Ubiquitination and Degradation of Homeodomain-interacting Protein Kinase 2 by WD40 Repeat/SOCS Box Protein WSB-1*

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Homeodomain-interacting protein kinase 2 (HIPK2) is a member of the nuclear protein kinase family, which induces both p53- and CtBP-mediated apoptosis. Levels of HIPK2 were increased by UV irradiation and cisplatin treatment, thereby implying the degradation of HIPK2 in cells under normal conditions. Here, we indicate that HIPK2 is ubiquitinated and degraded by the WD40-repeat/SOCS box protein WSB-1, a process that is blocked under DNA damage conditions. Yeast two-hybrid screening was conducted to identify the proteins that interact with HIPK2. WSB-1, an E3 ubiquitin ligase, was characterized as an HIPK2-interacting protein. The coexpression of WSB-1 resulted in the degradation of HIPK2 via its C-terminal region. Domain analysis of WSB-1 showed that WD40-repeats and the SOCS box were required for its interaction with and degradation of HIPK2, respectively. In support of the degradation of HIPK2 by WSB-1, HIPK2 was polyubiquitinated by WSB-1 in vitro and in vivo. The knockdown of endogenous WSB-1 with the expression of short hairpin RNA against WSB-1 increases the stability of endogenous HIPK2 and resulted in the accumulation of HIPK2. The ubiquitination and degradation of HIPK2 by WSB-1 was inhibited completely via the administration of DNA damage reagents, including Adriamycin and cisplatin. These findings effectively illustrate the regulatory mechanisms by which HIPK2 is maintained at a low level, by WSB-1 in cells under normal conditions, and stabilized by genotoxic stresses.

Homeodomain-interacting protein kinase 2 (HIPK2)§ is a member of a novel family of nuclear protein kinases and is well conserved from Drosophila to humans (1–4). HIPK2 interacts with a variety of transcription factors (5–8), p300/CBP coactivator (9, 10), and Groucho/TLE corepressor (11), thereby regulating target gene expression in a context-dependent manner. The loss of a functional HIPK2 allele induces a reduction of apoptosis and increased numbers of trigeminal ganglia, whereas HIPK2 overexpression in the developing sensory and sympathetic neurons promotes apoptosis in a caspase-dependent manner (11, 12). The HIPK1 and HIPK2 double-knock-out approach showed that HIPK2 performs overlapping functions with HIPK1 in the mediation of cell proliferation and apoptosis during mouse development (13). A number of key regulatory molecules, including p53, CtBP, Axin, Bm3, Sp100, TP53INP1, and PML, are known to be associated with the function of HIPK2 in the induction of apoptosis (14–18). UV-induced apoptosis is understood via the action of HIPK2 at the molecular level. HIPK2 is activated and stabilized by UV irradiation and selectively phosphorylates p53 at Ser-46. Thus, the kinase function of HIPK2 increases the expression of p53-target genes and enhances UV-induced apoptosis (7, 8). Additionally, HIPK2 phosphorylates the Ser-422 of CtBP, and phosphorylated CtBP is degraded via the 26 S proteasome pathway, resulting in apoptosis in p53-deficient cells (19). Endogenous HIPK2 protein is barely detected and is induced by UV irradiation or treatment with the chemotherapeutic drug, cisplatin, whereas the levels of HIPK2 mRNA remained unchanged under identical conditions (20). The levels of p53 and Bax increase concomitantly with the induction of HIPK2 (21). However, it remains to be determined whether HIPK2 is degraded at the protein level, nor is it understood which molecule is responsible for the tight control of HIPK2 levels in cells under normal conditions.

The highly controlled degradation of proteins via the ubiquitin-proteasome pathway represents a key mechanism for a variety of cellular activities, including cell cycle regulation and apoptosis. The ubiquitination of proteins requires three enzymes: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; and E3, ubiquitin ligase (22, 23). Among them, the ubiquitin ligase has specificity and recognizes the target protein. The majority of eukaryotes harbor a single E1 enzyme, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; aa, amino acid(s); HA, hemagglutinin; shRNA, short hairpin RNA.
several E2 enzymes, and a large and more diverse class of E3 enzymes. Several domains have been associated with E3 enzyme activity. The best known examples are the SCF (Skp1-Cullin-F-box) and the ECS (ElonginB/C-Cul2/5-SOCS) ubiquitin ligase complexes. The SCF ubiquitin ligases are a family of multisubunit RING finger E3 enzymes in which an F-box protein is responsible for substrate recognition. F-box proteins interact with the core SCF complexes via their common N-terminal F-box motif and harbor a C-terminal protein-protein interaction motif, typically WD40 repeats or leucine-rich repeats (24, 25). The ECS complex displays striking overall similarities with the SCF complex. The SOCS (suppressor of cytokine signaling) box-containing proteins function as adaptors that link substrates to the ElonginB/C-Cullin complex (26, 27).

