

Parkin Regulates Eg5 Expression by Hsp70 Ubiquitination-dependent Inactivation of c-Jun NH₂-terminal Kinase*

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Eg5 is a motor protein of the kinesin family that is critical for spindle assembly during mitosis and has recently been implicated in tumorigenesis. It is largely unknown how Eg5 expression is regulated in cells. In this study, we present the first evidence that the cellular Eg5 level is down-regulated by Parkin, an E3 ubiquitin ligase well known for its role in the development of Parkinson disease. Our data show that Parkin does not trigger Eg5 protein degradation through the ubiquitin-proteasome pathway. Instead, Parkin represses Eg5 gene transcription by blocking c-Jun binding to the activator protein 1 site present in the Eg5 promoter. Our data further show that Parkin inactivates c-Jun NH₂-terminal kinase (JNK), resulting in decreased phosphorylation of c-Jun. The inactivation of JNK is further mediated by multiple monoubiquitination of Hsp70. Importantly, both the ubiquitination of Hsp70 and the subsequent inactivation of the JNK-c-Jun pathway are crucial for Parkin to down-regulate Eg5 expression. These results thus uncover a novel function for Parkin in modulating the expression of Eg5 through the Hsp70-JNK-c-Jun signaling pathway.

Chromosome segregation during cell division is orchestrated by the mitotic spindle, a bipolar apparatus composed of microtubules and their associated proteins. The bipolar organization of the spindle is crucial for accurate segregation of chromosomes and relies on microtubule-dependent motor proteins (1, 2). Eg5, a member of the kinesin family, is one of the best characterized motor proteins that function in bipolar spindle assembly (3, 4). Eg5 has a highly conserved NH₂-terminal catalytic motor domain and can hydrolyze ATP to move processively toward the plus ends of microtubules (5). Compelling evidence demonstrates that Eg5 functions as a homotetramer, with two motor domains at each end of a central stalk, and

contributes to spindle assembly by cross-linking and pushing apart adjacent interpolar microtubules (4). In addition, Eg5 can tether microtubule plus ends and condense microtubules into bundles, which may also contribute to spindle morphogenesis (4).

Besides its role in spindle formation, Eg5 has recently been implicated in tumorigenesis (6–8). For example, activation of Eg5 and Hex by retroviral insertion has been shown to contribute to mouse B-cell leukemia (7). Microarray analysis has identified the up-regulation of Eg5 in blast crisis chronic myeloid leukemia (8). In addition, this motor protein has been shown to cause genomic instability in transgenic mice (6). It remains elusive how Eg5 expression is regulated in cells. In this study, we have identified Parkin, an E3 ubiquitin ligase that regulates the ubiquitination and proteasome-dependent degradation of a wide range of proteins (9–11), as a molecule capable of regulating Eg5 expression. Our data demonstrate that Parkin down-regulates Eg5 at the transcriptional level, through Hsp70 ubiquitination-dependent inactivation of c-Jun NH₂-terminal kinase (JNK)² and inhibition of c-Jun binding to the activator protein 1 (AP1) site located in the Eg5 promoter.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—The proteasome inhibitor MG132 and the protein synthesis inhibitor cycloheximide were purchased from Calbiochem. The following antibodies were used in this study: Parkin, HA, and Myc antibodies (Cell Signaling); Eg5 antibody (BD Biosciences); JNK, c-Jun, and phosphorylated c-Jun antibodies (Calbiochem); Hsp70 and CDCrel-1 antibodies (Stressgen); β -actin and FLAG antibodies (Sigma); antibodies against poly-ubiquitinated proteins (clone FK1) and mono-/poly-ubiquitinated proteins (clone FK2) (Affiniti Research); and horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (Pierce Biotechnology).

Cell Culture and Treatment—EPP85 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO₂. Plasmid transfections were performed using the polyethyleneimine reagent (Sigma). Small interfering RNAs (siRNAs)

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² The abbreviations used are: JNK, c-Jun NH₂-terminal kinase; AP1, activator protein 1; RT, reverse transcription; siRNA, small interfering RNA; HA, hemagglutinin.

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were synthesized by Ribobio (Guangzhou, China) and transfected to cells with the Lipofectamine 2000 reagent (Invitrogen). Adenoviruses were prepared and amplified in low-passage 293 cells as described previously (12, 13). The multiplicity of infection was defined as the ratio of infectious units divided by the number of cells.

Western Blot Analysis—Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% fat-free dry milk and incubated first with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent (Pierce Biotechnology). The intensity of protein bands was determined by the ImageJ software.

