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Molecules and Cells

Parkin Suppresses c-Jun N-Terminal Kinase-Induced Cell Death via Transriptional Regulation in *Drosophila*

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Parkin is the most prevalent genetic factor in the onset of autosomal recessive juvenile parkinsonism (AR-JP), and mutations in *parkin* has been reported to cause motor defects, which result from dopamine deficiency caused by dopaminergic neuronal cell death. Activation of c-Jun N-terminal kinase (JNK) has also been implicated in neuronal cell death in Parkinson's disease (PD). Moreover, *Drosophila* models for AR-JP, loss of function mutants of *Drosophila parkin*, also show dopaminergic neural degeneration associated with hyperactivation of JNK, increased apoptosis, and mitochondrial defects. However, the molecular mechanism by which Parkin protects cells from apoptosis remains unclear. In the present study, we tested whether *Drosophila* Parkin suppressed the JNK signaling pathway in developing tissues. Ectopically expressed *parkin* strongly suppressed the constitutively active form of Hemipterous (Hep^{CA}), a *Drosophila* JNK kinase that induces an eye degeneration phenotype and apoptosis in the eye imaginal disc. Moreover, *parkin* also suppressed extra vein formation induced by Basket (Bsk), a *Drosophila* JNK. Interestingly, the *bsk* mRNA level was markedly reduced by *parkin* over-expression, suggesting that the effect of *parkin* on the phenotype induced by activation of JNK signaling was achieved by transcriptional regulation. Furthermore, we found that the expression level of JNK target genes was reduced by *parkin* over-expression. Taken together, these results suggest that *Drosophila* Parkin suppresses JNK signaling by reducing *bsk* transcription.

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative motor disorder, the major symptoms of which are rigidity, tremor, bradykinesia of the limbs, and postural instability (Lang and Lozano, 1998). These motor defects result from dopamine deficiency caused by the degeneration of dopaminergic neurons in the subtantia nigra pars compacta and striatum, the neuropathological hallmark of PD (Rinne, 1993).

To date, several genes have been implicated in Mendelian-form inherited PD (Biskup et al., 2008). Among them, *parkin*, a major causative gene, is the most commonly mutated in familial PD (Kitada et al., 1998). *parkin* encodes an E3 ubiquitin ligase, which controls the level of other proteins and itself, by regulated protein degradation (Shimura et al., 2000). Indeed, several studies have shown that Parkin ubiquitinates and degrades several proteins, including CDCrel-1 (Zhang et al., 2000), parkin-associated endothelin receptor-like (Pael) receptor (Imai et al., 2001), α-synuclein (Shimura et al., 2001), synphilin-1 (Chung et al., 2001), and cyclin E (Staropoli et al., 2003). In addition to protein degradation, Parkin has also been implicated in transcriptional regulation of several genes including *p53* (da Costa et al., 2009; Liu et al., 2008; Unschuld et al., 2006). Supporting a role of Parkin in transcriptional regulation, Parkin has been shown to be localized in the nucleus as well as the cytoplasm (da Costa et al., 2009).

c-Jun N-terminal kinase (JNK) has been implicated in the neurodegenerative processes in PD pathogenesis as a mediator of stress-induced apoptosis (Cassarino et al., 2000; Cha et al., 2005; Wang et al., 2004; Xia et al., 2001). For example, JNK is activated by 1-methyl-4-phenylpyridinium ion (MPP^+), a parkinsonian neurotoxin, in human neuroblastoma cells (Cassarino et al., 2000), and inhibiting the JNK signaling pathway through over-expression of JNK interacting protein-1, a scaffold protein, or treatment with SP600125, a specific JNK inhibitor, suppresses MPP^+-induced cell death in dopaminergic neurons (Wang et al., 2004; Xia et al., 2001). Additionally, JNK is associated with Parkin in both mammalian cells and a *Drosophila* model (Cha et al., 2005; Liu et al., 2008). Parkin suppresses JNK activity (Cha et al., 2005; Liu et al., 2008), and JNK is strongly activated in the dopaminergic neurons of *parkin* mutants (Cha et al., 2005). However, the molecular mechanism by which Parkin regulates JNK activity is unknown.

