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Fbxw7 Acts as an E3 Ubiquitin Ligase That Targets c-Myb for Nemo-like Kinase (NLK)-induced Degradation*[S]

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The c-*myb* proto-oncogene product (c-Myb) is degraded in response to Wnt-1 signaling via a pathway involving TAK1 (transforming growth factor-β-activated kinase 1), HIPK2 (homeodomain-interacting protein kinase 2), and NLK (Nemo-like kinase). NLK directly binds to c-Myb, which results in the phosphorylation of c-Myb at multiple sites, and induces its ubiquitination and proteasome-dependent degradation. Here, we report that Fbxw7, the F-box protein of an SCF complex, targets c-Myb for degradation in a Wnt-1- and NLK-dependent manner. Fbxw7α directly binds to c-Myb via its C-terminal WD40 domain and induces the ubiquitination of c-Myb in the presence of NLK *in vivo* and *in vitro*. The c-Myb phosphorylation site mutant failed to interact with Fbxw7α, suggesting that the c-Myb/Fbxw7α interaction is enhanced by NLK phosphorylation of c-Myb. Treatment of M1 cells with Fbxw7 small interfering RNA (siRNA) rescued the Wnt-induced c-Myb degradation and also the Wnt-induced inhibition of cell proliferation. NLK bound to Cul1, a component of the SCF complex, while HIPK2 interacted with both Fbxw7α and Cul1, suggesting that both kinases enhance the c-Myb/SCF interaction. In contrast to c-Myb, the v-*myb* gene product (v-Myb) encoded by the avian myeloblastosis virus was resistant to NLK/Fbxw7α-induced degradation. Thus, Fbxw7 is an E3 ubiquitin ligase of c-Myb, and the increased c-Myb levels may contribute, at least partly, to transformation induced by mutation of Fbxw7.

The c-*myb* proto-oncogene product (c-Myb) is the cellular progenitor of the v-*myb* oncogene of the avian myeloblastosis virus (1). c-*myb* is involved in the proliferation of immature hematopoietic cells (2), as well as hematopoietic cell differentiation (3). c-Myb has three functional domains responsible for DNA binding, transcriptional activation, and negative regulation (4). The DNA-binding domain in the N terminus consists of three imperfect tandem repeats of 51–52 amino acids and binds to the DNA sequence 5′-AACNG-3′ (5). The centrally located transcriptional activation domain binds to the transcriptional coactivator CBP (6, 7). The negative regulator domain in the C-terminal portion of c-Myb interacts with the corepressors TIF1β and BS69 (8, 9), and its deletion or mutation increases c-Myb activity (4, 10). Various c-Myb target genes have been identified, including c-*myc* (11), *bcl-2* (12, 13), and *GBX2* (14), which are involved in cell cycle control, blockage of apoptosis, and growth and differentiation control, respectively.

Although the specific signaling pathways that regulate c-Myb activity remain largely unknown, we recently found that c-Myb is phosphorylated and degraded in response to Wnt-1 signaling (15). The Wnt-1 signal is transduced to c-Myb via a pathway involving TAK1(TGF-β-activated kinase), HIPK2 (Homeodomain-interacting protein kinase 2), and NLK (Nemo-like kinase). HIPK2 and NLK directly bind to c-Myb, and NLK phosphorylates c-Myb at multiple sites, resulting in its ubiquitination and proteasome-dependent degradation. Wnt signals control differentiation or apoptosis in many cell types, including hematopoietic cells (16), suggesting that Wnt-induced c-Myb degradation plays an important role in the proliferation and differentiation of hematopoietic cells. Because v-Myb encoded by the avian myeloblastosis virus is resistant to Wnt-1/TAK1/HIPK2/NLK-induced degradation due to the lack of critical NLK phosphorylation sites (17), which are located in the C-terminal region of c-Myb, v-Myb may escape Wnt-dependent negative regulation. NLK also phosphorylates A-Myb, another member of the vertebrate Myb proteins family, but does not induce A-Myb degradation (18). Instead, NLK inhibits the association between A-Myb and the coactivator CBP, thus blocking A-Myb-induced transactivation. This difference may be because the specific E3 ubiquitin ligase recognizes NLK-phosphorylated c-Myb, but not A-Myb.

Covalent attachment of multiple ubiquitin molecules to a protein substrate is catalyzed by three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (19). One of the two major classes of E3s, the SCF (Skp1-Cul1-F-box protein) complex, consists of three common subunits (Skp1, Cul1, and Rbx1) and a variable substrate recognition subunit (an F-box protein) (19). Fbxw7 (F-box and WD40 domain protein 7; also known as hCDC4,

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hAGO, or SEL10) is a member of the F-box family of proteins and functions as a substrate recognition subunit of the SCF complex (20). The *Fbxw7* gene encodes three protein isoforms (α, β, and γ) (21–24), each of which has a unique N-terminal region followed by a common region encoded by unique 5′-exons and shared exons, respectively. Fbxw7α and Fbxw7β are predominantly localized in the nucleus and cytoplasm, respectively, whereas Fbxw7γ is a nucleolar protein (25, 26). Substrate recognition by Fbxw7 is mediated through interaction with eight adjacent WD40 protein-binding motifs located in the Fbxw7 C-terminal region (27). Fbxw7 targets multiple proteins for proteasome degradation, including important regulators of cell proliferation such as cyclin E (21–23), c-Myc (28, 29), c-Jun (30), Aurora-A (31), and Notch (32). The role of Fbxw7 as a negative regulator of several oncoproteins is consistent with reports that mutations of human Fbxw7 have been detected in several human tumor types (24, 33), and *trans*-heterozygous mutant mice of Fbxw7 and p53 are predisposed to a variety of epithelial tumors (31). Here, we report that Fbxw7 acts as an E3 ubiquitin ligase that targets c-Myb for Wnt/HIPK2/NLK-induced degradation.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmids containing the chicken β-actin promoter and various forms of c-Myb and HIPK2 have been described previously (15, 17). A PCR-based method was used to construct plasmids expressing His-tagged c-Myb, His-tagged Fbxw7α, and various Lys mutants of c-Myb. The Skp1, Cul1, and Rbx1 expression plasmids have been described previously (34). Plasmids expressing various Fbxw proteins and Skp2 were also reported previously (28).

Co-immunoprecipitation Assays—For co-immunoprecipitation experiments (see Figs. 1*A*, 5*B*, and 7, *A* and *B*), 293T cells (1×10^6 cells/100-mm dish) were transfected using the $CaPO_4$ method with mixtures of various plasmids, as described in the figure legends. At 40 h post-transfection, cells were lysed by mild sonication in 0.5 ml of NET buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40, 10% glycerol protease inhibitor mixture) containing 150 mM NaCl. The lysates were immunoprecipitated with various antibodies, as described in the figure legends, and the immunocomplexes were subjected to SDS-PAGE, followed by Western blotting using the antibodies described in the figure legends and the ECL Western blotting System (Amersham Biosciences). Aliquots of the lysates were also directly used for Western blotting.

