comparison, far less caspase activity was induced by cytochrome *c* or granzyme B in extracts prepared from *parkin*−/− livers (Figure 4c). To investigate the anti-apoptotic features of *parkin*−/− hepatocytes, we established cultured primary hepatocytes from *parkin*−/− mice and compared levels of apoptosis induced by cisplatin (CDDP), doxorubicin (DOX) or etoposide (VP-16) between *parkin*−/− hepatocytes and wild-type cells. As shown in Figure 4d, significantly less cell death was induced by CDDP, DOX or VP-16 in the *parkin*−/− hepatocytes compared to the normal hepatocytes, consistent with the finding that *parkin* deficiency caused suppression of caspase activation. Annexin-V assay revealed significantly reduced numbers of apoptotic *parkin*−/− hepatocytes after treatment with DOX compared to those of the hepatocytes derived from the wild-type mice (Figure 4e).

To determine whether upregulation of *follistatin* is involved in reducing apoptosis in *parkin*−/− cells, we used small-interfering RNA (siRNA) to inhibit the expression of endogenous *follistatin* in primary mouse hepatocytes. Induction of *follistatin*-specific, but not control, double-strand synthetic RNAs reduced endogenous *follistatin* transcript levels in both normal and *parkin*−/− mouse hepatocytes, although only trace amounts of *follistatin* were detectable in hepatocytes derived from wild-type mice (Figure 5a). In the primary hepatocytes derived from *parkin*−/− mice, treatment with DOX was more effective in inducing apoptosis in hepatocytes treated with *follistatin* siRNA compared to those treated with control siRNA (Figure 5b). In contrast, a far less suppressive effect of knockdown of endogenous *follistatin* was observed on apoptosis of normal hepatocytes. These findings suggest that upregulation of *follistatin* is closely associated with suppression of caspase activation and apoptosis in *parkin*−/− hepatocytes.

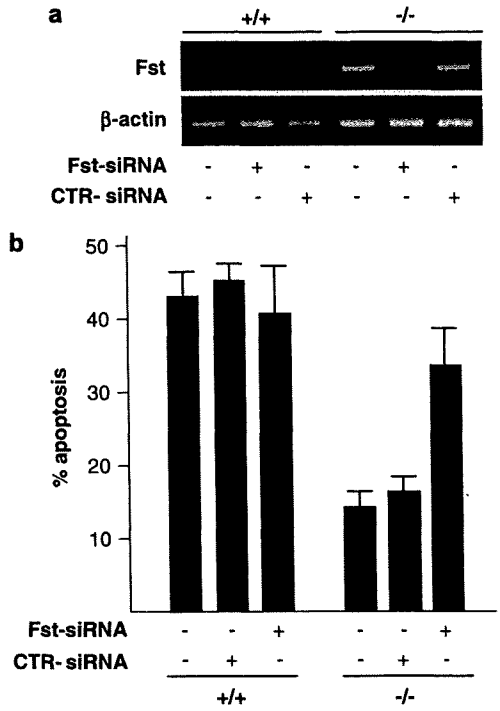

Figure 5 Regulation of apoptosis by endogenous follistatin in *parkin*-deficient cells. (**a**) Primary hepatocytes established from wild-type (+/+) and *parkin*−/− mice were transfected with small-interfering RNA (siRNA) targeting *follistatin*-siRNA (Fst-siRNA) or control siRNA (CTR-siRNA). The expression levels of *follistatin* (Fst, upper panel) or β-*actin* (lower panel) were examined by RT–PCR analyses. (**b**) The percentage of apoptotic cells (mean ± s.d.; $n=3$) was determined by a trypan blue dye exclusion assay following the culture of Fst-siRNA or control (CTR-siRNA)-transfected mouse primary hepatocytes with 10 μM DOX.