In the present study, to examine the mechanism by which Parkin regulates the JNK signaling pathway, we investigated the effects of *parkin* over-expression on JNK kinase and the JNK-induced phenotype of developing tissues of *Drosophila*, and on the transcript level of *basket* (*bsk*), a JNK-encoding

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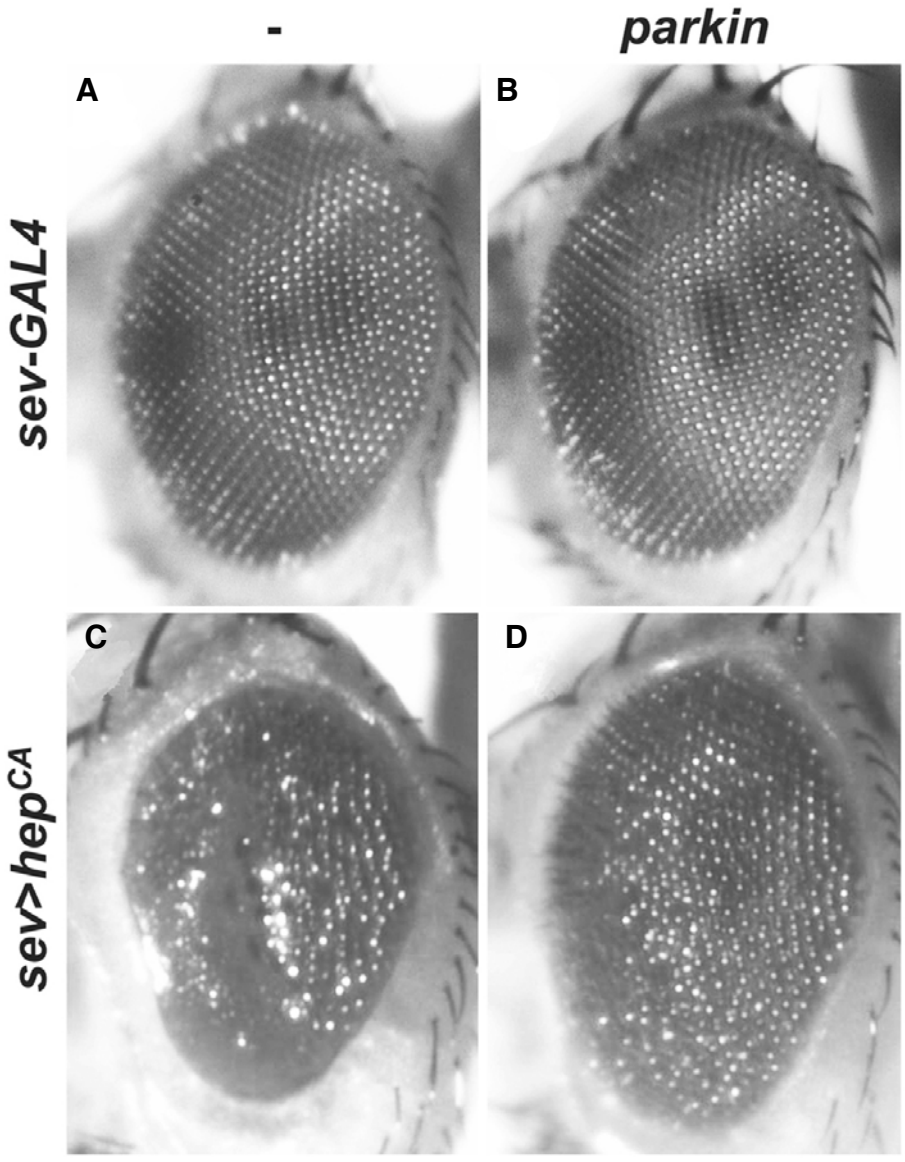