Immunoprecipitation—Cell lysate was incubated with specific antibodies at 4 °C for 2 h, and protein A/G-agarose beads (Pierce Biotechnology) were then added to incubate for another 3 h. The beads were washed extensively and boiled in SDS loading buffer, and the precipitated proteins were detected by SDS-PAGE and Western blotting.

Quantitative Real-time RT-PCR—Total cellular RNA was prepared using the TRIzol reagent (Invitrogen). Quantitative real-time RT-PCR was performed in triplicate as described (14). Glyceraldehyde-3-phosphate dehydrogenase was used as a control to normalize the reading for Eg5 in each sample.

Luciferase Reporter Assay—Eg5 promoter activity was measured by using the Eg5 promoter-driven luciferase reporter plasmid pEg5-Luc and the β -galactosidase-expressing plasmid pcDNA3-LacZ. In the pEg5-Luc plasmid, the Eg5 promoter (from -1264, -1223, -1186, or -1121 to the major site of transcriptional initiation) was used to drive the luciferase gene. The luciferase activity was measured using the FB12 luminometer (Berthold Detection Systems) and normalized to β -galactosidase activity.

Protein Turnover Assay—Cells were treated with cycloheximide (20 μ g/ml) for 0, 6, 12, 24, or 36 h. The level of Eg5 or CDCrel-1 at different time points was examined by Western blot analysis and quantified by densitometric analysis of the Western blot bands.

Protein-Oligonucleotide Binding Assay—Nuclear extracts were incubated in 96-well plates with immobilized oligonucleotide sequences that contain the AP1 site. The plates were incubated in succession with c-Jun antibody, horseradish peroxidase-conjugated secondary antibody, and SigmaFast peroxidase substrate (Sigma). The absorbance at a wavelength of 450 nm was then measured to quantify c-Jun/AP1 binding site activity.

Immunocomplex Kinase Assay—JNK activity was measured using the immunocomplex JNK kinase assay kit according to the manufacturer's instructions (Calbiochem).

RESULTS

Parkin Down-regulates Eg5 in a Ubiquitin Ligase-dependent Manner—To identify proteins regulated by the E3 ubiquitin ligase Parkin, we analyzed the protein expression profiles of cells treated with Parkin adenoviruses and compared with those treated with control adenoviruses. Such an analysis

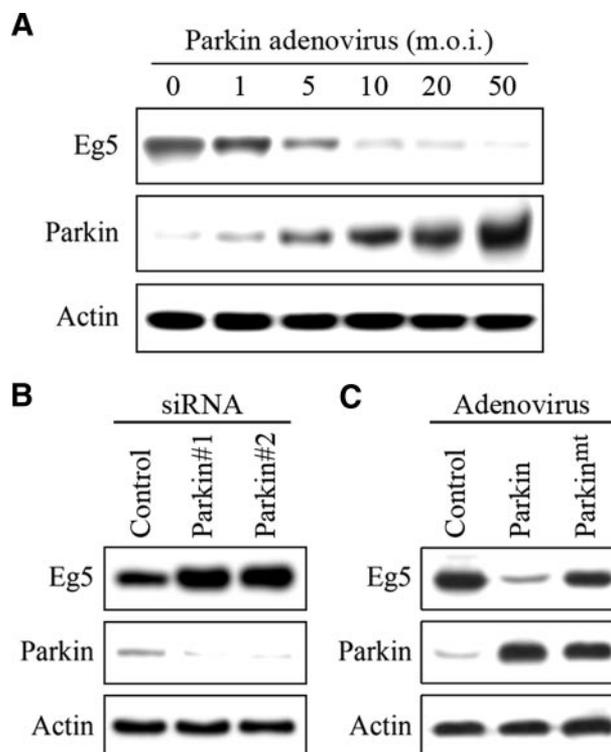


FIGURE 1. Parkin down-regulates Eg5 in a ubiquitin ligase-dependent manner. A, Western blot analysis of the expression of Eg5, Parkin, and actin in EPP85 cells treated for 24 h with different doses of Parkin adenoviruses. B, Western blot analysis of the expression of Eg5, Parkin, and actin in cells transfected with two different Parkin siRNAs or luciferase control siRNA. C, Western blot analysis of the expression of Eg5, Parkin, and actin in cells treated with 10 multiplicity of infection (*m.o.i.*) of Parkin, ligase-dead mutant Parkin (*Parkin^{mt}*), or β -galactosidase control adenoviruses.

revealed significant down-regulation of the mitotic kinesin Eg5, along with several known substrates of Parkin such as CDCrel-1 (10). The down-regulation of Eg5 by Parkin was confirmed by Western blot analysis; as shown in Fig. 1A, Parkin adenoviruses decreased cellular Eg5 level in a dose-dependent manner. In addition, siRNA-mediated knockdown of endogenous Parkin increased Eg5 expression (Fig. 1B). We also found that the down-regulatory effect of Parkin on Eg5 expression disappeared when ligase-dead mutant Parkin adenoviruses were used (Fig. 1C), suggesting that the ubiquitin ligase activity of Parkin is required for its inhibition of Eg5 level.