Discussion

The molecular events underlying the development of human HCC are not well understood. However, it is believed that a multistep process of genetic alterations is responsible for hepatocarcinogenesis (Feitelson *et al.*, 2002; Laurent-Puig and Zucman-Rossi, 2006). The development and progression of cancers is characterized by inactivation of tumor suppressor genes and by amplification of selected oncogenes (Farazi and DePinho, 2006). Although several transgenic mouse models have been shown to develop liver cancers, the key tumor suppressor molecules involved in hepatocarcinogenesis are unclear. In the present study, we demonstrated for the first time that parkin is important in the regulation of hepatocyte proliferation and apoptosis, and that the loss of parkin expression results in the development of HCC. There is a paucity of data on whether genes associated with Parkinson's disease are important in the biochemical pathways essential for carcinogenesis (West *et al.*, 2005). In contrast to epidemiologic studies indicating a negative association between Parkinson's disease and cancer, recent reports suggest that parkin is involved in the development of various human cancers (Cesari *et al.*, 2003; Denison *et al.*, 2003; Picchio *et al.*, 2004; Wang *et al.*, 2004). Our present findings, which show that *parkin*−/− mice are susceptible to HCC, provided the evidence suggesting that *parkin* is important as a tumor suppressor gene.

Several other groups independently generated *parkin*−/− mice with various targeted deletions of the *parkin* gene (Goldberg *et al.*, 2003; Itier *et al.*, 2003; Palacino *et al.*, 2004; Von Coelln *et al.*, 2004; Perez and Palmiter, 2005). Two groups described exon 3-deleted mice models similar to ours and reported that they had no dopaminergic neuronal loss (Goldberg *et al.*, 2003; Palacino *et al.*, 2004). A recent report also demonstrated negligible deficits in neurological function, learning or memory in exon 2-deleted models (Perez and Palmiter, 2005). These findings suggest that *parkin*−/− mice do not recapitulate signs central to Parkinsonism. However, all these previous studies focused on phenotypic analyses of the central nervous system. It is thus unclear whether these *parkin*−/− mice developed hepatic

tumors. Given that other *parkin*−/− mice did not develop HCC, the inconsistent phenotype observed by different laboratories might be due to differences in the genetic backgrounds of strains. Alternatively, the discrepancy between our results and those of others may have arisen from the timing of the analyses for the liver phenotype. Our *parkin*−/− mice model showed no evidence of hepatic tumors at 48 weeks. The majority of tumors were detected at an age of 72 weeks or older, whereas some of the previous analyses of *parkin*−/− mice were carried out when the mice were less than 72 weeks of age (Goldberg *et al.*, 2003; Palacino *et al.*, 2004). Further analyses are necessary to determine whether *parkin* deficiency is always responsible for enhanced hepatocyte proliferation and the development of HCC in various *parkin*−/− mouse models. In contrast to hepatomegaly, we found that the body weight of the *parkin*−/− mice was significantly lower than the wild-type mice by unknown reason. One possibility is to assume that changes in the levels of metabolism-related molecules were involved in the low body weights of *parkin*−/− mice.

Importantly, we demonstrated that *follistatin* is commonly upregulated in both nontumorous and tumorous liver tissues of *parkin*−/− mice. Our findings also showed that *parkin* expression resulted in the decrease in the expression levels of *follistatin* transcripts *in vitro*. The molecular mechanism responsible for regulation of *follistatin* transcription by parkin is unclear at present. However, it was shown that parkin regulates the gene transcription of monoamine oxidase in various cells (Jiang *et al.*, 2006). It is of note that the ability of parkin to suppress monoamine oxidase expression does not appear to be dependent on its ubiquitin ligase activity. Parc, a parkin-like ubiquitin ligase, also has a RING-IBR-RING motif and acts as a cytoplasmic anchor for the p53 protein, resulting in suppression of *p53* gene-dependent transcription (Nikolaev *et al.*, 2003). In the current study, reporter plasmid assay revealed that parkin expression induced the substantial decrease of promoter activity of *follistatin* gene. Thus, it might be possible to assume that parkin is involved in the transcriptional regulation of *follistatin* gene expression, either independent of its E3 ligase function or by the proteosomal degradation of the target protein related to the transcriptional regulation of *follistatin*. In addition to the possible transcriptional regulation of *follistatin* by parkin, the activation of β-catenin observed in the HCC tissues might be attributable to enhanced expression of the *follistatin* gene, because it has been demonstrated that activation of β-catenin results in the induction of *follistatin* expression in various cancer cells (Willert *et al.*, 2002; Germann *et al.*, 2003).