Western Blotting—293T or CV-1 cells were transfected with mixtures of plasmids, as described in legends to Figs. 1*B*, 2, *A–D*, 3, and 6. At 40 h post-transfection, cells were lysed in SDS sample buffer with mild sonication and subjected to SDS-PAGE, followed by Western blotting using the antibodies described in the figure legends and detection by ECL. The transfection efficiency was determined by measuring β-galactosidase activity in aliquots of cells, and the amounts of lysate used for Western blotting were normalized to the β-galactosidase activity. In some cases, Western blot signals were quantified using NIH Image.

RNA Interference Experiments—The previously reported siRNAs for human Fbxw7 mRNA (34) were used. The sense strand sequences used for each Fbxw7 siRNA (Wako, Nippon Gene) are as follows (the siRNA consists of the complementary oligonucleotide): siRNA #1, 5′-GUGAAGUUGUUGGAGUAGAdTdT-3′; siRNA #2, 5′-ACAGGACAGTGUUUACAAAdTdT-3′; and siRNA #3, 5′-CCUUCUCUGGAGAGAGAAAdTdT-3′. An siRNA specific for green fluorescent protein was used as a control. To examine the silencing effect (Supplementary Fig. S1*B*), 293T cells were transfected with a mixture of 0.3 μg of HA-Fbxw7 expression plasmid, 0.05 μg of pact-β-gal, and 10 or 25 nM (final concentration in 10-ml culture) oligonucleotide siRNAs using the $CaPO_4$ method. At 24 h post-transfection, cell lysates were subjected to Western blotting with anti-HA antibody 12CA5 as described above. To investigate the effect of siRNA on c-Myb degradation, 293T cells were transfected with mixtures of plasmids as described in the legend to Supplementary Fig. S1*C* using the $CaPO_4$ method. At 24 h post-transfection, cell lysates were subjected to Western blotting with an anti-c-Myb (αCT5) polyclonal antibody.

Isolation of Fz1-expressing M1 Cell Clones and Bromodeoxyuridine Incorporation—The retroviral expression vector for rat Fz1 was constructed using the murine stem cell virus-based retroviral vector, and virus was prepared by transfecting it into BOSC23 packaging cells. M1 cells were infected with this virus (multiplicity of infection = 1) for 24 h, and clones were selected using neomycin. Anti-Fz1 antibody (R&D Systems, AF1120) was used for Western blotting.

To measure cell proliferation, a Biotrak ELISA kit (Amersham Biosciences), based on the incorporation of BrdUrd,[2] was used, and the assay was performed following the manufacturer's guidelines. In brief, M1 cells were transfected with 25 nM Fbxw7 siRNA using Lipofectamine 2000 (Invitrogen), and 3 days after transfection, BrdU was incorporated for 15 h. After fixation and blocking, a peroxidase-labeled anti-BrdU antibody was added. The substrate reaction was performed with tetramethylbenzidine, and the color was read at 450 nm in a spectrophotometer.

Ubiquitination Assays—For the *in vivo* ubiquitination assay, 293T cells were transfected with 4 μg of pact-FLAG-c-Myb, 2 μg of pcDNA3-Myc-Ub, 0.5 μg of pCMV-NLK, and 1 μg of pCGN-HA-Fbxw7α or empty vector. At 24 h post-transfection, the cells were treated with MG132 (15 μM) for 4 h. Cells were then scraped into 100 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2% SDS), heated for 10 min at 95 °C, diluted with buffer lacking SDS to reduce the SDS concentration to 0.2%, and sonicated mildly on ice. After pre-adsorption with protein G-Sepharose, an anti-FLAG M2 monoclonal antibody was used to precipitate immunocomplexes, which were then examined by Western blotting with an anti-Myc monoclonal antibody (MBL).

In vitro ubiquitination assays were performed essentially as described previously (22, 35). Components of the SCF complex (FLAG-Skp1, HA-Cul1, Myc-Rbx1, and HA-Fbxw7α) were coexpressed in transfected 293T cells and immunopurified on

[2] The abbreviations used are: BrdUrd, bromodeoxyuridine; E3, ubiquitin-protein isopeptide ligase; siRNA, small interfering RNA; HA, hemagglutinin; T-ALL, T cell acute lymphoblastic leukemia; CBP, cAMP-responsive element-binding protein-binding protein.

protein G beads using anti-HA monoclonal antibody 12CA5. His-c-Myb and NLK were separately expressed in transfected 293T cells and purified using a HIS-select cobalt affinity gel (Sigma). NLK contains eight His repeats in its N-terminal region and can be purified using the cobalt affinity gel. His-c-Myb was phosphorylated by incubating with His-NLK and 2 mM ATP for 1 h at room temperature in kinase buffer (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol). The SCF immuno complexes were mixed with phosphorylated c-Myb and 2 mM ATP for 30 min on ice to allow binding in the presence of 2 μM MG132, 10 μM proteasome inhibitor I (Calbiochem), and protease inhibitor mixture (Complete, Roche Applied Science). Ubiquitination reactions were carried out essentially as described (22, 35, 36).

RESULTS

Fbxw7α Binds to Wild-type c-Myb but Not to the c-Myb-15A Mutant—We previously found that TRAF7 binds to c-Myb via its WD40 domain (36–37), suggesting that WD40 repeat-containing proteins are good candidates for c-Myb-binding proteins. To identify the E3 ubiquitin ligase that targets c-Myb in an NLK-dependent manner, we focused on the F-box and WD40 repeat-containing proteins, which are components of the cullin-based E3 ubiquitin ligases. Previously, we demonstrated that coexpression of NLK or HIPK2 with c-Myb induces c-Myb degradation in CV-1 cells (15). However, we found that coexpression of neither NLK nor HIPK2 induced c-Myb degradation in 293T cells, suggesting that the level of some component(s) in the signaling pathway that induces c-Myb degradation is low in 293T cells. Therefore, we used 293T cells to examine the interaction between c-Myb and various members of the Fbxw family. Interestingly, all five Fbxw proteins examined co-immunoprecipitated with c-Myb (Fig. 1*A*). We also previously found that the c-Myb-15A mutant, in which all the 15 putative NLK Ser/Thr phosphorylation sites are replaced by Ala, was not degraded by coexpression of NLK or HIPK2 (15). Four of the Fbxw proteins examined co-precipitated with c-Myb-5A, whereas Fbxw7α did not, suggesting that Fbxw7α binds to c-Myb in a phosphorylation-dependent manner. The Fbxw7α mutant that lacked the N-terminal F-box but retained the C-terminal WD40 repeats (Fbxw7ΔNF) bound to c-Myb, suggesting that Fbxw7α interacts with c-Myb via its C-terminal WD40 repeats. Skp2, which was previously reported to bind to and induce the degradation of B-Myb (38), bound to both wild-type c-Myb and c-Myb-15A, suggesting that the interaction between c-Myb and Skp2 is phosphorylation-independent.