Fig. 1. Genetic interaction between *hep* and *parkin* in the developing eye. (A, B) Eye images of *sev-GAL4* flies without (A) and with (B) *UAS-parkin*. Normal morphology was seen in these flies. (C, D) Eye images of *sev* > hep^{CA} flies without (C) and with (D) *UAS-parkin*. The rough eye phenotype induced by hep^{CA} (C) was rescued by co-expression of *parkin* (D). The genotypes of the samples are *sev-GAL4*/+ (A), *UAS-parkin*^{WT}/+; *sev-GAL4*/+ (B), *sev-GAL4*, *UAS-*hep^{CA}/+ (C), and *UAS-parkin*^{WT}/+; *sev-GAL4*, *UAS-*hep^{CA}/+ (D). CA, constitutively active; *hep*, *hemipterous*; *sev*, *sevenless*.

gene. Our results show that Parkin inhibited JNK activity by down-regulating the expression of *bsk*.

MATERIALS AND METHODS

Drosophila strains

The *UAS-parkin*^{WT} fly line has been described previously (Cha et al., 2005). *Sevenless* (*sev*)-*GAL4* and *UAS-hemipterous*^{CA} (hep^{CA}, the constitutively active form of *Drosophila* JNKK) were obtained from the Bloomington Drosophila Stock Center and Dr. K. Matsumoto (Nagoya University, Japan), respectively. *UAS-basket* (*bsk*, *Drosophila* JNK) and *MS1096-GAL4* driver line were gifts from Dr. M. Mlodzik (EMBL-Heidelberg, Germany) and Dr. M. Freeman (MRC Laboratory of Molecular Biology, UK), respectively. All *Drosophila* were maintained with standard cornmeal-bean flour-yeast-agar medium at 25°C.

Ectopic gene expression with the UAS-GAL4 system

To ectopically over-express specific genes, including *hep*, *bsk*, and *parkin*, we used the modular mis-expression system, based on a P-element vector carrying a GAL4-regulated promoter (Brand and Perrimon, 1993; Nicolai et al., 2003; Rorth, 1996). *sev-GAL4* and *MS1096-GAL4* direct gene expression to the 7th photoreceptor of the eye and the whole wing, respectively.

Observation of *Drosophila* eyes and wings

External eye morphologies were observed under a dissecting microscopy (Carl Zeiss, Germany). To observe the wing vein, we isolated wings from fly's body by cutting the proximal portion. These wings were mounted in Gary's Magic Mountant solution (1.5 g of Canada balsam in 1 ml of methylsalicylate) on a slide glass. Then, it was coverslipped and examined under a light microscope (100× magnification).

Acridine orange staining

The eye imaginal discs of stage L3 larvae were dissected in phosphate-buffered saline (PBS). The discs were then incubated for 5 min in 1.6×10^{-6} M acridine orange (Sigma-Aldrich, USA), and rinsed briefly in PBS. The samples were examined