Our data showed that *parkin*−/− hepatocytes were significantly more resistant to cell death in association with the upregulation of *follistatin*. Follistatin is secreted, and is sometimes membrane associated, and exerts its effects as the most potent endogenous inhibitor of activin (Harrison *et al.*, 2005). Accumulating evidence suggests that the activin/follistatin system is involved in the regulation of hepatocyte proliferation and apoptotic cell death (Chen *et al.*, 2002; Harrison *et al.*, 2005; Rodgarkia-Dara *et al.*, 2006). Several animal models and analyses of clinical specimens revealed that deregulation of activin signaling, mainly achieved by follistatin upregulation, contributes to pathologic conditions such as hepatic inflammation, fibrosis and liver cancer (Rossmanith *et al.*, 2002; Grusch *et al.*, 2006; Patella *et al.*, 2006). Overexpression of activins induces hepatocyte apoptosis *in vitro* and *in vivo* (Schwall *et al.*, 1993; Hully *et al.*, 1994), whereas follistatin administration decreased hepatocyte apoptosis in a hepatitis mouse model (Patella *et al.*, 2006). Furthermore, follistatin administration by intraportal infusion or adenovirus-mediated overexpression stimulates DNA synthesis and liver growth in normal rat livers (Kogure *et al.*, 2000; Takabe *et al.*, 2003). Experimentally induced rodent liver tumors, as well as human HCCs, show upregulated expression of *follistatin* (Grusch *et al.*, 2006). These findings suggest that deregulation of the balance between the expression of activins and their antagonist, follistatin, may contribute to the growth advantage of hepatocytes and result in liver cancer. Therefore, it is likely that upregulation of *follistatin* is associated with the development of HCC in *parkin*−/− mice.

In conclusion, our findings demonstrate that parkin is important in the regulation of hepatocyte proliferation and apoptosis, and that the loss of parkin expression might contribute to the overproliferation of hepatocytes, leading to hepatocarcinogenesis. Because β-catenin activation was specifically observed in the tumor, but not in the nontumorous region of the *parkin*-deficient livers, additional events for genetic changes might be required in the *parkin*-deficient liver to develop the HCC.

Materials and methods

Generation of parkin−/− *mice*

The mutant *parkin* allele lacking approximately 2 kb of the *parkin* exon 3 genomic sequence was generated in a mixed 129SV/C57BL6 (50/50) genetic background (Kitao *et al.*, 2007). Mice received humane care according to the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23).

Isolation and culture of mouse primary hepatocytes

Mouse primary hepatocytes were obtained from 10- to 11-week-old mice by the two-step collagenase perfusion method (Seglen, 1976). In brief, hepatocyte suspensions were obtained by passing collagenase type II (Gibco BRL, Life Technologies Inc., Rockville, MD, USA) digested liver through a 70 μm cell strainer, followed by centrifugation to collect the mature hepatocytes. After isolation, hepatocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum and seeded on collagen type 1-coated dishes at a density of 8×10^4 cells/cm^2. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

siRNA duplexes composed of 25 nucleotide sense and antisense strands used for targeting follistatin were obtained

from Invitrogen (Carlsbad, CA, USA). siRNA (20 μM) in 60 μl of Trans-IT-TKO reagent (Mirus Bio Corporation, Madison, WI, USA) was incubated in serum-free Opti-MEM medium (Qiagen, Valencia, CA, USA) for 10 min, followed by the addition of transfection mixture into the cells.

Quantitative real-time RT–PCR
RNA was extracted using Sepasol-RNA 1 Super (Nacalai Tesque, Kyoto, Japan). For the RT reaction, total RNA was reverse-transcribed into cDNA using the Superscript III first strand synthesis system and oligo-dT_{12-18} primers (Invitrogen; Matsumoto *et al.*, 2006). PCR amplification was performed using Takara Ex Taq DNA polymerase (Takara, Tokyo, Japan). The oligonucleotide primers used in this study are shown in Supplementary Table 4. Quantification of gene expression was performed by quantitative real-time RT–PCR using a 7300 Real-Time PCR system (PE Applied Biosystems, Foster City, CA, USA) and Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) as described (Kou *et al.*, 2006). To assess the quantity of isolated RNA, as well as the efficiency of cDNA synthesis, target cDNAs were normalized to the endogenous mRNA levels of the housekeeping reference gene, *18S rRNA* (Matsumoto *et al.*, 2006). For simplicity, the expression levels of the target gene were expressed relative to those of the control specimen. Human liver tissues for RT–PCR analyses were obtained from biopsy specimens of tumor tissues at the proximal edge of freshly resected specimens and frozen immediately in liquid nitrogen.