In addition to the SOCS box, SOCS box proteins harbor a variety of protein-protein interaction domains, including the β-domain, WD40 repeats, and ankyrin motifs that bind to substrates. Thus far, at least twenty proteins have been determined to harbor a C-terminal SOCS box. These proteins fall into five classes based on the protein motifs found in the N-terminal region of the SOCS box (28). WSB-1 and WSB-2 have been classified as part of a new family; members of this family harbor seven WD40 repeats and an SOCS box in the N terminus and C terminus of a protein, respectively. Although the domain organization and the interactions of WSB-1 with ElonginB/C are very well understood, investigators have only recently undertaken studies to determine whether WSB-1 can function as an E3 ubiquitin ligase, and to investigate the potential target molecules of WSB-1 (29–31).

In this study, we show that WSB-1 is an E3 ubiquitin ligase that specifically targets HIPK2 for degradation via the 26 S proteasome pathway. We determined that WSB-1 promotes the ubiquitination and degradation of HIPK2, a process in which both WD40 repeats and the SOCS box are required for the recognition and degradation of HIPK2. WSB-1-mediated degradation of HIPK2 was blocked in DNA-damaged cells, which provide regulatory mechanisms by which the HIPK2 levels are tightly controlled in cells under normal conditions, and DNA damage stresses overcome the degradation of HIPK2 by WSB-1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—U2OS, RKO, and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For immunoblot analysis, HEK293 cells were seeded onto 6-well plates, and DNA transfection was carried out using the calcium phosphate procedure. U2OS and RKO cells were transfected with Fugene6 reagent (Roche Molecular Biochemistry) as described above, on 6-well plates.

**Plasmid Construction and Site-directed Mutagenesis**—For bait construction, a DNA fragment was inserted into EcoRI and XhoI sites of pEntr3C. Various WSB-1 deletion mutants were constructed by insertion of each PCR-amplified DNA fragments into the EcoRI and XhoI sites of pEntr3C. Various HIPK2 deletion constructs and GFP-HIPK2 plasmid have been described previously. The pET3E-Ubc5C plasmid expressing the His-Ubc5C was kindly provided by Yue Xiong (University of North Carolina). The Myc-WSB-1, GFP-WSB-1, His-WSB-1, and GST-WSB-1 plasmids were constructed by Gateway Technology (Invitrogen) with pEntr-WSB-1. The point mutant of WSB-1 for the WD40 domain was generated using the QuikChange mutagenesis kit (Stratagene) according to the recommendations of the manufacturer. The mutations were verified by DNA sequencing. Mutagenesis was conducted on the pEntr-derived WSB-1 plasmid, and GST-WSB-1 mutant expression plasmid was generated using Gateway Technology (Invitrogen). WSB-1 shRNA plasmid was constructed by inserting double-stranded oligonucleotides, which harbor the WSB-1 sequence (5′-GCTGTAAAGTGCAAGGAAATT-3′), into the BglII and HindIII sites of pSUPER (OligoEngine), in accordance with the manufacturer’s recommendations.

**Yeast Two-hybrid Screening**—For bait construction, a DNA fragment encoding amino acids 503–1189 of HIPK2 was subcloned into the EcoRI and SalI sites of pGBK7 (Clontech) and transformed into the yeast strain, AH109, followed by mating with yeast Y187 cells pretreated with mouse embryonic day 11 cDNA library (Clontech). Approximately 10³ transfor-
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**FIGURE 2. Identification of WSB-1 as an HIPK2-interacting protein.** A, the expression plasmids encoding full-length, N-terminal, and C-terminal HIPK2 were transfected into HEK293 cells, and the protein levels in the presence or absence of MG132 were determined by Western blotting using anti-Myc antibody. B, the specific interactions of WSB-1, which was identified as an HIPK2-interacting protein by yeast two-hybrid screening, with HIPK2 (503-1189) were confirmed by streaking of yeast cells onto synthetic drop-out plates lacking TL (Trp/Leu) or TLH (Trp/Leu/His). Yeast cells were transformed with empty vectors, and plasmids encoding WSB-1 yeast clone is depicted under the schematic. C, the HA-WSB-1 expression plasmid was transfected into HEK293 cells together with or without Myc-HIPK2 expression plasmid, the cell lysates were immunoprecipitated using anti-HA antibody, and the co-immunoprecipitated WSB-1 was detected by Western blotting using anti-Myc antibody. D, in vitro translated Myc-HIPK2 was subjected to GST pull-down analysis using GST (lane 2) or GST-WSB-1 (lane 3). The bound proteins were detected by Western blotting using anti-Myc antibody. E, RKO cell lysates were immunoprecipitated with preimmune serum (lane 3) or anti-HIPK2 antibody (lane 4), and the precipitates were detected by Western blotting with anti-WSB-1 antibody.