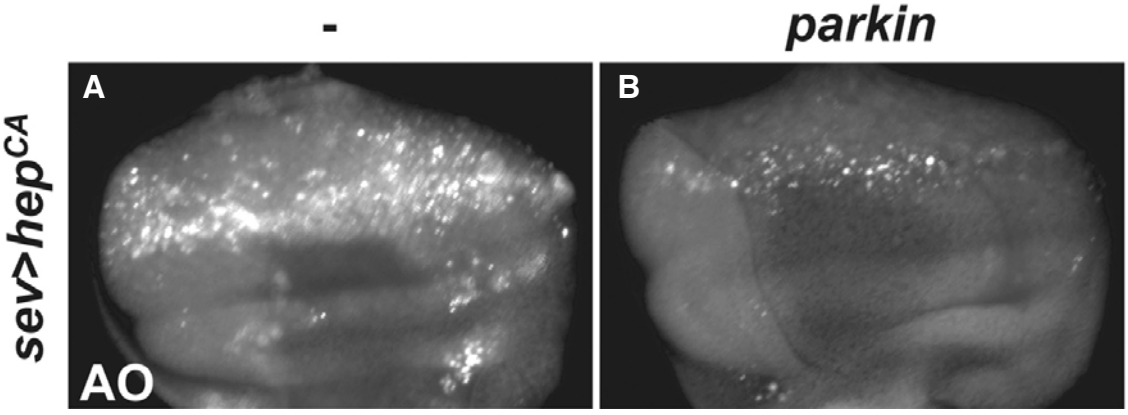

Fig. 2. Inhibition of *hep*-induced apoptosis by *parkin*. AO-stained images of the eye imaginal discs of *sev* > hep^{CA} (A) or *sev* > hep^{CA}+*parkin* (B) larva. The genotypes of the samples are *sev-GAL4*, *UAS-*hep^{CA}/+ (A) and *UAS-*$parkin^{WT}$/+; *sev-GAL4*, *UAS-*hep^{CA}/+ (B). AO, acridine orange.

under an Axiophot2 fluorescence microscope (Carl Zeiss, Germany).

Preparation of RNA and RT-PCR

Total RNAs from the eye discs of larva were isolated using the TRIzol reagent (Invitrogen, USA). Then, for RT-PCR, cDNA was synthesized using the Maxime kit (iNtRON Biotechnology, Korea) according to the manufacturer's protocol. RT-PCR was performed using the following primer pairs: *actin* (5′-TTAGCTCAGCCTCGCCACTTGCG-3′ and 5′-GAGCACAGCCTGGATGGCC-3′), *bsk* (5′-AAATGCTCGCCACTTTGAGT-3′ and 5′-TGGCTGTAACCGTTGCATAA-3′), *dFOXO* (5′-GCCTGGAGGTGCTCAATAAC-3′ and 5′-GTGGCCAGCGGTATATTGAT-3′), *Chic* (5′-TGCTTTGCGTTGTTGTTCTC-3′ and 5′-TTTGGTTGCATTTGCAGTTC-3′), and *hid* (5′-GCGAGTACCAGAGCGATCAT-3′ and 5′-TTATTGCTGCTGCTCGAGTG-3′).

RESULTS

***Drosophila* Parkin attenuates malformation of the eye, induced by the constitutively active form of hemipterous**

A previous study showed that Parkin was involved in the regulation of the JNK pathway and apoptosis (Cha et al., 2005). Based on this, we assessed whether Parkin could suppress JNK signaling in the developing eye. To do this, the effect of *parkin* over-expression on the phenotype induced by *hemipterous* (*hep*), a gene for *Drosophila* JNKK, was examined. When the constitutively active form of *hep* was over-expressed in the developing eye, using a *sevenless* (*sev*)-*GAL4* driver, severe eye degeneration was induced (Fig. 1C), while *sev-GAL4* itself did not affect eye development (Fig. 1A). This phenotype of hep^{CA} ectopic expression was probably the result of apoptosis, as strong activation of JNK signaling often causes cell death (Igaki, 2009). Moreover, co-expression of *Drosophila parkin* strongly suppressed the eye degenerative phenotype induced by hep^{CA} (Fig. 1D), although expression of *parkin* alone under a *sev-GAL4* driver resulted in no apparent change (Fig. 1B). This suggests that Parkin functions as a suppressor of the JNK signaling pathway in the developing eye.