Parkin Does Not Trigger Eg5 Degradation through the Ubiquitin-Proteasome Pathway—We then examined whether Parkin down-regulates Eg5 by promoting Eg5 degradation through the ubiquitin-proteasome pathway. This possibility was tested by blocking proteasome function with a specific inhibitor, MG132. We found that MG132 prevented the activity of Parkin to down-regulate CDCrel-1, but not Eg5 (Fig. 2A), indicating that the decrease of Eg5 by Parkin might not be mediated by proteasome-dependent protein degradation. In addition, we found that Parkin adenoviruses did not trigger the ubiquitination of Eg5, although CDCrel-1 was clearly ubiquitinated (Fig. 2B). Immunoprecipitation experiments further revealed that Parkin was able to interact with CDCrel-1 but not with Eg5 (Fig. 2C). Thus, Eg5 is not a direct substrate for the ubiquitin ligase activity of Parkin.

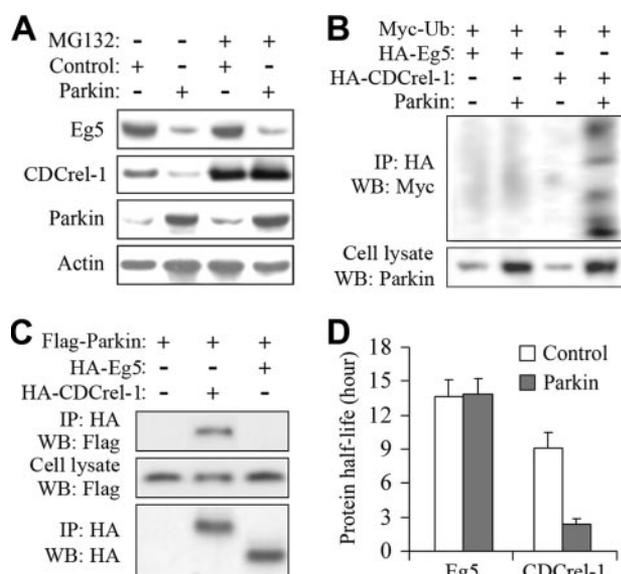


FIGURE 2. Parkin does not trigger Eg5 degradation through the ubiquitin-proteasome pathway. *A*, Western blot analysis of the expression of Eg5, CDCrel-1, Parkin, and actin in cells treated with control or Parkin adenoviruses in the presence or absence of MG132. *B*, Parkin triggers the ubiquitination of CDCrel-1 but not Eg5. Cells were transfected with pcDNA3-Myc-ubiquitin together with pRK5-HA-Eg5, pRK5-HA-CDCrel-1, or empty vector, and then treated with control or Parkin adenoviruses in the presence of MG132. Cell lysate was subjected to immunoprecipitation (IP) with HA antibody and Western blotting (WB) with Myc antibody. The expression of Parkin in cells was revealed by Western blot analysis of the cell lysate with Parkin antibody. *C*, Parkin interacts with CDCrel-1 but not with Eg5. Cells were transfected with pcDNA3-Flag-Parkin together with pRK5-HA-Eg5, pRK5-HA-CDCrel-1, or empty vector, and then treated with MG132. Cell lysate was subjected to immunoprecipitation with HA antibody and Western blotting with FLAG or HA antibody. The expression of FLAG-Parkin in cells was revealed by Western blot analysis of the cell lysate with FLAG antibody. *D*, determination of the half-life of Eg5 and CDCrel-1 by protein turnover assay in cells treated with control or Parkin adenoviruses. The values and error bars shown in the graph represent mean \pm S.D. of three independent experiments.

We also examined the effect of Parkin on Eg5 stability by measuring the half-life of Eg5 via protein turnover assay. Such an experiment revealed an Eg5 half-life of 13.6 h, which was not significantly changed by Parkin adenoviruses (Fig. 2*D*). In contrast, the half-life of CDCrel-1 was remarkably decreased by treatment with Parkin adenoviruses (Fig. 2*D*). Together, these data demonstrate that Parkin does not cause Eg5 degradation through the ubiquitin-proteasome pathway.