RNA preparation and hybridization to the microarray
Total RNA was extracted from mice liver tissues using a RNA assay mini kit (Qiagen). First-strand cDNA was synthesized from 500 ng of total RNA in the presence of Cy5 or Cy3 dCTP. The Cy3- and Cy5-labeled samples derived from wild-type and *parkin−/−* mice at 72 weeks of age were injected simultaneously into the same spot of the whole mouse 60-mer oligo microarray (Agilent Technologies, Palo Alto, CA, USA). After hybridization at 65 °C for 17 h, the slides were washed with 6× SSC containing 0.005% Triton X-102, and dried using a nitrogen-filled air gun. Array image acquisition and feature extraction was performed using an Agilent G2565AA Microarray Scanner with feature extraction software (version 8.5.1.1; Agilent Technologies). Statistical evaluation was performed using the algorithm developed by Agilent for the array analysis. Genes that were upregulated or downregulated in tumorous and nontumorous liver tissues by more than fivefold compared to normal liver tissues and had *P*-values less than 0.05 were considered.

Immunoblotting analysis
Protein samples were separated by SDS–polyacrylamide gel electrophoresis (PAGE) 10% (w/v) polyacrylamide gels and subjected to immunoblotting analysis as described previously (Endo *et al.*, 2007). The polyclonal antibodies against mouse follistatin and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Calbiochem (San Diego, CA, USA), respectively.

Immunohistochemical staining analysis
Immunohistochemical staining was carried out according to a previously described protocol (Toda *et al.*, 1999). The polyclonal antibodies for PCNA, AFP and β-catenin were purchased from Santa Cruz Biotechnology, Dako Cytomation (Glostrup, Denmark) and Transduction Labs (Palo Alto, CA, USA), respectively.

Measurement of caspase activity and apoptosis assay
Liver tissues from the mice were prepared in buffer A (1 M KCl, 1 M $MgCl_2$, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.5 M EDTA and 10% CHAPS) and normalized for total protein content. A 30 μg sample of protein was incubated with either 10 ng of granzyme B (Calbiochem) or 0.5 nmol of cytochrome *c* (Sigma, St Louis, MO, USA) and 1 mM dATP at 37 °C for 30 min as described (Marusawa *et al.*, 2003). Then, 5 μl of reaction mixture was incubated with caspase-3 substrate Ac-DEVD-pNA (10 mM), and caspase activity was assessed using a colorimetric CaspACE assay system (Promega, Madison, WI, USA). For the measurement of caspase-3 activity, the plates were read at 405 nm using microplate reader (Molecular Devices Co., Tokyo, Japan). For the evaluation of cell death, mice primary hepatocytes were cultured in DMEM on collagen-coated dishes. After 24 h of isolation, cells were treated with either CDDP (200 μM), DOX (10 μM) or VP-16 (400 nM) for 48 h. Cell viability was determined by the trypan blue exclusion test (Marusawa *et al.*, 2003) or the Annexin-V-Fluos apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instruction.

Flow cytometric analysis
A total of 1×10^5 cells were plated on 10 cm collagen-coated dishes. Two days after treatment with HGF (PeproTech, London, UK) at a concentration of 10 ng/ml, BrdU (20 μM; Roche, Basel, Switzerland) was added to the culture medium for 12 h. The cells were then fixed in 70% ice-cold ethanol and incubated at 4 °C overnight. Fluorescence was determined by using a FACScan flow cytometer (Becton Dickinson, Franklin Lake, NJ, USA) after adding propidium iodide. Data were acquired and analysed using CELLQuest software (Becton Dickinson).

Cell culture, transfection and reporter plasmid assay
Human hepatoma-derived cell lines, Huh-7 and Hep3B were maintained in DMEM containing 10% fetal bovine serum. For plasmid transfection, we used Lipofectamine (Invitrogen) for Huh-7 and Trans-IT 293 (Mirus Bio Corporation) for Hep3B. To determine the promoter activity of the *follistatin* gene, the reporter plasmids were generated by inserting the PCR-amplified 196- and 361-bp 5′-proximal promoter regions of the *follistatin* gene (de Groot *et al.*, 2000) into the pGL-3 luciferase vectors (Promega). The reporter construct and pRL-TK (Promega) were then co-transfected with an expression plasmid-encoding *parkin* or control vector into NIH3T3 cells. The cell lysates were analysed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega; Tanaka *et al.*, 2006).