**FIGURE 3. Ubiquitination of HIPK2 by WSB-1 in vitro and in vivo.** A, Myc-HIPK2 was expressed in HEK293 cells in the presence or absence of WSB-1, and the transfected cells were chased with cycloheximide to inhibit de novo protein synthesis. The levels of HIPK2 were analyzed at the indicated times after the introduction of cycloheximide. The levels of GFP were also determined as a negative control. B, the expression plasmid encoding either Myc-HIPK2, N-terminal, or C-terminal deletion mutants was transfected into HEK293 cells together with Myc-WSB-1 expression plasmid, and the protein levels of both Myc-HIPK2 and Myc-WSB-1 were determined by Western blotting using anti-Myc antibody. C, RKO cells were transfected with Myc-HIPK2 expression plasmid in combination with HA-Ub and/or HA-WSB-1 expression plasmids as indicated, and HIPK2 ubiquitination was analyzed by Western blotting with anti-Myc antibody. D, in vitro translated Myc-HIPK2 was incubated with a reaction mixture lacking either E1, E2, ubiquitin, or purified GST-WSB-1, as indicated. The reaction mixtures were resolved by SDS-PAGE, and polyubiquitinated Myc-HIPK2 was detected by Western blotting using anti-Myc antibody.

**In Vitro Ubiquitination—**For the in vitro ubiquitination assay, affinity-purified GST-WSB-1 was mixed with in vitro translated Myc-HIPK2 as a substrate, and mixtures were added to the ubiquitination reaction (final volume, 30 μl) containing 40 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 2 mM diithiothreitol, 2 mM ATP, 8 μg of purified ubiquitin (Sigma), 0.5 μg of E1 (Boston Biochem), and 1 μg of His-Ubc5c. The reactions were incubated for 90 min at 37 °C and were terminated by boiling for 7 min in SDS sample buffer. The reaction mixtures were resolved by 6% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The ubiquitinated HIPK2 proteins were detected by Western blotting with anti-Myc antibody.

**In Vitro Pull-down Assays—**The GST-fusion proteins were expressed in *E. coli* BL21(DE3) cells, and were purified with glutathione-Sepharose beads according to the instructions of the manufacturer. The domains of Myc-HIPK2 proteins were synthesized by using the coupled TNT in vitro transcription-translation system (Promega, Madison, WI). Synthesized proteins were incubated with GST-WSB-1 (aa 284-421) at 4 °C for 1 h in binding buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Triton X-100) and washed three times with phosphate-buffered saline with 0.5% Triton X-100. After washing, the bound proteins were resolved by SDS-PAGE and detected by Western blotting with anti-Myc antibody.

**Co-immunoprecipitation and Western Blotting—**The co-immunoprecipitation of endogenous proteins was performed after the lysis of 2 x 10⁶ cells in high salt lysis buffer (50 mM Hepes, 350 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA). After incubation on ice for 10 min and centrifugation for 10 min at 4 °C, equal volumes of protein were diluted with lysis buffer lacking NaCl (dilution buffer), then incubated overnight with antibody and protein A/G-Sepharose beads at 4 °C on a rotating wheel. The beads were washed three times with lysis buffer. The whole cell lysates and immunoprecipitates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes.
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RESULTS