Fbxw7α Is Responsible for the Wnt/NLK Pathway-induced Degradation of c-Myb—Although coexpression of NLK alone with c-Myb did not induce c-Myb degradation in 293T cells, coexpression of NLK and Fbxw7α dramatically reduced the c-Myb level (Fig. 1*B*). A decrease in the c-Myb level was not observed with the Fbxw7α mutant lacking the N-terminal F box (Fbxw7ΔNF) or with the other four Fbxw proteins tested. Coexpression of various amounts of Fbxw7α, NLK, and HIPK2 with c-Myb indicated that these three factors cooperatively reduced the c-Myb levels in 293T cells (Fig. 2*A*). Coexpression of all three factors dramatically reduced the c-Myb level, and the decrease in c-Myb level was abrogated by MG132 treatment of

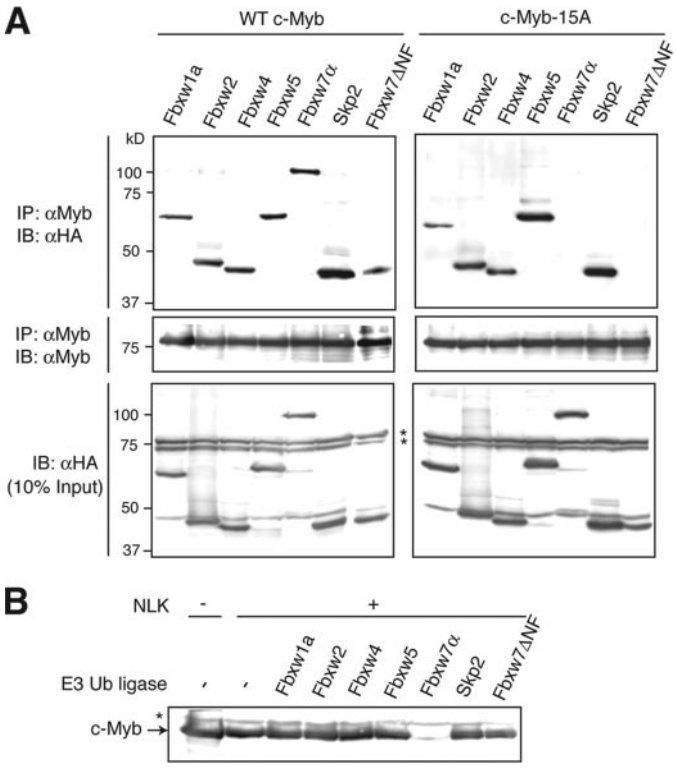

FIGURE 1. **Fbxw7α binds to and induces degradation of c-Myb in an NLK-dependent manner in 293T cells.** *A*, Fbxw7α binds to wild-type (*WT*) c-Myb, but not to c-Myb-15A. 293T cells were transfected with a mixture of 4 μg of pact-c-Myb and 4 μg of HA-Fbxw or HA-Skp2 expression plasmids. Lysates from the transfected cells were immunoprecipitated (*IP*) with anti-c-Myb antibody (αCT5), followed by Western blotting (*IB*) with anti-HA (12CA5) (*top*) or anti-c-Myb (αCT5) (*middle*) antibody. Lysates were also directly used for Western blotting with anti-HA antibody (12CA5) (*bottom*). *Asterisks* indicate nonspecific bands. *B*, Fbxw7α induces c-Myb degradation in the presence of NLK. 293T cells were transfected with a mixture of 1.5 μg of the FLAG-c-Myb expression plasmid, 0.3 μg of the NLK expression plasmid, 0.3 μg of Fbxw or Skp2 expression plasmids, and 0.02 μg of pact-β-gal. Lysates from the transfected cells were used for Western blotting with anti-c-Myb antibody (αCT5). *Ub*, ubiquitin.

transfected cells (Fig. 2*B*). These data indicate that Fbxw7α, NLK, and HIPK2 synergistically induce c-Myb degradation in 293T cells. Degradation of c-Myb-15A was not induced by coexpression of Fbxw7α and NLK (Fig. 2*C*). Coexpression of NLK with Skp2, which bound to c-Myb-15A as shown in Fig. 1*A*, also did not induce the degradation of c-Myb-15A.

To investigate whether endogenous Fbxw7 is responsible for the Wnt-1-induced degradation of c-Myb, we first used the 293T cell system in which the components of the Wnt/NLK pathway were exogenously expressed. In 293T cells, coexpression of Wnt-1 and Fz did not affect the c-Myb levels, whereas coexpression of Wnt-1, Fz1, TAK1, and TAB1 slightly reduced the c-Myb levels (Supplementary Fig. S1*A*, *lanes 1–3*). When c-Myb was coexpressed with TAK1, TAB1, HIPK2, and NLK, the c-Myb levels were significantly reduced (Supplementary Fig. S1*A*, *lane 4*). Furthermore, when six components (Wnt-1, Fz1, TAK1, TAB1, HIPK2, and NLK) of the Wnt/NLK pathway were coexpressed with c-Myb, almost no c-Myb signal was detected (Supplementary Fig. S1*A*, *lane 5*).

We then investigated the effect of Fbxw siRNA on the Wnt-1-induced degradation of c-Myb. Three different siRNAs against human Fbxw7 mRNA were previously reported to

