Degradation of Tob1 Mediated by SCF^{Skp2}-Dependent Ubiquitination

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Abstract

Tob1, a member of the Tob/BTG family, is involved in the control of G_1-S progression by suppressing cyclin D1 expression and acts as a tumor suppressor gene. Tob1 was reported to have a quick turnover through the ubiquitin-proteasome pathway, but proteins involved in this process are still unknown. We showed that Skp2, a substrate-targeting subunit of the SCF (Skp1/Cul1/F-box protein) ubiquitin ligase complex, was involved in ubiquitin-dependent degradation of Tob1. Skp2 interacted with Tob1 and facilitated ubiquitination of Tob1 in intact cells as well as *in vitro*. Skp2 mutants without the F-box or leucine rich repeat were not able to bind to Tob1 and did not enhance ubiquitination of Tob1. Tob1 was stabilized in both Skp2$^{-/-}$ mouse fibroblasts and Skp2 knockdown HeLa cells. Moreover, cyclin D1 expression was suppressed in Skp2 knockdown HeLa cells. These data suggest that Tob1 is a novel target for degradation by the SCF-Skp2 ubiquitin ligase. (Cancer Res 2006; 66(17): 8477-83)

Introduction

Tob1, a member of the Tob/BTG family, is an antiproliferative protein. Exogenous overexpression of Tob1 protein induces G_0-G_1 arrest (1, 2). The antiproliferative function of Tob1 is attributed to its role as a transcriptional corepressor and suppressor of cyclin D1 promoter activity (2). It has been reported that development of tumors in lungs, liver, and lymph nodes was observed in mice lacking *Tob1* (3). Furthermore, *TOB1* expression is often decreased in human cancers (3). These results suggest that *TOB1* acts as a tumor suppressor gene. Recently, Tob1 protein has been reported to have a quick turnover through the ubiquitin-proteasome pathway (4), but the ubiquitin ligase involved in this proteolytic process remains unknown.

The ubiquitin-proteasome system controls the abundance of several cellular proteins (5), particularly short-lived and tightly regulated proteins, such as oncoproteins, tumor suppressors, transcription factors, and cell cycle regulators (6). Polyubiquitinated proteins conjugated as a result of collaboration between a ubiquitin-activating enzyme (E1), a ubiquitin-conjugation enzyme (E2), and a ubiquitin ligase (E3) are selectively recognized and hydrolyzed by the 26S proteasome (5). Specificity of target protein selection is defined by ubiquitin ligases (7, 8).

Cell cycle transitions are driven by waves of ubiquitin-dependent degradations of key cell cycle regulators, such as SCF (Skp1/Cullin/F-box protein) complexes and anaphase-promoting complexes, which represent two major classes of ubiquitin ligases whose activities are thought to primarily regulate G_1-S and metaphase/anaphase cell cycle transitions, respectively (9, 10). The SCF complexes consist of one variable component F-box protein and three invariable components, including Skp1, Cul1, and Rbx1/Roc1. Skp2, an F-box protein containing a leucine rich repeat (LRR), promotes degradation of the cyclin-dependent kinase (CDK) inhibitor p27 (11–13). Skp2 knockout (KO) mice show remarkable accumulation of p27. Moreover, several tumor suppressor proteins, including p21, p57, p130, and FOXO1 are targeted by Skp2 for degradation (14–17). Skp2 is an oncoprotein that often shows increased expression in human cancers and correlates with prognosis (18, 19). Because Tob1 is a negative regulator of G_1-S progression and is suggested to be down-regulated by the ubiquitin-proteasome system, we investigated whether Skp2 was involved in regulation of Tob1. Here, we provide evidence that Skp2 interacts with Tob1 and promotes ubiquitin-dependent degradation of Tob1.

Materials and Methods

Cell culture and synchronization. HEK293 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum. For arrest in the M phase, HeLa cells were incubated with 100 ng/mL nocodazol for 16 hours (Sigma, St. Louis, MO), washed with PBS, and subsequently incubated in nocodazol-free medium for indicated periods. DNA content of HeLa cells synchronized by nocodazol treatment was examined by flow cytometry (Beckman Coulter, Fullerton, CA), as described previously (20). Mouse embryonic fibroblasts (MEF) were prepared from Skp2$^{-/-}$ mice as described previously (13).