***Drosophila* Parkin suppresses apoptosis induced by the constitutively active form of hemipterous**

Next, we checked whether the suppression of the hep^{CA}-induced eye degeneration phenotype by *parkin* was the result of decreased apoptosis. Previously, activation of JNK signaling by the over-expression of hep^{CA} was shown to induce strong apoptosis in the developing wing imaginal disc (Adachi-Yamada et al., 1999). Consistently, large numbers of dead cells were detected in the eye imaginal disc of hep^{CA}-expressing animals (Fig. 2A). As expected, co-expression of *parkin* and hep^{CA} strongly suppressed hep^{CA}-induced apoptosis (Fig. 2B), suggesting that *parkin* prevented tissue degeneration by suppressing the pro-apoptotic activity of the JNK pathway.

Parkin acts as a suppressor of *bsk*-induced wing vein formation

As *parkin* suppresses the hep^{CA}-induced phenotype, Parkin presumably inactivates Hep activity or suppresses downstream of *hep*. To investigate whether Parkin acted downstream of Hep, we examined the genetic interaction between *parkin* and *basket* (*bsk*), a gene for a *Drosophila* JNK. When *bsk* was over-expressed in the developing wing, using the *MS1096-GAL4* driver, the wings of adults carrying one copy each of *MS1096-GAL4* and *UAS-bsk* showed ectopic vein formation in the distal portion of the fourth and fifth longitudinal veins, as previously reported (Hwang and Cho, 2006; Figs. 3A, 3C, and 3E). Co-expression of *parkin* with *bsk* abolished the extra veins on the wing induced by *bsk* almost completely (Figs. 3B, 3D, and 3F). This result indicates that Parkin acted as a suppressor of JNK.

The level of the *bsk* transcript is reduced by *Drosophila* Parkin

Next, to clarify how Parkin suppressed JNK activity, we measured the transcript level of *bsk* in *parkin*-over-expressing eye discs by quantitative RT-PCR. As Parkin is an E3 ubiquitin ligase that functions in protein degradation (Tanaka et al., 1998; Takahashi et al., 2003), Parkin is supposed to regulate one of the JNK pathway components by posttranslational modification. However, unexpectedly, we found that the level of *bsk* transcript was reduced by more than 50% by *parkin* over-expression, versus the control (Fig. 4A), suggesting that Parkin regulates JNK pathway by suppressing *bsk* transcription.

Expression levels of JNK target genes was reduced by *Drosophila* Parkin

To confirm that the decrease in *bsk* transcription resulted in a reduction of transcriptional activity of JNK, we checked the transcriptional level of JNK targets. As expected, over-expression of *parkin* suppressed the transcription of representative