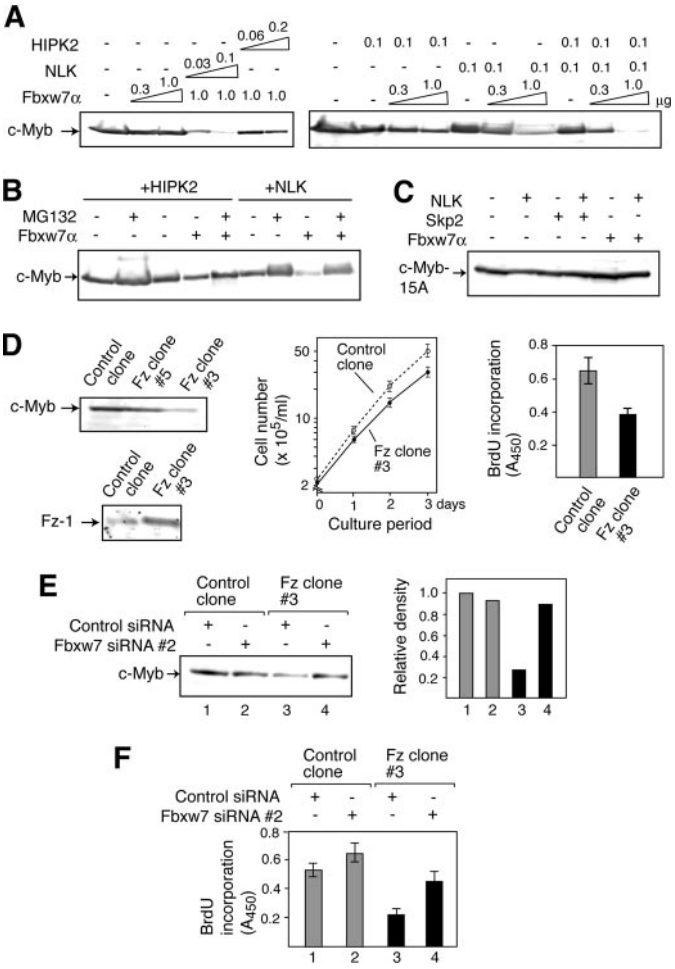

FIGURE 2. **Fbxw7 is responsible for Wnt-1-induced c-Myb degradation in 293T cells.** *A*, Fbxw7α, NLK, and HIPK2 cooperatively reduce the c-Myb level. 293T cells were transfected with a mixture of 1 μg of the FLAG-c-Myb expression plasmid, the indicated amounts of the Fbxw7α, NLK, and HIPK2 expression plasmids, and 0.07 μg of pact-β-gal. Lysates from the transfected cells were used for Western blotting with anti-FLAG M2. *B*, Fbxw7α, together with NLK and HIPK2, induces c-Myb degradation. 293T cells were transfected with a mixture of 1 μg of the FLAG-c-Myb expression plasmid, 1 μg of the Fbxw7α expression plasmid, 0.1 μg of the NLK expression plasmid, or 0.2 μg of the HIPK2 expression plasmid and 0.07 μg of pact-β-gal. In some cases, transfected cells were treated with MG132 (15 μM) for 4 h before preparation of lysates. Western blotting was performed as described above. *C*, Fbxw7α and NLK cannot induce degradation of c-Myb-15A. 293T cells were transfected with a mixture of 1 μg of the FLAG-c-Myb-15A expression plasmid, 0.4 μg of the Fbxw7α expression plasmid, 0.4 μg of the Skp2 expression plasmid, or 0.2 μg of the NLK expression plasmid and 0.02 μg of pact-β-gal. Western blotting was performed as described above. *D*, ectopic expression of Fz1 down-regulates c-Myb in M1 cells. *Left*, M1 cells were infected with the Fz1-encoding retrovirus, and 20 clones were isolated. Cell lysates from two clones and the control clone were used for Western blotting with anti-c-Myb and anti-Fz1 antibodies. *Middle*, the growth curves of clone 3 and the control clone are shown. The mean ± S.E. of three experiments is shown. *Right*, the degree of BrdUrd (*BrdU*) incorporation of clone 3 and the control clone was measured. The mean ± S.E. of three experiments is indicated in a bar graph. *E*, Fbxw7 siRNA rescues Wnt-induced c-Myb degradation. Clone 3 and the control clone were treated with the indicated siRNA (25 nM), and lysates were used for Western blotting with anti-c-Myb antibodies. The density of each band was measured, and relative values are indicated in a bar graph on the right. *F*, Fbxw7 siRNA rescues the Wnt-dependent inhibition of cell proliferation. Clone 3 and the control clone were treated with siRNA as described above, and the degree of BrdU incorporation was measured. The mean ± S.E. of three experiments is indicated in a bar graph.

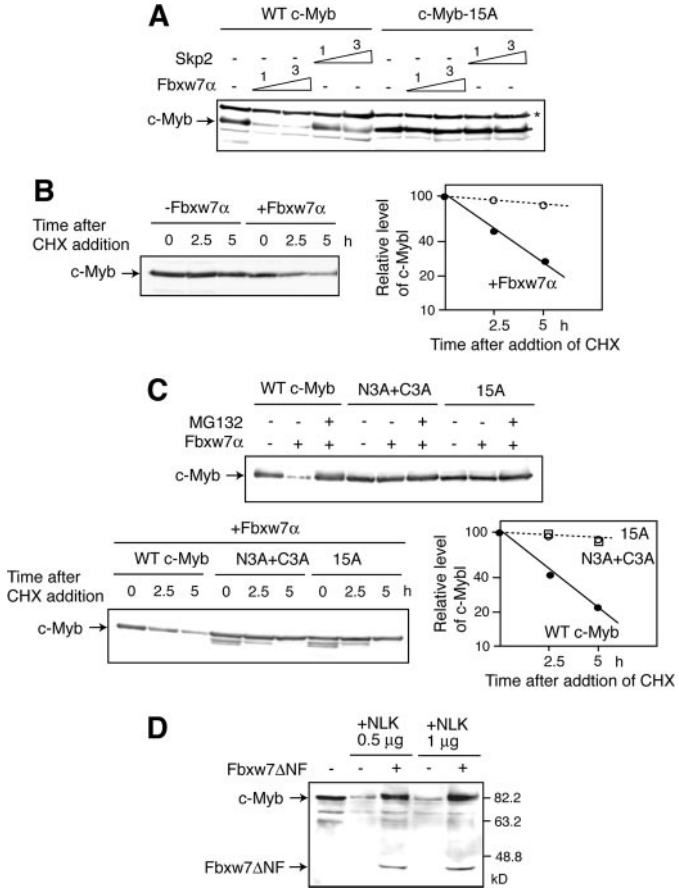

FIGURE 3. **Fbxw7α induces degradation of c-Myb but not c-Myb-15A in CV-1 cells.** *A*, decrease in the c-Myb level induced by Fbxw7α. CV-1 cells were transfected with a mixture of 4 μg of the wild-type (*WT*) or c-Myb-15A expression plasmid, the indicated amounts of the Fbxw7α or Skp2 expression plasmid, and 0.4 μg of pact-β-gal. Lysates from the transfected cells were used for Western blotting with anti-c-Myb (αCT5). *B*, Fbxw7α induces degradation of c-Myb. CV-1 cells were transfected with a mixture of 7.5 μg of the FLAG-c-Myb expression plasmid, 2 μg of the Fbxw7α expression plasmid, and 0.4 μg of pact-β-gal. After addition of cycloheximide (*CHX*) to the cell cultures, lysates were prepared at the indicated times and used for Western blotting with anti-FLAG M2. The density of c-Myb band was quantified and plotted on the *right*. *C*, Fbxw7α induces degradation of wild-type c-Myb, but not the c-Myb phosphorylation site mutants. CV-1 cells were transfected with a mixture of 6 μg of the indicated c-Myb expression plasmids, 2 μg of the Fbxw7α expression plasmid, and 0.4 μg of pact-β-gal. *Upper*, in some cases, transfected cells were treated with MG132 (5 μM) for 7 h before lysate preparation. *Lower*, after addition of cycloheximide (Calbiochem, 50 μg/ml) to the cell culture, lysates were prepared at the indicated times. Western blotting was performed with anti-FLAG M2. The density of c-Myb band was quantified and plotted on the *right*. *D*, the F-box deletion mutant of Fbxw7α blocks the NLK-induced degradation of c-Myb. CV-1 cells were transfected with a mixture of 6 μg of the FLAG-c-Myb expression plasmid, 8 μg of the plasmid expressing the FLAG-tagged Fbxw7 mutant lacking whereas N-terminal F-box (Fbxw7ΔNF), the indicated amounts of the NLK expression plasmid, and 0.5 μg of pact-β-gal. Western blotting with anti-FLAG M2 was performed.

reduce the levels of Fbxw7 (34). We also confirmed this by analyzing their effect on the levels of exogenously expressed Fbxw7α, although the efficiency differed slightly among siRNAs (Supplementary Fig. S1*B*). Reduction of c-Myb by coexpression of six components of the Wnt/NLK pathway was partly but significantly abrogated by these siRNAs (Supplementary Fig. S1*C*), suggesting that endogenous Fbxw7 is responsible for the Wnt-1-induced degradation of c-Myb.