Antibodies. The antibodies used in this study were as follows: anti-Myc antibody 9B11 (Cell Signaling, Danvers, MA), anti-Myc antibody 9E10 (Roche, Basel, Switzerland), anti-Flag antibody M2 (Sigma), anti-HA antibody 12CA5 (Roche), anti-Skp2 monoclonal antibody 2B12 (Zymed, San Francisco, CA), anti-Skp2 polyclonal antibody H435 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Tob1 monoclonal antibody 4B1 (IBL, Takasaki, Japan), anti-Tob polyclonal antibody (2), anti-p27 antibody (BD Transduction Laboratories, San Jose, CA), anti-cyclin D1 antibody 553 (MBL, Nagoya, Japan), anti-cyclin A antibody H431 (Santa Cruz Biotechnology), anti-p130 antibody (TDL), anti-ubiquitin chain antibody FK2 (Nippon Bio-Test Laboratories, Tokyo, Japan), and anti-β-actin antibody AC-15 (Sigma).

Transfection, immunoprecipitation, Western blotting, and *in vivo* ubiquitination assay. Expression plasmids of full-length (FL) Tob1 and its mutant (pME-Tob1, pME-Tob-Myc, and pME-Tob-D4) have been described (1, 2). The expression vector of the COOH-terminal truncated Tob1 (1-165), pME-Tob-ΔC, and NH_2-terminal truncated Tob1 (166-end), pME-Tob-ΔN-Flag, were generated by overlap extension PCR. Expression vectors of Flag-Skp2, Flag-Skp2-NF, Flag-Skp2-ΔLRR, Flag-Skp2-ΔNF, and Myc-Skp2 have been described (13, 15). Vectors were transiently transfected into HEK293

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-1603

cells by the calcium phosphate method. After 42 hours, cells were either left untreated or treated with 20 μmol/L MG132 (Peptide Institute, Minoh, Japan) or DMSO for 6 hours and subsequently lysed in lysis buffer consisting of 50 mmol/L Tris-HCl (pH 8.0), 300 mmol/L NaCl, 0.3% Triton X-100, and protease inhibitors. For immunoprecipitation, cell lysates were incubated with 2 μg antibodies and protein G-Sepharose 4FF (GE Healthcare Bioscience, Piscataway, NJ) at 4°C for 1 hour, respectively. Immunocomplexes were washed four times with lysis buffer. For double immunoprecipitation, the first immunocomplexes were denatured by treatment with SDS sample buffer at 100°C for 10 minutes. Then, ubiquitinated Tob1 was reimmunoprecipitated. Cell lysates or immunoprecipitated samples were separated by SDS-PAGE followed by immunoblotting. Proteins were visualized using an enhanced chemiluminescence system (Perkin-Elmer, Wellesley, MA).

***In vitro* ubiquitination assay.** SCF-Skp2 components, such as Myc-Cul1, HA-Skp1, HA-Roc1/Rbx1, Myc-Skp2 (FL or ΔLRR), and HA-Nedd8 were transiently transfected into HEK293 cells by the calcium phosphate method. After 48 hours, cells were harvested and lysed in lysis buffer. SCF-Skp2 complex immunoprecipitated with anti-Myc antibody-conjugated agarose (Santa Cruz Biotechnology) was used as E3 ligase. Tob-Flag proteins overexpressed in HEK293 cells were immunoprecipitated with anti-Flag antibody cross-linked beads (M2-beads, Sigma) and subsequently eluted by Flag-peptide (Sigma). Eluted Tob-Flag proteins were used as the substrates. The SCF-Skp2 complex and Tob-Flag proteins were incubated with E1 (Boston Biochem, Cambridge, MA), E2 (UbcH3 and UbcH5b; Boston Biochem), and ubiquitin (Sigma) for 30 minutes at 30°C in reaction buffer consisting of 0.05 mol/L Tris-HCl (pH 8.3), 5 mmol/L $MgCl_2$, 2 mmol/L DTT, 10 mmol/L phosphocreatine, 0.2 units/mL phosphocreatine kinase, 5 mmol/L ATP, 0.05 μg/μL ubiquitin aldehyde, 0.25 mmol/L MG132, and protease inhibitors. After denature, incubated samples were analyzed by immunoblotting with anti-Tob antibody or reimmunoprecipitated M2-beads following immunoblotting with horseradish peroxidase–conjugated anti-ubiquitin chain antibody or anti-Tob1 antibody.

RNA interference. HeLa cells grown at 30% to 50% confluence were incubated in DMEM containing 10% fetal bovine serum. A mixture of small interfering RNA (siRNA) for Skp2 or nonspecific control siRNA was transfected by Oligofectamine (Invitrogen, Carlsbad, CA), according to protocols suggested by the manufacturer. Nucleotide sequences of siRNAs for Skp2 were 5′-(CUGCGGGUUUCGGAUCCCATT)d(TT)-3′ (reverse) and 5′-(GCAUGUACAGGUGGCUGUU)d(TT)-3′ (reverse).