Identification of WSB-1 as HIPK2-interacting Protein—It is known that the levels of HIPK2 protein are increased as the result of UV irradiation and cisplatin treatment, both of which are genotoxic stresses that induce DNA damage (7, 8, 20). However, it remains to be determined whether HIPK2 is stabilized in protein levels by escaping from proteolysis, and which molecule is involved in this process. In an effort to ascertain the degradation of HIPK2 in cells under normal conditions, the cells expressing GFP-HIPK2 were exposed to UV, and the levels of HIPK2 were observed by GFP fluorescence. The intensity of GFP fluorescence from GFP-HIPK2 was increased via UV irradiation in a time-dependent manner (Fig. 1A, upper panel). The administration of DNA damage reagents, such as Adriamycin or cisplatin, to cells also resulted in induction of the endogenous HIPK2 protein level (Fig. 1A, middle panel and B, lanes 1–4). Furthermore, the treatment of cells with the proteasome inhibitor, MG132, resulted in the accumulation of HIPK2 (Fig. 1B, lane 6). Taken together, our results show that HIPK2 may be degraded by proteolysis in cells under normal conditions.

A yeast two-hybrid screening was conducted in an effort to identify potential cellular proteins that target HIPK2 for degradation. First, HIPK2 domains for proteolytic degradation were determined to construct a bait plasmid for yeast two-hybrid screening. Expression plasmids for either wild-type HIPK2, N-terminal HIPK2, or C-terminal HIPK2 were transfected into the HEK293 cells, and the protein levels were determined either prior to or after treatment with the proteasome inhibitor, MG132. The protein levels of wild-type HIPK2 and C-terminal HIPK2, but not N-terminal HIPK2, were increased as the result of MG132 treatment (Fig. 2A, lanes 2 and 6). This result indicates that HIPK2 is degraded by proteasome via its C terminus. Therefore, the C terminus of HIPK2 (aa 503–1189) was utilized as bait for the screening of mouse embryonic extract using cDNA libraries. Among the several cellular proteins that were identified and sequenced, a clone showed identity to WSB-1, a subunit of E3 ubiquitin ligase (Fig. 2B). The isolated WSB-1 clone harbored a C-terminal domain (aa 284–421) that included two WD40 repeats and a SOCS box. The specific interactions between WSB-1 and HIPK2 were verified in the yeast two-hybrid and co-immunoprecipitation assays in cultured cells (Fig. 2, B and C). GST pull-down analysis showed that WSB-1 interacted physically with HIPK2 (Fig. 2D). The association of endogenous HIPK2 with WSB-1 was also verified via co-immunoprecipitation with anti-HIPK2 antibody followed by Western blotting using anti-WSB-1 antibody (Fig. 2E). Collectively, our results show that WSB-1 is an HIPK2-interacting protein in vitro and in vivo and is associated with HIPK2 in cultured cells.

Ubiquitination and Degradation of HIPK2 by WSB-1—In an effort to determine whether WSB-1 functions as an E3 ubiquitin ligase against HIPK2, Myc-HIPK2 expression plasmids were transfected into HEK293 cells either with or without the WSB-1 expression plasmid. Transfected cells were chased with cycloheximide to inhibit de novo protein synthesis. At the indicated times after the addition of cycloheximide, the levels of the HIPK2 proteins were analyzed by Western blotting. The stability of HIPK2 was markedly reduced by the coexpression of WSB-1 (Fig. 3A). Because the C terminus of HIPK2 was utilized as bait for yeast two-hybrid screening for the identification of WSB-1, HIPK2 domain for degradation by WSB-1 was determined. As had been expected, the C terminus (aa 503–1189) of HIPK2, but not the N terminus, was degraded by the coexpression of WSB-1 (Fig. 3B, lane 6). To ascertain the functional
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interaction of WSB-1 with HIPK2, ubiquitinations of HIPK2 by WSB-1 were explored under in vitro and in vivo conditions. HIPK2 was efficiently polyubiquitinated when HA-WSB-1 was expressed in cells along with HA-Ub (Fig. 3C, lane 3). Consistent with the above observations, affinity-purified GST-WSB-1 ubiquitinated Myc-HIPK2 only when all of E1, E2, ubiquitin, and WSB-1 were contained in the reaction mixture (Fig. 3D, lane 5). These results indicate that WSB-1 could function as an E3 ubiquitin ligase against HIPK2.