To further confirm that Fbxw7 is responsible for the Wnt-induced degradation of c-Myb, we then used the mouse myeloid leukemic M1 cell line, which expresses endogenous c-Myb. M1 cells were infected with the Fz1-encoding retrovirus, and among 20 clones isolated, clone 3 expressed a significantly

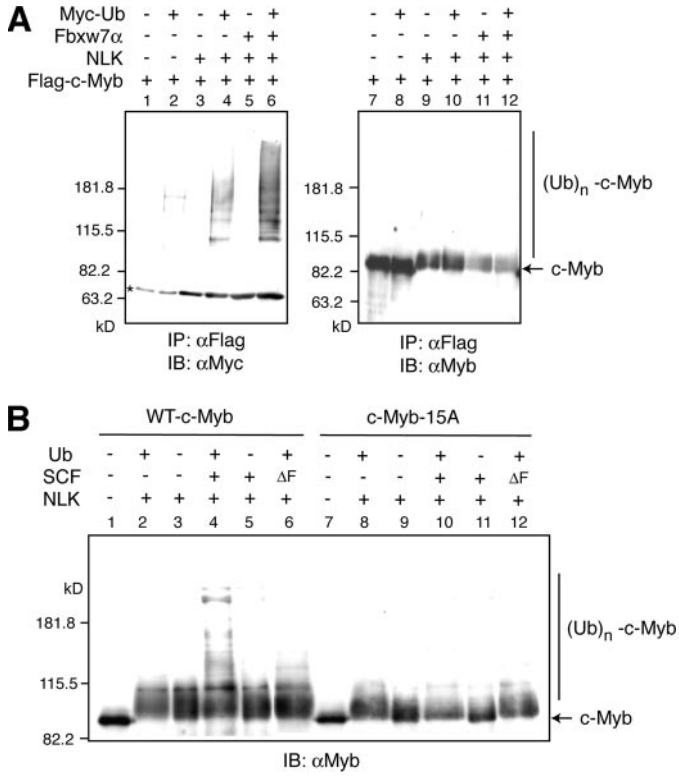

FIGURE 4. **Fbxw7α induces ubiquitination of c-Myb in the presence of NLK.** *A*, *in vivo* ubiquitination. 293T cells were transfected with a mixture of plasmids to express the proteins indicated above each lane and pact-β-gal as described under "Experimental Procedures" and treated with MG132 (15 μM) for 4 h before lysate preparation. Lysates were immunoprecipitated (*IP*) with anti-FLAG M2, and the immunocomplexes were subjected to Western blotting (*IB*) with anti-Myc to detect Myc-tagged ubiquitin (*Ub*)-conjugated c-Myb (*left*), or with anti-Myb antibody (mAb1-1) (*right*). *B*, *in vitro* ubiquitination. Myb, NLK, and the SCF complex were prepared as described under "Experimental Procedures." Myb was phosphorylated by NLK, incubated with the indicated proteins in ubiquitination buffer, and subjected to Western blotting with anti-c-Myb (αCT5). *WT*, wild-type.

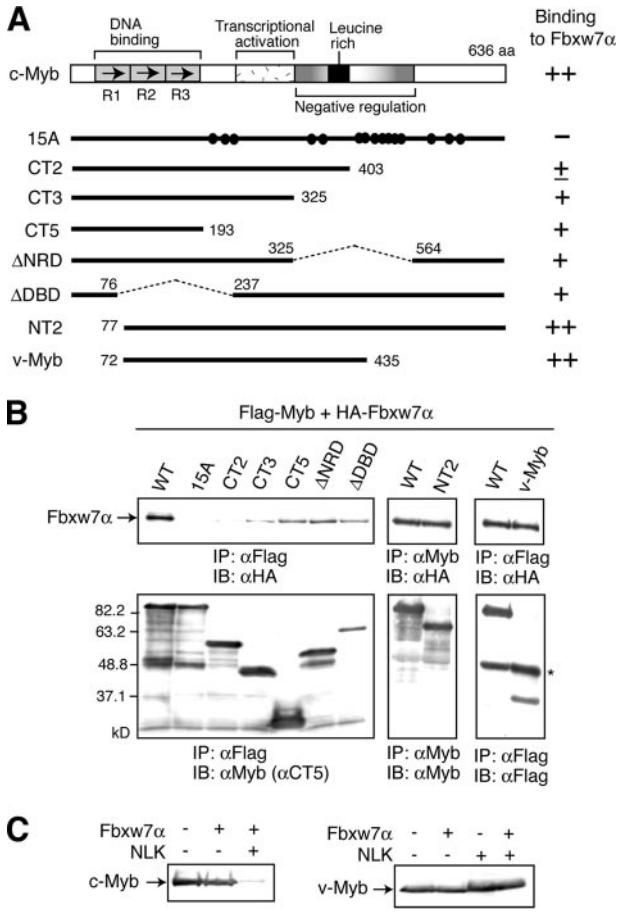

FIGURE 5. **Interaction between c-Myb and Fbxw7.** *A* and *B*, co-immunoprecipitation of Fbxw7α and various c-Myb mutants. The c-Myb mutants used and v-Myb are shown (*A*). 293T cells were transfected with a mixture of 3 μg of plasmids to express various forms of FLAG-c-Myb, 3 μg of the HA-Fbxw7α expression plasmid, and 0.04 μg of pact-β-gal. Lysates were immunoprecipitated (*IP*) with anti-FLAG M2 or anti-c-Myb (mAb1-1), and subjected to Western blotting with anti-HA 12CA5 (*upper*), anti-Myb (αCT5) (*lower left*), anti-Myb (mAb1-1) (*lower middle*), or anti-FLAG M2 (*lower right*). The *asterisk* indicates a nonspecifically recognized band. *aa*, amino acids; *WT*, wild-type. *C*, Fbxw7α and NLK cannot induce the v-Myb degradation. 293T cells were transfected with a mixture of 1 μg of the FLAG-v-Myb expression plasmid, 0.3 μg of the NLK expression plasmid, 0.3 μg of the Fbxw7α expression plasmid, and 0.02 μg of pact-β-gal. Lysates were used for Western blotting with anti-FLAG M2.

lower level of c-Myb than the control cell line (Fig. 2*D*, *left*). Clone 3 exhibited a slightly slower growth rate than the control cell line (Fig. 2*D*, *middle*). Consistent with this, the degree of BrdU incorporation of clone 3, which reflects the rate of DNA synthesis, was lower than that of control cells (Fig. 2*D*, *right*). Thus, Wnt signal, which was activated by overexpression of Fz, induced down-regulation of endogenous c-Myb in M1 cells and suppressed their growth rate. We then treated clone 3 and the control clone using Fbxw7 siRNA#2. The sequence corresponding to this siRNA is completely conserved between human and mouse Fbxw7. This Fbxw7 siRNA rescued the Fz1-induced down-regulation of c-Myb in clone 3 (Fig. 2*E*). Furthermore, the Fz1-induced decrease in BrdU incorporation was also partly rescued (Fig. 2*F*). These results suggest that Fbxw7 is responsible for Wnt-induced degradation of c-Myb, which affects cell proliferation.