Eg5 Is Down-regulated by Parkin at the Transcriptional Level—We then investigated whether the down-regulation of Eg5 by Parkin occurs at the messenger level by measuring Eg5 mRNA expression. Quantitative real-time RT-PCR analysis revealed that Parkin, but not its ligase-dead mutant, reduced Eg5 mRNA expression in cells (Fig. 3*A*). In addition, siRNA-mediated knockdown of endogenous Parkin could increase Eg5 mRNA expression (Fig. 3*B*). Luciferase reporter assay using the Eg5 promoter further showed that Parkin, but not its ligase-dead mutant, significantly decreased Eg5 promoter activity (Fig. 3*C*), indicating that Parkin might down-regulate Eg5 at the transcriptional level.

To further analyze the repression of Eg5 gene transcription by Parkin, we transfected cells with a series of luciferase reporter plasmids, including pEg5(-1264)-Luc, pEg5(-1223)-Luc, pEg5(-1186)-Luc, and pEg5(-1121)-Luc, which are

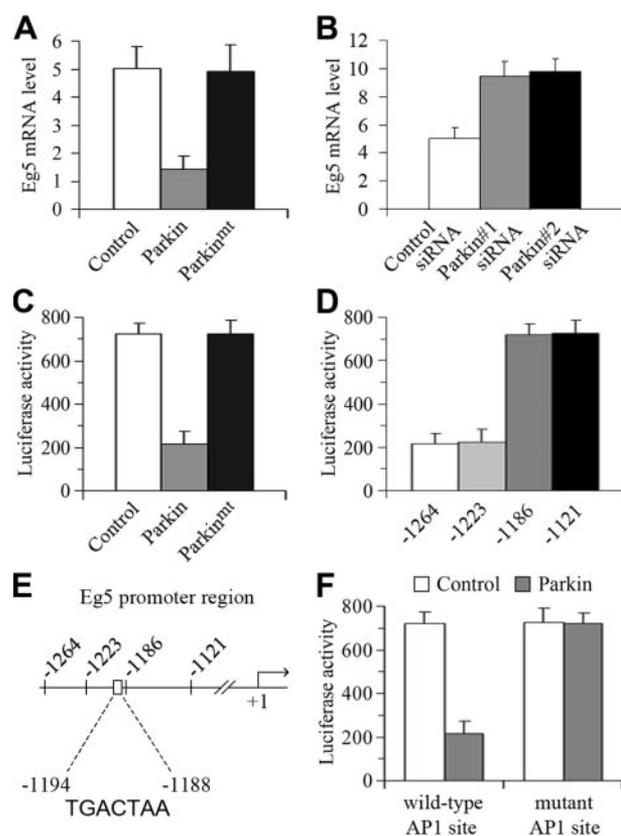


FIGURE 3. Eg5 is down-regulated by Parkin at the transcriptional level.

A, quantitative real-time RT-PCR analysis of Eg5 mRNA expression in cells treated for 24 h with 10 multiplicity of infection of control, Parkin, or Parkin^{mt} adenoviruses. *B*, quantitative real-time RT-PCR analysis of Eg5 mRNA expression in cells transfected with control or Parkin siRNAs. *C*, cells were transfected with pcDNA3-LacZ and pEg5(-1264)-Luc, a luciferase reporter plasmid driven by the Eg5 promoter (from -1264 to the major site of transcriptional initiation), together with pcDNA3-Flag-Parkin, pcDNA3-Flag-Parkin^{mt}, or empty vector (control). Eg5 promoter-driven luciferase activity was then determined and normalized to β -galactosidase activity. *D*, luciferase activities in cells transfected with pcDNA3-Flag-Parkin, pcDNA3-LacZ, and a series of luciferase reporter plasmids, including pEg5(-1264)-Luc, pEg5(-1223)-Luc, pEg5(-1186)-Luc, and pEg5(-1121)-Luc, which are driven by different sizes of Eg5 promoter (from -1264, -1223, -1186, or -1121 to the major site of transcriptional initiation). *E*, a scheme showing the Eg5 promoter region. The open box indicates the AP1 site (from -1194 to -1188 relative to the major site of transcriptional initiation). *F*, luciferase activities in cells transfected with pcDNA3-Flag-Parkin (or empty vector), pcDNA3-LacZ, and luciferase reporter plasmids driven by the Eg5 promoter harboring a wild-type or mutant AP1 site. The values and error bars shown in the graphs represent mean \pm S.D. of three independent experiments.

driven by different sizes of Eg5 promoter (from -1264, -1223, -1186, or -1121 to the major site of transcriptional initiation) (Fig. 3, *D* and *E*). Cells transfected with pEg5(-1264)-Luc and pEg5(-1223)-Luc together with Parkin had comparable, low luciferase activities (Fig. 3*D*). Strikingly, in cells transfected with pEg5(-1186)-Luc or pEg5(-1121)-Luc, the Parkin-induced decrease in Eg5 luciferase activity was largely blocked (Fig. 3*D*). These results indicate that the nucleotide sequence between -1223 and -1186 in the Eg5 promoter might contain a cis-regulatory DNA element that mediates the transcriptional down-regulation of Eg5 by Parkin.