Knockdown of Endogenous WSB-1 Increases HIPK2 Stability—Next, we assessed the effects of WSB-1 knockdown on the stability of HIPK2. RKO cells were transfected with increasing quantities of expression plasmids for shRNA against WSB-1, and WSB-1 levels were analyzed by Western blotting with anti-WSB-1 antibody. As shown in Fig. 4A, endogenous WSB-1 was silenced efficiently by the expression of shRNA against WSB-1 in a dose-dependent manner. The knockdown of endogenous WSB-1 concomitantly resulted in the elevation of endogenous HIPK2 levels (Fig. 4B, middle panel). Furthermore, HIPK2 stabilization was assessed in a single cell. To this end, U2OS cells were transfected with WSB-1 shRNA expression plasmids coupled with the GFP expression plasmid, and the expression levels of endogenous HIPK2 were observed by immunostaining using anti-HIPK2 antibody. The WSB-1 shRNA-expressing cells could be monitored indirectly by co-transfection of the GFP expression plasmid at a high ratio, of 1.5 μg (WSB-1 shRNA) to 0.05 μg (EGFP-C2). As shown in Fig. 4C, the GFP-positive cells expressed endogenous HIPK2 at a high level (middle panel, arrows), whereas the cells expressing either control shRNA (upper panel) or no GFP/shWSB-1 (middle panel, arrowhead) displayed relatively low HIPK2 levels. The administration, however, of MG132 to the cells resulted in a high level of endogenous HIPK2 expression, regardless of WSB-1 shRNA expression (lower panel). Taken together, these results showed that WSB-1 could degrade HIPK2 constantly in the cells under normal conditions.

WSB-1 Interacts with HIPK2 through WD40 Repeats—WSB-1 is composed of seven WD40 repeats and an SOCS box in the C terminus (Fig. 5A). WD40 repeats have been shown to participate in diverse cellular functions, and the SOCS box is known to be involved in the protein degradation in the ECS ubiquitin ligase complex (34–36). To determine whether the WD40 domain...
residues, including a typical tryptophan residue of WD40 repeats, are conserved from the second to seventh WD40 repeats of WSB-1. To determine whether the WD40 repeat of WSB-1, but not the SOCS box, is involved in its interaction with HIPK2, we substituted conserved Phe-359, Asp-362, and Leu-366 residues with alanines on the WSB-1 sequence (aa 339–421), which contained a seventh WD40 repeat and the SOCS box. The point mutant of WSB-1 (339–421, AAA) interacted less efficiently with GST-HIPK2 than did wild-type WSB-1 (339–421, FDL), even though the SOCS box was not altered (Fig. 5E, lane 4). These data show that WSB-1 interacts physically with HIPK2 via its multiple WD40 repeats.

Both WD40 Repeats and the SOCS Box of WSB-1 Are Required for Degradation of HIPK2—Next, we determined the WSB-1 domains required for HIPK2 degradation. A series of WSB-1 deletion mutants was transfected into HEK293 cells together with the Myc-HIPK2 expression plasmid, and HIPK2 degradation was analyzed by Western blotting using anti-Myc antibody (Fig. 6A). Full-length WSB-1 and deletion mutants lacking two N-terminal WD40 repeats (first and second WD40) were shown to degrade HIPK2. However, further deletion to the third and fifth WD40 repeats gradually abolished degradation of HIPK2 by WSB-1 mutants (Fig. 6A, lanes 4 and 5). WSB-1 devoid of the SOCS box proved unable to degrade HIPK2, although its interaction with HIPK2 remained unaltered (Fig. 5B). The ability of WSB-1 to degrade HIPK2 was also determined with the ubiquitination of the HIPK2 C terminus in cultured cells. Consistent with the above findings, the WSB-1 mutant lacking either five N-terminal WD40 repeats or the C-terminal SOCS box could not ubiquitinate HIPK2, whereas wild-type WSB-1 markedly ubiquitinated HIPK2 (Fig. 6B). In addition, the affinity-purified GST-WSB-1 mutant lacking the SOCS box could not ubiquitinate HIPK2 in vitro (Fig. 6C). These results indicate that WD40 domains and the SOCS box are required for the recognition of HIPK2 by WSB-1 and the constitution of the E3 ligase complex for the degradation of HIPK2, respectively.