Fbxw7α Is Responsible for the NLK-induced Degradation of c-Myb in CV-1 Cells—Coexpression of Fbxw7α and c-Myb reduced the c-Myb level in CV-1 cells, but decreases in c-Myb level were not observed with the c-Myb-15A mutant (Fig. 3*A*). In the control experiment, coexpression of Skp2 did not induce a decrease in the c-Myb level. Measurement of the c-Myb level at various times after cycloheximide treatment demonstrated that Fbxw7α reduced c-Myb stability (Fig. 3*B*). A decrease in the c-Myb level induced by coexpression of Fbxw7α was abrogated by MG132 treatment of transfected cells (Fig. 3*C*), further supporting the hypothesis that Fbxw7α induces c-Myb degradation. Fbxw7α did not induce degradation of the two c-Myb mutants, in which 15 (15A) or six (N3A+C3A) putative NLK phosphorylation sites in the c-Myb molecule were mutated to Ala (Fig. 3*C*). A previous study showed that these mutants were not degraded by coexpression with NLK (17). The lower molecular weight bands detected in the two mutants could be generated by inhibition of the ribosomal translocation or induction of specific proteases by cycloheximide. These bands disappeared rapidly, suggesting that they were degraded by a mechanism that is different from that of the proteasome- and NLK-dependent degradation. Thus, c-Myb degradation by Fbxw7α in CV-1 cells requires the NLK phosphorylation sites. When an Fbxw7α mutant that lacks the N-terminal F-box (Fbxw7ΔNF)

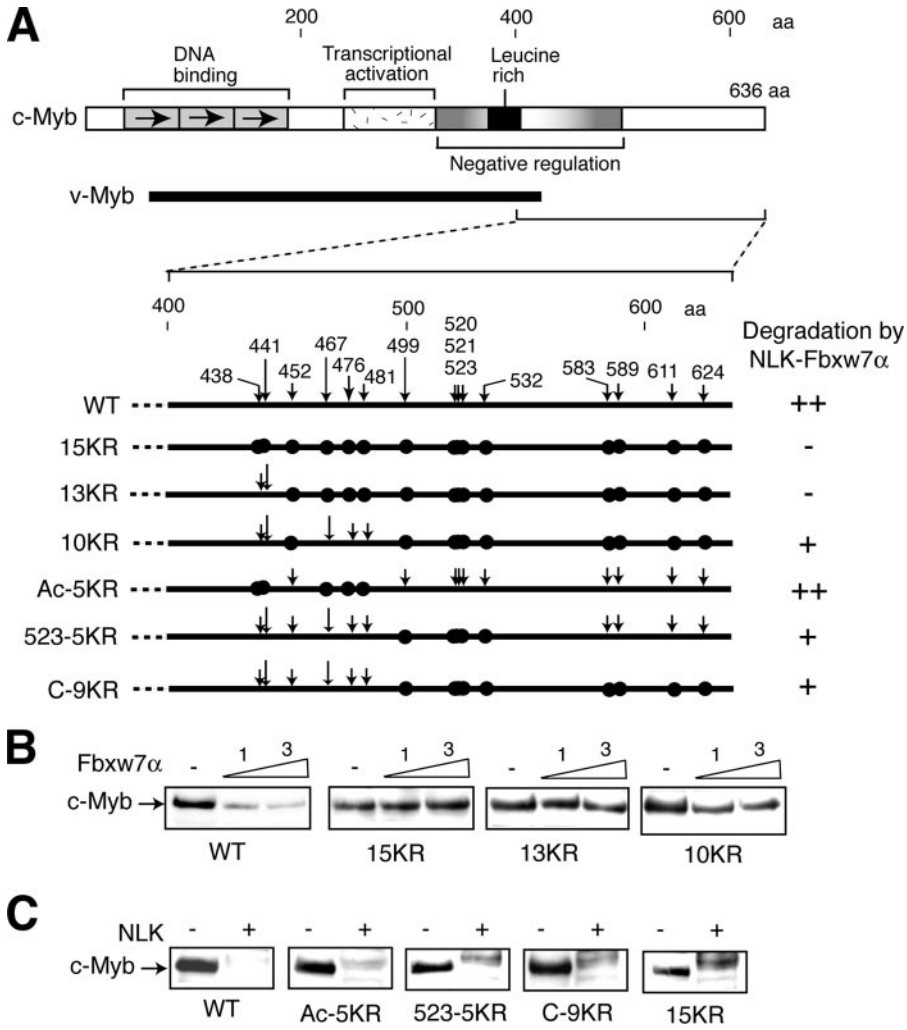

FIGURE 6. **Putative ubiquitination sites in the C-terminal region of c-Myb are critical for Fbxw7α/NLK-induced degradation.** *A*, Lys residue mutants in the C-terminal region of c-Myb. A series of c-Myb mutants in which multiple Lys residues were mutated to Arg are shown. The degree of degradation of each mutant by Fbxw7α and NLK is summarized on the right. *B* and *C*, mutation of some Lys residues in the C-terminal region of c-Myb blocks the Fbxw7α- or NLK-induced degradation. CV-1 cells were transfected with a mixture of 4 μg of the FLAG-c-Myb expression plasmid, 1 or 3 μg of the Fbxw7α expression plasmid, and 0.4 μg of pact-β-gal (*B*) or 6 μg of the FLAG-c-Myb expression plasmid, 2 μg of the NLK expression plasmid, and 0.4 μg of pact-β-gal (*C*). Lysates from the transfected cells were used for Western blotting with anti-FLAG M2. *aa*, amino acids; *WT*, wild-type.

was coexpressed with c-Myb, NLK-induced degradation of c-Myb in CV-1 cells was almost completely abrogated (Fig. 3*D*). These results suggest that Fbxw7α is required for the NLK-induced degradation of c-Myb in CV-1 cells.