A web-based navigation through transcription factor binding databases, using the Parkin response sequence -1223 to -1186 as bait, identified a putative binding site for the heterodimeric transcription factor AP1 (notably *c-Jun/c-Fos*,

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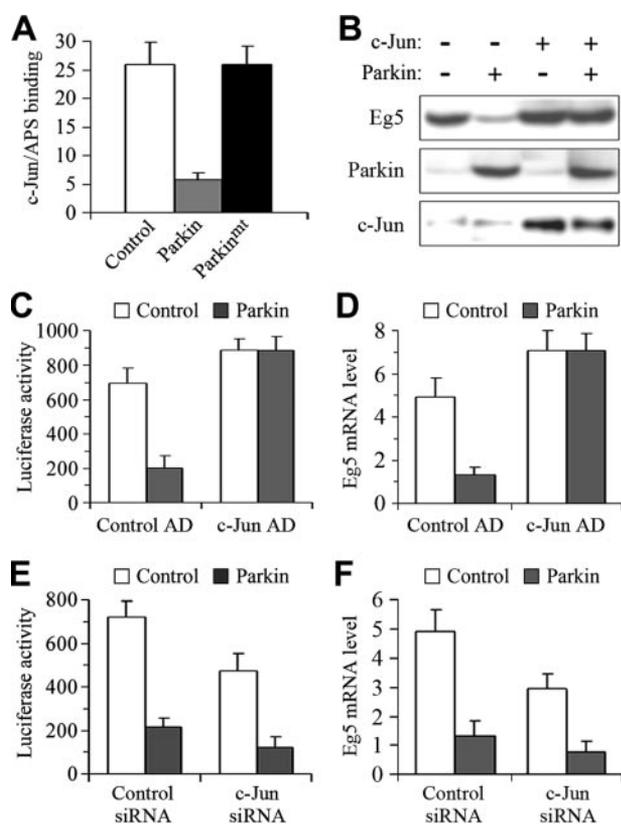


FIGURE 4. Parkin decreases Eg5 expression by blocking c-Jun binding to the AP1 site in the Eg5 promoter. *A*, cells were treated for 24 h with 10 multiplicity of infection of control, Parkin, or Parkin^{mt} adenoviruses, and the c-Jun/AP1 site (AP5) binding activity was then determined. *B*, Western blot analysis of the expression of Eg5, Parkin, and c-Jun in cells treated with control or Parkin adenoviruses, together with control or c-Jun adenoviruses. *C*, Eg5 promoter-driven luciferase activities in cells treated with control or Parkin adenoviruses, together with control or c-Jun adenoviruses. *D*, quantitative real-time RT-PCR analysis of Eg5 mRNA expression in cells treated with control or Parkin adenoviruses, together with control or c-Jun adenoviruses. *E*, Eg5 promoter-driven luciferase activities in cells transfected with control or c-Jun siRNAs and then treated with control or Parkin adenoviruses. *F*, quantitative real-time RT-PCR analysis of Eg5 mRNA expression in cells transfected with control or c-Jun siRNAs and then treated with control or Parkin adenoviruses. The values and error bars shown in the graphs represent mean \pm S.D. of three independent experiments.

(15)), located from -1194 to -1188 in the Eg5 promoter (Fig. 3E). To examine whether the repression of Eg5 gene transcription by Parkin is mediated by the AP1 binding site, luciferase reporter assay was performed using an Eg5 promoter harboring mutations in the AP1 site. We found that Parkin-induced reduction of Eg5 promoter activity was completely blocked by AP1 site mutations (Fig. 3F), indicating a critical role for the AP1 site in mediating Eg5 down-regulation by Parkin.

Parkin Decreases Eg5 Expression by Inactivation of the JNK-c-Jun Pathway and Inhibition of c-Jun Binding to the AP1 Site in the Eg5 Promoter—Using a protein-oligonucleotide binding assay, we further found that Parkin, but not its ligase-dead mutant, significantly inhibited c-Jun binding to the AP1 site (Fig. 4A). To further investigate the role of c-Jun inhibition in mediating Eg5 down-regulation by Parkin, we overexpressed c-Jun and then examined the effect of Parkin on Eg5 expression. We found that adenovirus-mediated c-Jun overexpression completely blocked the ability of Parkin to decrease the Eg5 level (Fig. 4B). In addition, c-Jun overexpression could inhibit the ability