DNA Damage Blocks Degradation of HIPK2 by WSB-1 in Vivo—Next, we attempted to determine whether the polyubiquitination of HIPK2 is blocked by administration of DNA damage reagents such as Adriamycin and cisplatin, as HIPK2 was shown to be stabilized under these conditions. Myc-WSB-1 and Myc-HIPK2 expression plasmids were transfected into HEK293 cells, and the cells were then exposed to either Adriamycin or cisplatin. As shown in Fig. 7A, the coexpression of WSB-1 resulted in the degradation of HIPK2 (lane 2). However, HIPK2 was not degraded by WSB-1 in the cells treated with Adriamycin, cisplatin, or MG132 (lanes 3–5). It was determined that the catalytic activity of HIPK2 was increased following DNA damage, such that both the autophosphorylation of HIPK2 and the phosphorylation of the substrate were increased (8, 21). In an attempt to gain insight into the blockage of HIPK2 degradation under DNA damage conditions, a GST pulldown assay was employed to determine whether the binding of WSB-1 to HIPK2 is dependent on the phosphorylation status of HIPK2. The pull-down of HIPK2 wild-type and deletion mutants showed that kinase-dead full-length HIPK2 and kinase-dead HIPK2(aa 1–629) bound strongly to GST-WSB-1, but catalytically active HIPK2 and HIPK2(aa 1–629) did not (Fig. 7B). Consistent with the above observation, the dephosphorylation of wild-type HIPK2 by alkaline phosphatase treatment caused a faster migration of HIPK2 in the gel, and the concomitant enhanced binding of HIPK2 to GST-WSB-1 but not of the catalytically inactive HIPK2 K221R mutant (Fig. 7D, lanes 5–8). These results strongly indicated that WSB-1 was not capable of recognizing HIPK2 after cells were exposed to genotoxic stresses, which caused conditions in which HIPK2 could be activated and stabilized following its release from E3 ubiquitin ligase complexes containing WSB-1.

DISCUSSION

HIPK2 is a key regulator for UV-induced apoptosis. During this process, the activated and stabilized HIPK2 inhibits the progression of the cell cycle and induces apoptosis via phosphorylation of target proteins, such as p53 and CtBP (6–8). There-
HIPK2, WSB-1 is capable of assembling with the Cul5/Rbx1 or Cul5/Rbx2 module to form ubiquitin ligase complexes via its conserved BC motif in the SOCS box (29, 44). Therefore, it could be speculated that the three-dimensional β-propeller structure of WD40 repeats is required for either high affinity recognition or the correct positioning of substrate to the Cul5-Rbx1-ElonginB/C-based E3 ligase complex. Further structural analysis of HIPK2-EC$^{\text{WSB-1}}$ complex would provide a molecular mechanistic basis for the recognition of HIPK2 by WSB-1 and information regarding key residues of WSB-1 WD40 repeats in the course of dephosphorylation-dependent HIPK2 recognition.

Post-translational modifications such as ubiquitination and phosphorylation play important roles in the regulation of cellular protein function. A vast amount of evidence has established the interplay between phosphorylation and ubiquitination in protein degradation (45–47). The substrates for SCF HIPK2, WSB-1 should be regulated both prior to and after the exposure of cells to the extracellular stresses that induce DNA damage. In this report, we provide evidence that HIPK2 is ubiquitinated and degraded by WSB-1, WD40 repeats/SOCS box protein in cells under normal conditions, but the exposure of cells to UV or cisplatin prevents the destruction of HIPK2 by WSB-1. Consequently, HIPK2 remains active and stable for the induction of apoptosis.

WSB-1 is a protein that contains N-terminal WD40 repeats and a C-terminal SOCS box (28). WD40 repeats have been implicated in protein–protein interactions. WD40 repeats were originally recognized in the β subunit of G proteins and have since been described in a wide variety of cytoplasmic proteins, many of which are involved in signal transduction (34, 37). The regions of WD40 repeats have been reported to function as domains responsible for targeting of a protein to the ubiquitin E3 ligase complex (25, 38). Domain analysis of WSB-1 showed that WD40 repeats are sufficient for the recognition of HIPK2, but the SOCS box is required for the degradation of HIPK2 as is the case with other WD40-containing proteins (24, 39–43). WSB-1 lacking a C-terminal SOCS box interacted with HIPK2 in vitro and in cultured cells (Fig. 5) but did not ubiquitinate HIPK2 in vitro and in cultured cells (Fig. 6). In addition, a serial deletion of the N-terminal WD40 repeats of WSB-1 gradually abolished the WSB-1 activity for HIPK2 degradation, in proportion to the degree of WD40 repeat deletion and independent of HIPK2 recognition (Figs. 5 and 6). These results strongly suggest that, in addition to HIPK2 recognition, the WD40 repeats of WSB-1 play an additional role in the degradation of complexes in cell cycle regulation harbor so-called phosphodegrons, short peptide sequence elements that are targeted by certain kinases. Once a substrate is phosphorylated, SCF complexes recognize and degrade the phosphorylated substrate. In the case of APC complex, the formation of active APC complex is regulated by phosphorylation. In mitotic prophase, APC becomes phosphorylated on multiple sites by Cdns, and these phosphorylations are a prerequisite for interaction with and activation by Cdc20 (39, 40, 48). In the cases of both substrate phosphorylation and the phosphorylation of destruction machinery, the degradation of target proteins is accomplished at the most appropriate time during the cell cycle. The opposite rationale could be applied to the degradation of HIPK2 by WSB-1. Under normal conditions, the synthesized HIPK2 is degraded constitutively by WSB-1. After the cells are exposed to genotoxic stresses, including UV or cisplatin, HIPK2 is phosphorylated, which allows it to escape from destruction by WSB-1. These results show that the regulation of the HIPK2 levels is dependent on the inverse relationship between the phosphorylation and ubiquitination of HIPK2.