Fbxw7α Induces Ubiquitination of c-Myb—We then asked whether Fbxw7α can induce ubiquitination of c-Myb. Coexpression of NLK with c-Myb induced ubiquitination of c-Myb in CV-1 cells, whereas coexpression of both NLK and Fbxw7α further enhanced c-Myb ubiquitination (Fig. 4*A*). The ubiquitination signals in cells expressing both NLK and Fbxw7α (*lane 6*) were apparently stronger than in cells expressing only NLK (*lane 4*), despite the c-Myb signal being weaker in the former (compare *lanes 11* and *9*). This result suggests that coexpression of NLK and Fbxw7α strongly enhanced the degree of ubiquitination per c-Myb molecule. In *in vitro* ubiquitination assays, the SCF complex, which is composed of three components (Skp1, Cul1, and Fbxw7α), and Rbx1 enhanced the ubiquitination of c-Myb in the presence of NLK (Fig. 4*B*). Furthermore, the SCF complex and NLK failed to induce the ubiquitination of c-Myb-15A. These results indicate that the SCF complex including Fbxw7α acts as an E3 ubiquitin ligase of c-Myb in the presence of NLK.

Fbxw7α Interacts with the DNA-binding Domain and the C-terminal Half of c-Myb—To investigate the interaction between c-Myb and Fbxw7α more precisely, we used a series of c-Myb mutants in co-immunoprecipitation assays. When c-Myb and Fbxw7α were coexpressed in 293T cells in the absence of NLK, wild-type c-Myb was efficiently co-precipitated with Fbxw7α (Fig. 5, *A* and *B*). In contrast, the c-Myb-15A mutant did not co-precipitate with Fbxw7α as shown in Fig. 1*A*. The results of the co-immunoprecipitation assays using various c-Myb mutants indicated that the removal of the C-terminal 233 or 311 amino acids of c-Myb (CT2 and CT3) significantly reduces the interaction with Fbxw7α. Furthermore, a deletion of two repeats in the DNA-binding domain also significantly decreases Fbxw7α binding. These results indicate that Fbxw7α interacts with c-Myb via the DNA-binding domain and the C-terminal half of c-Myb.

Fbxw7α Cannot Induce the Degradation of v-Myb Lacking the NLK Phosphorylation Sites and the Putative Ubiquitination Sites—Interestingly, v-Myb encoded by avian myeloblastosis virus efficiently bound to Fbxw7α with an affinity similar to that of wild-type c-Myb (Fig. 5, *A* and *B*). However, coexpression of NLK and Fbxw7α did not induce the degradation of v-Myb (Fig. 5*C*). This result may reflect the lack of several critical NLK phosphorylation sites in the C-terminal region that are absent in v-Myb as we previously demonstrated (17). However, NLK can phosphorylate multiple sites in v-Myb, and Fbxw7α can still bind to v-Myb, which led us to hypothesize that the C-terminal region missing in v-Myb may contain not only the NLK phosphorylation sites but also the ubiquitination sites critical for proteasome-dependent degradation. When 15 or 13 Lys residues in the C-terminal region of c-Myb, which is deleted in v-Myb, were replaced by Arg (15KR and 13KR), the Fbxw7α- or NLK-induced degradation of c-Myb was almost completely abrogated (Fig. 6, *A* and *B*). We have previously reported that Lys-438 and Lys-441 are acetylated by CBP and contribute to