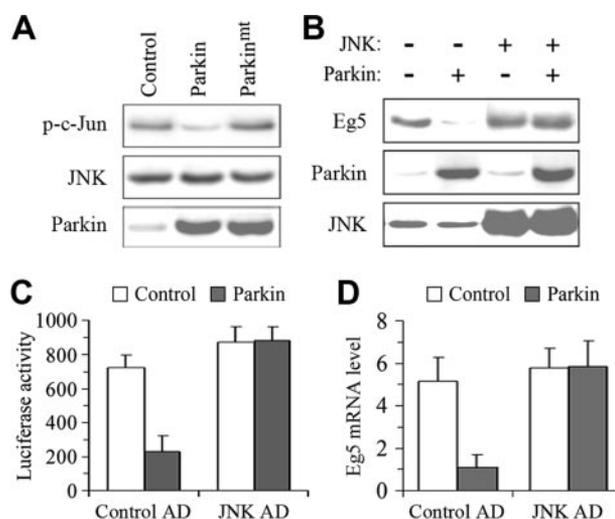


FIGURE 5. Parkin decreases Eg5 expression by inactivation of JNK. *A*, cells were treated with control, Parkin, or Parkin^{mt} adenoviruses, and c-Jun phosphorylation (p-c-Jun) was determined by immunocomplex kinase assay as a measure of JNK activity. The expression of JNK and Parkin was determined by Western blot analysis. *B*, Western blot analysis of the expression of Eg5, Parkin, and JNK in cells treated with control or Parkin adenoviruses, together with control or JNK adenoviruses. *C*, Eg5 promoter-driven luciferase activities in cells treated with control or Parkin adenoviruses, together with control or JNK adenoviruses. *D*, quantitative real-time RT-PCR analysis of Eg5 mRNA expression in cells treated with control or Parkin adenoviruses, together with control or JNK adenoviruses. The values and error bars shown in the graphs represent mean \pm S.D. of three independent experiments.

of Parkin to decrease the Eg5 transcription and mRNA level (Fig. 4, C and D). Interestingly, overexpression of c-Jun *per se* could increase Eg5 expression (Fig. 4, B–D), supporting a role for this transcription factor in promoting Eg5 gene expression. We also found that siRNA-mediated knockdown of c-Jun could facilitate the down-regulatory effect of Parkin on the Eg5 transcription and mRNA levels (Fig. 4, E and F). Collectively, these results reveal that Parkin decreases Eg5 expression by inhibiting c-Jun binding to the AP1 site in the Eg5 promoter.

We then wanted to identify the link between Parkin and c-Jun in the context of Eg5 down-regulation. A natural hypothesis was that Parkin might inactivate JNK, which in turn decreases c-Jun phosphorylation and inhibits c-Jun binding to the AP1 site in the Eg5 promoter. To test this hypothesis, JNK activity upon Parkin overexpression was examined by immunocomplex kinase assay. We observed a significant decrease of JNK activity by Parkin, but not by the ligase-dead mutant (Fig. 5A). In addition, we found that blocking proteasome function with MG132 did not affect the ability of Parkin to inactivate JNK (data not shown).

To study the role for JNK inactivation in the down-regulation of Eg5 by Parkin, we overexpressed JNK and then examined the effect of Parkin on Eg5 expression. We found that adenovirus-mediated overexpression of JNK, like the overexpression of c-Jun, could entirely inhibit the activity of Parkin to decrease Eg5 transcription and its protein and mRNA levels (Fig. 5, B–D). In addition, overexpression of JNK *per se* was also able to increase Eg5 expression (Fig. 5, B–D). Together, these data demonstrate a critical role for JNK inactivation in mediating Eg5 down-regulation by Parkin.

Hsp70 Ubiquitination Is Critical for Parkin to Inactivate JNK and to Decrease Eg5 Expression—We next investigated the molecular mechanism by which Parkin leads to JNK inactivation. It is clearly linked to the ubiquitin ligase-associated function of Parkin because the ligase-dead mutant could not inactivate JNK. Given that the JNK signaling is known to be regulated by ubiquitination events (16), it was possible that Parkin might inactivate JNK by triggering the ubiquitination of cellular proteins involved in the regulation of JNK activity. The failure of MG132 to prevent Parkin-induced JNK inactivation and Eg5 down-regulation further suggested that Parkin-mediated ubiquitination of such proteins does not result in their degradation by the proteasome. Potential targets include Hsp70, NEMO, and Traf2, which undergo degradation-independent ubiquitination by Parkin and are also implicated in the regulation of JNK activity (17–20).