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ORIGINAL ARTICLE

Accumulation of HtrA2/Omi in Neuronal and Glial Inclusions in Brains With α-Synucleinopathies

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Abstract

HtrA2/Omi is a mitochondrial serine protease that is released into the cytosol and promotes apoptotic processes by binding to several members of the inhibitors of apoptosis protein family. HtrA2/Omi knockout mice show a parkinsonian phenotype, and mutations in the gene encoding HtrA2/Omi have been identified as susceptibility factors for Parkinson disease (PD). These results suggest that HtrA2/Omi may be involved in the pathogenesis of PD. We performed immunohistochemical studies of HtrA2/Omi on brains from patients with α-synuclein-related disorders, including PD, dementia with Lewy bodies (DLB), and multiple-system atrophy (MSA); patients with other neurodegenerative diseases; and controls. HtrA2/Omi is expressed in normal brain tissue, and there was some anti-HtrA2/Omi immunostaining of neurons in normal brains as well as those with other neurodegenerative diseases. In PD and DLB brains, both classic (i.e. brainstem-type) and cortical Lewy bodies were intensely immunostained; pale bodies were also strongly immunopositive for HtrA2/Omi. In MSA brains, numerous glial cytoplasmic inclusions, neuronal cytoplasmic inclusions, and dystrophic neurites were also intensely immunoreactive for HtrA2/Omi. These results suggest that widespread accumulation of HtrA2/Omi may occur in pathologic α-synuclein-containing inclusions in brains with PD, DLB, or MSA and that HtrA2/Omi may be associated with the pathogenesis of α-synucleinopathies.

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Key Words: α-Synucleinopathy, Glial cytoplasmic inclusions, HtrA2/Omi, Immunohistochemistry, Lewy bodies.

INTRODUCTION

Missense mutations of the α-synuclein gene (*PARK1*) (1–3) and multiplications of the α-synuclein gene (*PARK4*) (4–6) have been reported to be implicated in autosomal dominant familial forms of Parkinson disease (PD). Lewy bodies (LBs) are neuronal inclusions characteristic for PD and dementia with LBs (DLB) (7–9), and α-synuclein has been confirmed to be a major constituent of LBs in PD and DLB (10–12). Multiple-system atrophy (MSA) is a single nosologic entity that encompasses olivopontocerebellar atrophy, striatonigral degeneration, and Shy-Drager syndrome (13). Specific oligodendroglial cytoplasmic inclusions, referred to as *glial cytoplasmic inclusions* (GCIs), are the major pathologic features of brains with MSA (14, 15). α-Synuclein is also a major component of GCIs (12, 16, 17). Therefore, PD, DLB, and MSA are now collectively referred to as *α-synucleinopathies* (18).

The inhibitor of apoptosis protein (IAPs) play an important role in regulating apoptosis; some IAP family proteins, such as X chromosome-linked IAP, have the ability to bind to and directly inhibit selected caspases (19–21). HtrA2/Omi was identified as a mitochondrial serine protease that interacts with IAPs and contributes to the progression of apoptosis (22–25). HtrA2/Omi is released from the mitochondrial intermembrane space into the cytosol upon receiving various apoptotic stimuli, and this released HtrA2/Omi induces apoptotic cell death by binding to IAPs, including X chromosome-linked IAP, and blocking their caspase-inhibitory activities (22–27). HtrA2/Omi also enhances caspase activity by inducing permeabilization of the mitochondrial outer membrane, which leads to the release of cytochrome c (28). Furthermore, HtrA2/Omi knockout mice were reported to show a parkinsonian phenotype, with selective neuronal loss in the striatum (29). Together, these data suggest that, like cytochrome c, HtrA2/Omi is important for cell survival within the mitochondria and that, when it is released from the mitochondria, HtrA2/Omi promotes apoptotic cell death.

Recently, a G399S mutation of the *HtrA2/Omi* gene was identified in some patients with PD, and an A141S