It should be noted that the expression of chick WSB-1 is induced by the Hedgehog signaling pathway during development (49). Chick WSB-1 is expressed in somitic mesoderm and developing limb buds, as well as in other embryonic structures in which Hedgehog signaling has been shown to play a role. Sonic Hedgehog has been studied extensively, particularly in the ventral patterning of the neural tube, where the expressions and activity of HIPK2 target proteins, including Nkx1.2, Nkx1.6, Pax6, and Groucho/TLE, are regulated by Shh signal-

![Image](https://example.com/image1.png)

**Figure 7.** Inhibition of HIPK2 degradation by WSB-1 by administration of cells with DNA damage reagents. A, HEK293 cells were transfected with Myc-HIPK2 and Myc-WSB-1 expression plasmids. Thirty-six hours after transfection, the cells were treated with either MG132 (lane 3), Adriamycin (lane 4), or cisplatin (lane 5). HIPK2 and WSB-1 were detected by Western blotting using anti-Myc antibody. B, Myc-tagged wild-type HIPK2 (WT), kinase-dead HIPK2 K221R mutant (KD), and HIPK2 deletion mutants (KD, aa 1–629; K221R, aa 1–629; K221R; and, aa S03–1189) were in vitro translated with a TnT-coupled rabbit reticulocyte lysate system, and were subjected to a GST pulldown assay using affinity purified GST-WSB-1. The bound proteins were eluted and resolved by 8% SDS-PAGE and were detected by Western blotting using anti-Myc antibody. Input denotes 5% of the protein used in the assay. C, in vitro translated wild-type Myc-HIPK2 or catalytically inactive Myc-HIPK2 K221R mutant was subjected to GST pulldown assay prior to or after calf intestinal alkaline phosphatase (CIAP) treatment. The bound proteins were analyzed as described in B. Note that the migration of HIPK2 is faster, and binding affinity to GST-WSB-1 was increased after alkaline phosphatase treatment (lane 4). D, Myc-tagged wild-type HIPK2 or kinase-dead HIPK2 K221R mutant expression plasmids were transfected into HEK293 cells along with HA-WSB-1 expression plasmids. The transfected cells were cultured for 12 h in the presence or absence of Adriamycin prior to collection for further analysis. HA-WSB-1 was precipitated from the cell lysates using anti-HA antibody. The co-precipitated Myc-HIPK2 was detected by Western blotting using anti-Myc antibody.
ing (50, 51). Members of the NK/NKx family homeoproteins function as a transcriptional activator or repressor in a context-dependent manner (32, 52). Groucho/TLE corepressor is a crucial cofactor for the NK/NKx family homeoproteins and its corepressor activities are regulated by HIPK2-mediated phosphorylation (1). Combinatorial actions of these transcription factors and cofactors upon external signal such as Shh are believed to be underlying mechanism of proper patterning of neural tube. Therefore, it would be interesting to study the regulation of HIPK2 by WSB-1 induction through Hedgehog signaling during neural tube development.

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31. Groucho/TLE corepressor is a crucial cofactor for the NK/NKx family homeoproteins and its corepressor activities are regulated by HIPK2-mediated phosphorylation (1). Combinatorial actions of these transcription factors and cofactors upon external signal such as Shh are believed to be underlying mechanism of proper patterning of neural tube. Therefore, it would be interesting to study the regulation of HIPK2 by WSB-1 induction through Hedgehog signaling during neural tube development.

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