To examine the involvement of Hsp70, NEMO, and Traf2 in Parkin-induced JNK inactivation and Eg5 down-regulation, cells were treated with control or Parkin adenoviruses together with adenoviruses encoding Hsp70, Hsp70 antisense RNA, NEMO, NEMO antisense RNA, Traf2, or Traf2 antisense RNA. We found that Hsp70 adenoviruses could facilitate the inhibitory effects of Parkin on JNK activity and the Eg5 level, whereas adenoviruses encoding Hsp70 antisense RNA largely blocked the above effects (Fig. 6A). In contrast, adenoviruses encoding NEMO, NEMO antisense RNA, Traf2, or Traf2 antisense RNA did not alter the effects of Parkin on JNK activity and Eg5 level (Fig. 6A). These results indicate the importance of Hsp70 in Parkin-induced inactivation of JNK and down-regulation of Eg5.

Using antibodies that specifically recognize poly-ubiquitinated proteins (clone FK1) or both mono- and poly-ubiquitinated proteins (clone FK2), we further found that Parkin, but not the ligase-dead mutant, induced multiple mono-ubiquitination of Hsp70 (Fig. 6B). This result was in agreement with the previous finding achieved with SH-SY5Y neuroblastoma cells (19). We next studied whether Hsp70 mono-ubiquitination is linked to the effects of Parkin on JNK activity and the Eg5 level. Cells were treated with control or Parkin adenoviruses, together with adenoviruses encoding wild-type or the lysine-less mutant Hsp70. As shown in Fig. 6C, Parkin-mediated Hsp70 mono-ubiquitination was significantly enhanced upon treatment with wild-type Hsp70 adenoviruses. In contrast, the mono-ubiquitination of Hsp70 was entirely abolished upon treatment with lysine-less mutant Hsp70 adenoviruses. This result suggests that the lysine-less mutant functions in a dominant-negative manner in cells. On the other hand, the result supports an essential role for lysine residues in mediating the ubiquitination of Hsp70 similar to the ubiquitination of many other proteins (21). Most importantly, we found that whereas wild-type Hsp70 facilitated the inhibitory effects of Parkin on JNK activity and Eg5 level, lysine-less mutant Hsp70 abolished the above effects. These data thus reveal that Hsp70 ubiquitination is critical for Parkin to inactivate JNK and to decrease the Eg5 level.

DISCUSSION

Eg5 is a microtubule-dependent motor protein of the kinesin family and plays an important role in mitotic spindle assembly (3, 4). Chemical compounds that inhibit Eg5 activity have