***In vivo* degradation assay.** HeLa cells were transfected with siRNA oligos or MEFs grown at 50% to 70% confluence and treated with cycloheximide (12.5 mg/mL; Sigma) for indicated times. Cell lysates were analyzed by Western blotting. Band intensities were quantitated using the image analysis software Image Gauge 4.21 (Fujifilm, Tokyo, Japan) and each signal intensity was normalized against individual levels of β-actin.

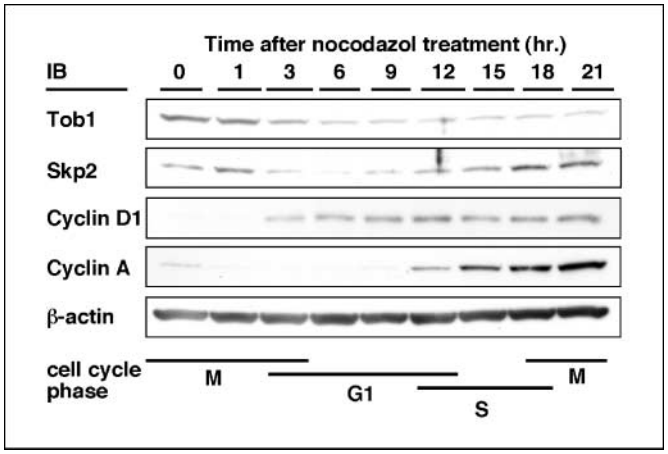

Figure 1. Expression of Tob1 during the cell cycle. HeLa cells were synchronized at the prometaphase by nocodazol (100 μg/mL) treatment for 16 hours. They were then harvested at indicated times after release from nocodazol treatment. Cell extracts were immunoblotted with Tob1, Skp2, cyclin D1, cyclin A, and β-actin antibodies. *IB,* immunoblot.