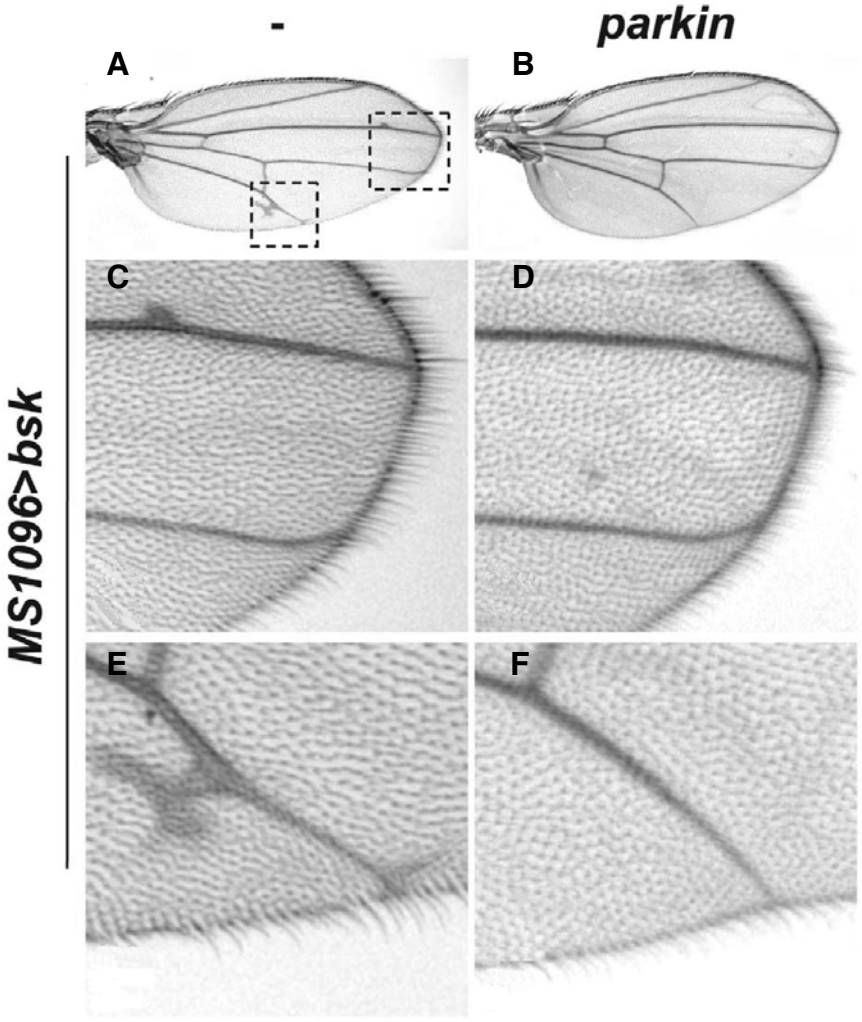

Fig. 3. Genetic interaction between *bsk* and *parkin* in developing wing. Wing images of *MS1096-GAL4* flies without (A, C, and E) and with (B, D, and F) *UAS-parkin*. Boxes drawn with dotted lines indicate extra wing veins. The middle (C, D) and lower (E, F) pictures are the magnified extra wing veins from the upper pictures (A, B). Extra wing veins induced by *bsk* (A, C, and E) were diminished by co-expression of *parkin* (B, D, and F). The genotypes of the samples are *MS1096-GAL4*/+; *UAS-bsk*/+ (A, C, and E) and *MS1096-GAL4*/*UAS-parkin*^{WT}; *UAS-bsk*/+ (B, D, and F). *bsk*, *basket*.

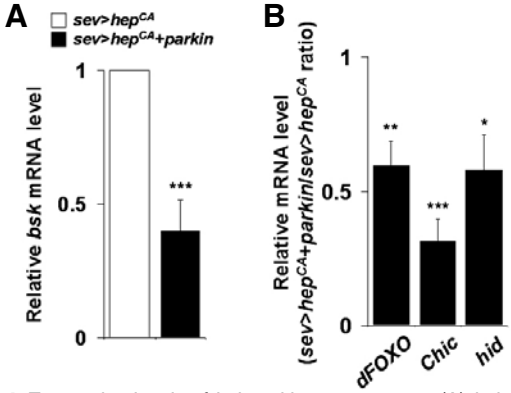

Fig. 4. Expression levels of *bsk* and its target genes. (A) *bsk* mRNA levels in the eye imaginal discs of *hep*^{CA}-over-expressing larva without (*sev* > *hep*^{CA}) and with (*sev* > *hep*^{CA}+*parkin*) *UAS-parkin*. (B) The relative level of JNK target gene transcripts in *sev* > *hep*^{CA} with *parkin* over-expression (*sev* > *hep*^{CA}+*parkin*) compared to the control (*sev* > *hep*^{CA}). All data are expressed as means ± SE of at least three independent experiments (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; Student's *t*-test). The genotypes of the samples are *sev-GAL4*, *UAS-hep*^{CA}/+ (*sev* > *hep*^{CA}) and *UAS-parkin*^{WT}/+; *sev-GAL4*, *UAS-hep*^{CA}/+ (*sev* > *hep*^{CA}+*parkin*). *Chic*, *chickadee*; *dFOXO*, *Drosophila forkhead box*, *subgroup O*; *hid*, *head involution defective*.