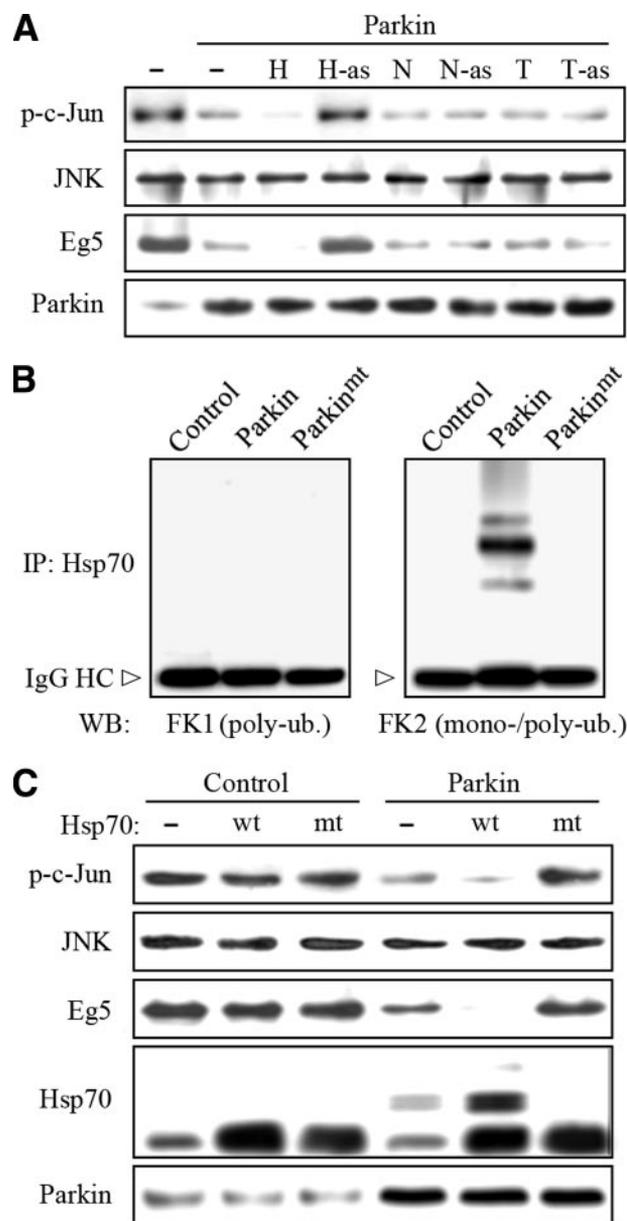


FIGURE 6. Mono-ubiquitination of Hsp70 is critical for Parkin to inactivate JNK and to decrease Eg5 expression. A, Hsp70 is important for Parkin-induced inactivation of JNK and down-regulation of Eg5. Cells were treated with control or Parkin adenoviruses together with adenoviruses encoding Hsp70 (H), Hsp70 antisense RNA (H-as), NEMO (N), NEMO antisense RNA (N-as), Traf2 (T), or Traf2 antisense RNA (T-as). Phosphorylation of c-Jun was determined by immunocomplex kinase assay as a measure of JNK activity. The expression of JNK, Eg5, and Parkin was determined by Western blot analysis. B, Parkin induces Hsp70 mono-ubiquitination. Cells were transfected with pRK5-Myc-ubiquitin and then treated with control, Parkin, or Parkin^{mt} adenoviruses in the presence of MG132. Cell lysate was subjected to immunoprecipitation (IP) with Hsp70 antibody and Western blotting (WB) with antibodies against poly-ubiquitinated proteins (clone FK1) or mono-/poly-ubiquitinated proteins (clone FK2). HC, heavy chain. C, cells were treated with control or Parkin adenoviruses, together with adenoviruses encoding wild-type (wt) or lysine-less mutant (mt) Hsp70. Phosphorylation of c-Jun was determined by immunocomplex kinase assay as a measure of JNK activity. The expression of JNK, Eg5, Hsp70, and Parkin was determined by Western blot analysis.

emerged as a new generation of anticancer agents that are currently under clinical investigation (22). Because Eg5 is hardly detectable in post-mitotic neurons and mainly acts in dividing cells (23), its inhibitors are expected to offer better specificity

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for cancer treatment than microtubule-targeted drugs, which often result in neurotoxicity (24). Recently, Eg5 has been implicated in tumorigenesis due to its activation in mouse B-cell leukemia, overexpression in blast crisis chronic myeloid leukemia, and triggering of genomic instability in transgenic mice (6–8).

Although the molecular mechanism underlying the regulation of Eg5 expression is largely unknown, our data presented in this study demonstrate that the cellular Eg5 level is down-regulated by the E3 ubiquitin ligase Parkin. In addition, we find that the ability of Parkin to decrease Eg5 is dependent on its ubiquitin ligase activity. These findings demonstrate a previously unrecognized role for Parkin in regulating Eg5 expression. Given the critical function of Eg5 in mitotic spindle formation, it would not be surprising if a mitotic role for Parkin is identified in the future, although the majority of research in the past decade has been focused on its involvement in Parkinson disease (25).

The present study also provides mechanistic insights into how Parkin down-regulates Eg5 expression in cells. Our data show that although this action of Parkin requires its ubiquitin ligase activity, it does not trigger proteasome-dependent Eg5 degradation. This feature is clearly different from the action of Parkin toward many of its substrate proteins such as CDCrel-1 (10). We find that Parkin down-regulates Eg5 by inhibiting Eg5 gene transcription, which is dependent on the AP1 binding site located in the Eg5 promoter. In addition, our data show that Parkin inactivates JNK and inhibits c-Jun binding to the AP1 site, and that inactivation of the JNK-c-Jun pathway is important for Parkin to down-regulate Eg5. These findings thus establish a critical role for the JNK-c-Jun signaling cascade in mediating the effect of Parkin on Eg5 gene expression.

It should be noted, however, that the connection between the JNK pathway and the kinesin family proteins is not unprecedented. For example, JNK-interacting proteins bind directly to kinesins to allow the loading of different cargoes as well as to control cargo release (26, 27). Recently, the JNK pathway has also been proposed to link Parkin mutations to oxidative stress-induced apoptosis in the development of Parkinson disease (28). Nevertheless, the present study provides the first evidence demonstrating a role for the JNK pathway in mediating the effect of Parkin on Eg5 expression.

Regarding the mechanisms by which Parkin inactivates JNK, our data demonstrate a critical requirement for Hsp70 mono-ubiquitination. This is supported by the finding that Hsp70 facilitates the inhibitory effects of Parkin on JNK activity and Eg5 level, whereas Hsp70 antisense RNA diminishes the above effects. Moreover, the lysine-less mutant Hsp70, which abrogates the ubiquitination of Hsp70 in a dominant-negative manner, also blocks the inhibitory effects of Parkin on JNK activity and Eg5 level. These findings reveal that Hsp70 mono-ubiquitination is crucial for Parkin to inactivate JNK and to decrease the

Eg5 level. Further studies are warranted to elucidate the molecular mechanisms of how Hsp70 mono-ubiquitination is involved in the above processes.

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