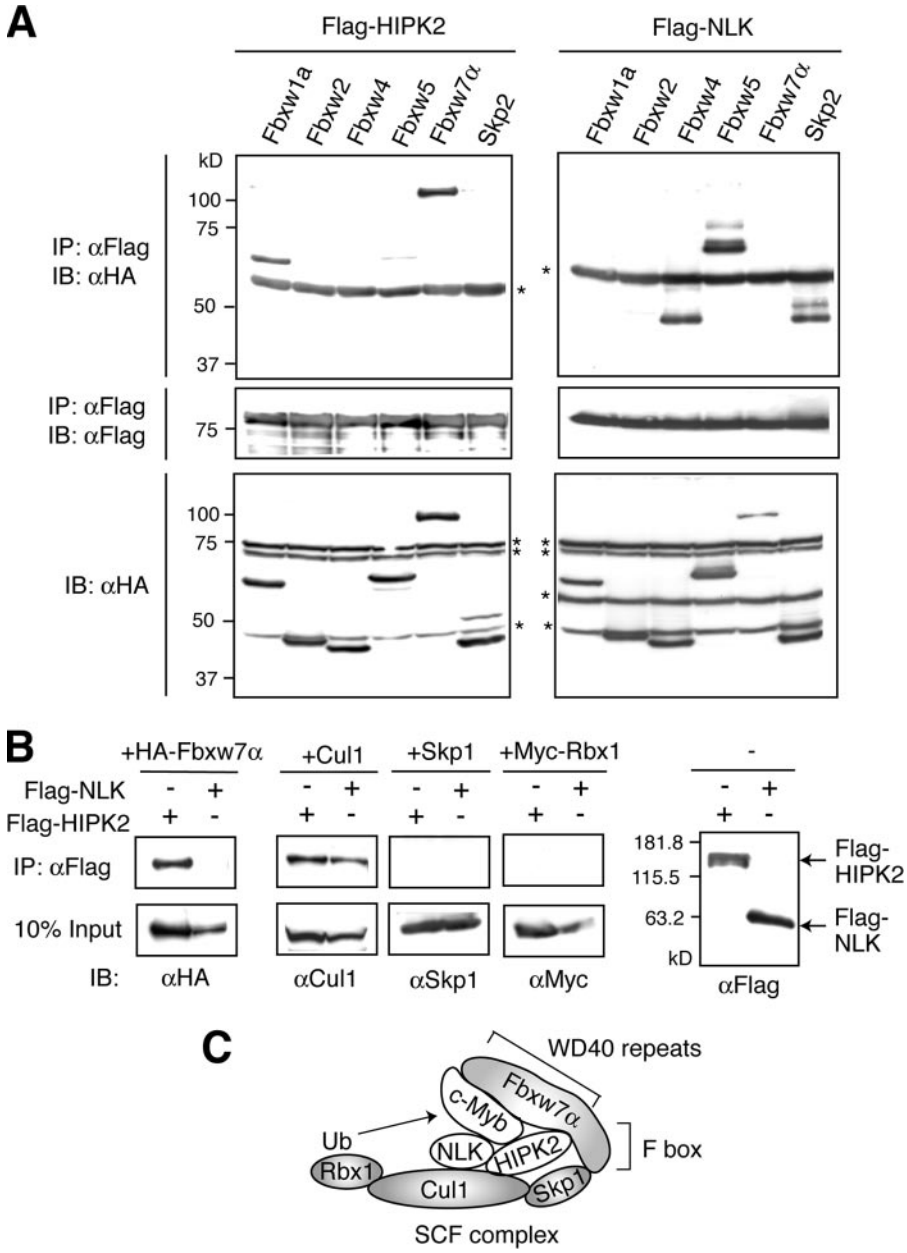

FIGURE 7. **NLK and HIPK2 interact with components of the SCF complex.** *A*, interaction of HIPK2 and NLK with various Fbxw proteins. 293T cells were transfected with a mixture of 4 μg of the FLAG-HIPK2 or 2 μg of the FLAG-NLK expression plasmid and 4 μg of plasmids to express HA-Fbxw proteins or HA-Skp2. Lysates from the transfected cells were immunoprecipitated (*IP*) with anti-FLAG M2 and subjected to Western blotting with anti-HA 12CA5 (*top panels*) or anti-FLAG M2 (*middle panels*). Lysates were also used for direct Western blotting (*IB*) with anti-HA 12CA5 (*bottom panels*). *B*, interaction of HIPK2 and NLK with components of the SCF complex. 293T cells were transfected with a mixture of 4 μg of the FLAG-HIPK2 or FLAG-NLK expression plasmids and 4 μg of the expression plasmids for the indicated SCF complex components. Cell lysates were immunoprecipitated with anti-FLAG M2 and subjected to Western blotting with the indicated antibodies. *C*, the interactions among c-Myb, HIPK2, NLK , and each component of the SCF complex are shown schematically. *Ub*, ubiquitin.

c-Myb-dependent transactivation (39). Mutation of five Lys residues, including these two residues, to Arg (Ac-5KR) did not affect NLK-induced degradation (Fig. 6, *A* and *C*). Replacement of another five Lys at positions 499, 520, 521, 523, and 532 by Arg (523-5KR) reduced NLK-induced degradation. Additional mutagenesis in the C-terminal region (C-9KR) did not further enhance the abrogation of NLK-induced degradation. These results suggest that specific Lys residues, such as those in the region between amino acids 499 and 532, and not the total number of Lys residues, are critical as ubiquitin acceptor sites for proteasome-dependent degradation. Thus, v-Myb may lack the critical ubiquitination sites for NLK/Fbxw7α-induced degradation.

NLK and HIPK2 Bind to Components of the SCF Complex—Two kinases HIPK2 and NLK directly bind to the DNA-binding domain in the N-terminal region of c-Myb (15). We therefore asked whether these two kinases also interact with Fbxw7α and other F-box proteins. In co-immunoprecipitation assays, Fbxw7α interacted with HIPK2, but not with NLK (Fig. 7*A*). Interestingly, HIPK2 bound to Fbxw1α, while NLK bound to Fbxw4, Fbxw5, and Skp2, suggesting that both these kinases may be involved in regulation of protein degradation mediated by these E3 ubiquitin ligases.

Using co-immunoprecipitation assays, we also examined whether NLK and HIPK2 bound to other components of the SCF complex. Cul1 co-precipitated with HIPK2 and NLK, but neither Skp1 nor Rbx1 co-precipitated (Fig. 5*B*). Thus, HIPK2 interacts with Fbxw7α and Cul1, and NLK also interacts with Cul1, suggesting that NLK and HIPK2 enhance the c-Myb/Fbxw7α interaction by their interactions with components of the SCF complex (Fig. 7*C*).

DISCUSSION

In this study, we have demonstrated that Fbxw7α directly binds to c-Myb via its C-terminal WD40 domain and induces the ubiquitination of c-Myb in the presence of NLK *in vivo* and *in vitro*. Thus, Fbxw7 targets c-Myb for degradation in response to Wnt-1 signals.

Although NLK overexpression led to c-Myb degradation in CV-1 cells (15), coexpression of NLK with c-Myb did not induce the degradation of c-Myb in 293T cells (Fig. 1*B*), suggesting that some components are expressed only at low levels in 293T cells. In fact, coexpression of NLK and Fbxw7α with c-Myb induced the efficient degradation of c-Myb in 293T cells. In the 293T cell co-immunoprecipitation assays, wild-type c-Myb was efficiently co-precipitated with Fbxw7α, whereas the c-Myb-15A mutant, in which all 15 putative NLK Ser/Thr phosphorylation sites were

replaced by Ala, was not (Fig. 1*A*). This result suggests that phosphorylation of c-Myb at some sites by a low level of endogenous NLK is required for interaction with Fbxw7α, although low level c-Myb phosphorylation is not sufficient to induce its degradation. Among the 15 putative NLK phosphorylation sites in c-Myb, the most critical sites are three sites in the C-terminal region (Thr-572, Ser-556, and Ser-528) because replacement of these sites by Ala considerably inhibited both Wnt-1- and NLK-induced degradation (17). The sequence surrounding Thr-572 (LMTPVSED) in c-Myb partially resembles to the phosphodegron sequence of cyclin E (^{378}LLTPPQSG^{385}), which is phosphorylated by GSK3β (40) or autophosphorylated by cyclin E/Cdk2 (41, 42) and is recognized by Fbxw7 in a phosphorylation-dependent manner (43), but the homology is very limited. In the case of c-Myb, therefore, Thr-572 and other multiple NLK phosphorylation sites may cooperatively contribute to the phosphorylation-dependent interaction with Fbxw7α, similar to recognition of the Cdk inhibitor Sic1 by the Cdc4 ubiquitin ligase (27, 44, 45). Recognition of Sic1 by Cdc4 requires phosphorylation of Sic1 on at least six of its nine Cdc4 phosphodegron sites.

In co-immunoprecipitation assays, not only the C-terminal region of c-Myb, which contains the critical NLK phosphorylation sites, but also the DNA-binding domain in the N-terminal region were responsible for the interaction with Fbxw7α (Fig. 5, *A* and *B*). The DNA-binding domain of c-Myb may support the c-Myb/Fbxw7α interaction via binding to HIPK2 because HIPK2 itself binds to the c-Myb DNA-binding domain (15) and also to Fbxw7α (Fig. 7, *B* and *C*). Thus, HIPK2 may support the c-Myb/Fbxw7α interaction. NLK may also enhance the affinity between c-Myb and the SCF complex via the c-Myb/Cul-1 interaction (Fig, 7, *B* and *C*). Direct interaction of NLK and HIPK2 with the components of the SCF complex suggests that these kinases may participate in degradation of other proteins mediated by the SCF complex.

Mutations of human Fbxw7 have been detected in several human tumor types (24, 33), which is consistent with the notion that Fbxw7 acts as a negative regulator of several oncoproteins. The increase in c-Myb levels may also contribute to human tumors caused by Fbxw7 mutations. Recently, a duplication of the c-*myb* gene and overexpression of c-Myb have been found in 8.4% of individuals with T cell acute lymphoblastic leukemia (T-ALL) and several T-ALL cell lines (46). Because knockdown of c-Myb expression induces differentiation of these T cell leukemias, over-expression of c-Myb may contribute to oncogenic processes. Furthermore, Fbxw7 mutations have recently been identified in a large fraction of human T-ALL lines (47). These results suggest that overexpression of c-Myb in T-ALL patients harboring Fbxw7 mutations may contribute to malignancies. Therefore, c-Myb could be a therapeutic target in human T-ALL.

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