JNK targets (Fig. 4B). Furthermore, the reduction in the levels of their transcripts was similar to that of *bsk* (compare Figs. 4A and 4B), indicating that the decreased *bsk* transcript level, as a result of *parkin* over-expression, was responsible for the reduction in the level of transcripts of these target genes.

DISCUSSION

Although *parkin* is a major causative gene of familial PD, the molecular mechanism by which *parkin* dysfunction causes PD is unknown. In particular, understanding the role of Parkin in protecting dopaminergic neurons from degeneration is essential in understanding the pathology of PD. As the JNK signaling pathway has been implicated in the dopaminergic neuronal degeneration in PD, studies about the role of Parkin in the controlling this pathway may provide important clues to the pathogenesis of *parkin*-associated PD.

In the present study, we showed that Parkin suppressed JNK activity in developing tissues. Over-expression of *Drosophila parkin* suppressed the constitutively active form of *hep* (*Drosophila JNKK*, *hep*^{CA})-induced eye degeneration and *bsk* (*Drosophila JNK*)-induced extra vein formation. Because activation of JNK is often associated with cell death, Parkin is assumed to function in protecting cells from JNK-dependent apoptosis.

Indeed, we found that Parkin reduced *hep*^{CA}-induced apoptosis and pro-apoptotic gene expression. Furthermore, our findings support the suppressive role of Parkin in JNK signaling not being restricted to dopaminergic neurons, but also in developing tissues, such as the eyes and wings. Consistent with this, our previous study showed that Parkin inhibited the JNK pathway during *Drosophila* thorax closure, and that JNK was hyperactivated in the dopaminergic neurons of a *Drosophila parkin* mutant (Cha et al., 2005). Collectively, our data suggest that Parkin suppresses the JNK signaling pathway, protecting cells from apoptosis in various tissues.

So, how might Parkin suppress JNK signaling? The JNK signaling pathway includes several evolutionarily well conserved proteins, the activity of which is regulated by phosphorylation (Igaki, 2009; Jeong et al., 2009). For example, Bsk is a substrate of its upstream Hep, and the Hep-Bsk cascade can be activated by several upstream JNKKKs, including dTAK1, DASK1, Slipper, and dMekk1, in *Drosophila* (Igaki, 2009). Among them, our data show that *parkin* could suppress a *bsk*-induced phenotype, suggesting that Parkin suppresses the function of Bsk or its downstream targets. From the biochemical function of Parkin, we initially expected that Parkin would regulate the Bsk protein level by controlling the polyubiquitination and degradation of Bsk. However, unexpectedly, we found that Parkin suppresses *bsk* transcription. Whether Parkin functions as a transcription factor and thereby directly regulates transcription of *bsk* is unclear. However, a recent study showed that Parkin can act as a transcription factor: over-expressed and endogenous Parkin interacted physically with the *p53* promoter and lowered *p53* mRNA levels and repressed *p53* promoter transactivation through its Ring1 domain (da Costa et al., 2009). Accordingly, the possibility exists that Parkin binds the *bsk* promoter and represses transactivation of the promoter. Alternatively, Parkin could control *bsk* transcription indirectly, by regulating transcription factor(s) involved in *bsk* gene expression. Supporting this idea, Parkin has been reported to repress Eg5 gene transcription by Hsp70 ubiquitination-dependent inactivation of JNK (Liu et al., 2008). To clarify the role of Parkin in the suppression of JNK signaling, further experiments on the molecular mechanism of the repression of *bsk* gene expression are needed.

In summary, we found that Parkin suppressed JNK signaling in developing tissues, thereby inhibiting JNK signaling-induced phenotypes, apoptosis, and JNK target gene expression. Furthermore, the regulation of JNK signaling by Parkin was achieved by repression of *bsk* transcription. These results suggest a novel role for Parkin as a transcriptional regulator of the JNK pathway, and provide insight into the pathogenesis of PD.

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