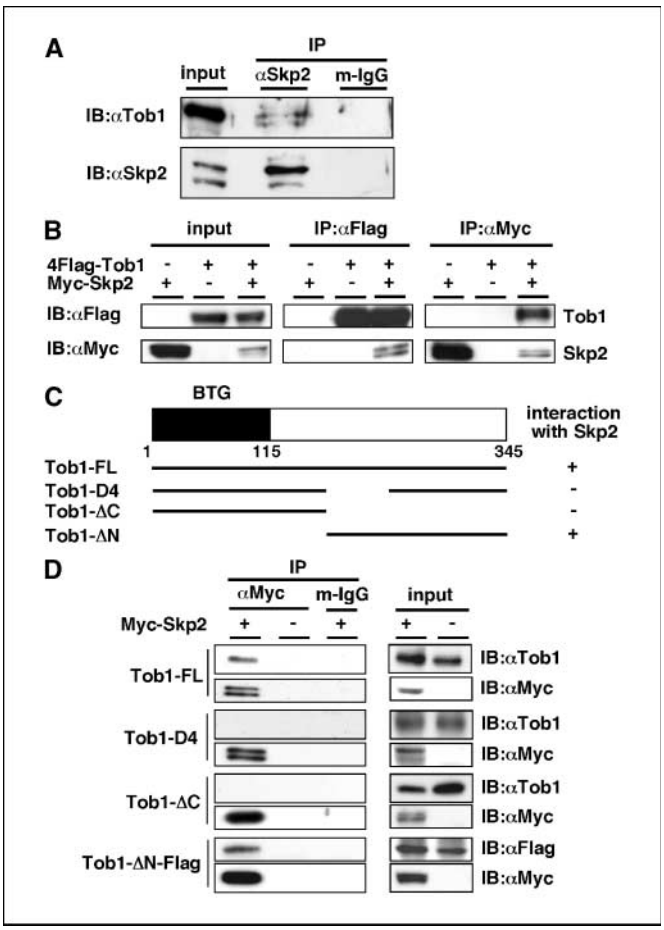

Figure 2. Interaction between Skp2 and Tob1. *A,* lysates prepared from HeLa cells treated with MG132 were immunoprecipitated (*IP*) with anti-Skp2 (α*Skp2*) antibody or mouse control IgG (*m-IgG*) following Western blotting with anti-Tob1 (*IB:*α*Tob1*) or Skp2 (*IB:*α*Skp2*) antibody. Endogenous Tob1 was coprecipitated with endogenous Skp2. *B,* Myc-tagged Skp2 and 4× Flag-tagged Tob1 were cotransfected into HEK293 cells, and cell lysates were subjected to immunoprecipitation following immunoblotting using appropriate antibodies. *C,* schematic representation of Flag-tagged Tob1 deletion mutants. *D,* FL or deletion mutants of Flag-tagged Tob1 were cotransfected with or without Myc-Skp2-FL into HEK293 cells. Lysates were subjected to immunoprecipitation with anti-Myc antibody (α*Myc*) or mouse control IgG following immunoblotting with anti-Tob1, anti-Flag (*IB:*α*Flag*), or anti-Myc (*IB:*α*Myc*) antibodies. Results of interactions between Tob1 mutants and Skp2 are summarized in (*C*).

Quantitative reverse transcription-PCR analysis. Total RNA was isolated from cultured cells using an Isogen kit (Wako, Osaka, Japan), and subjected to reverse transcription with random hexanucleotide primers and SuperScript Reverse Transcriptase II (Invitrogen). The resulting cDNA was subjected to real-time PCR using the Rotor-Gene 3000 System (Corbett Research, Mortlake, Australia) and a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). Primer sequences were 5′-GCAAAGGGAGTGACAAAGAC-3′ and 5′-GAGGCACAGACAGGAAAAGA-3′ for Skp2, 5′-GCTGCTGTAAGCCCTACCTT-3′ and 5′-CAAGCCCATACAGAGAGTGC-3′ for Tob1, and 5′-GCTCCTGTGCTGCGAAGT-3′ and 5′-TGTTCCTCtCAGACCTCCAG-3′ for cyclin D1. The abundance of transcripts of interest was normalized against that of 18S rRNA as an internal standard.

Results

Tob1 expression is inversely correlated with Skp2. It has been shown recently that Tob1 protein is rapidly degraded by the

ubiquitin-proteasome system (4). However, the ubiquitin ligase for Tob1 remains unidentified. We quantified the expression of Tob1 protein during the cell cycle. Following M phase arrest after nocodazol treatment, HeLa cells were released from cell cycle arrest and could proceed into the G_1 phase. Synchronized HeLa cells began to exit from mitosis within 2 hours after their release from arrest and entered the G_1 phase within 6 hours. Subsequently, the S phase began from about 12 hours after release from the nocodazol arrest phase (Supplementary Fig. S1). As shown in Fig. 1, Tob1 rapidly decreased from mid-G_1 phase to S phase in synchronized HeLa cells. Following a reduction in the Tob1 level, the expression of cyclin D1 that is negatively regulated by Tob1 was induced. Because SCF-Skp2 ubiquitin ligase plays important roles in G_1-S progression by targeting various tumor suppressor proteins for degradation, we investigated whether Skp2 correlated with Tob1 level. mRNA (data not shown) and protein (Fig. 1) level of Skp2 increased from G_1 phase. The expression pattern of Skp2 was inversely correlated with Tob1 protein during the HeLa cell cycle, thus we sought to determine whether Skp2 was involved in the degradation of Tob1.

Interaction of Tob1 with Skp2. To investigate whether Skp2 could bind Tob1, we did an immunoprecipitation study following the immunoblotting assay. Lysates prepared from HeLa cells treated with MG132 were immunoprecipitated with anti-Skp2 or mouse control IgG following Western blotting with anti-Tob1. As shown in Fig. 2*A*, endogenous Tob1 was coprecipitated with endogenous Skp2. Next, we confirmed the interaction between Tob1 and Skp2 *in vivo*-binding assay. Myc-tagged Skp2 and 4× Flag-tagged Tob1 expression vectors were transfected either alone or together into HEK293 cells, and cell lysates were immunoprecipitated and immunoblotted with an anti-Myc antibody or an anti-Flag antibody. As shown in Fig. 2*B*, Myc-Skp2 was detected in immunoprecipitates with the anti-Flag antibody, whereas it was not detected in immunoprecipitates without Flag-Tob1 expression. Alternatively, Flag-Tob1 was detected in immunoprecipitates with the anti-Myc antibody. These results showed an interaction between Skp2 and Tob1.

To identify the interacting region between Tob1 and Skp2, we did an immunoprecipitation/immunoblotting assay using truncation mutants of Tob1 or Skp2 (Figs. 2*C* and 3*A*). As shown in Fig. 2*D*, Tob1-D4 and ΔC mutants could not bind Skp2, whereas Tob1-ΔN

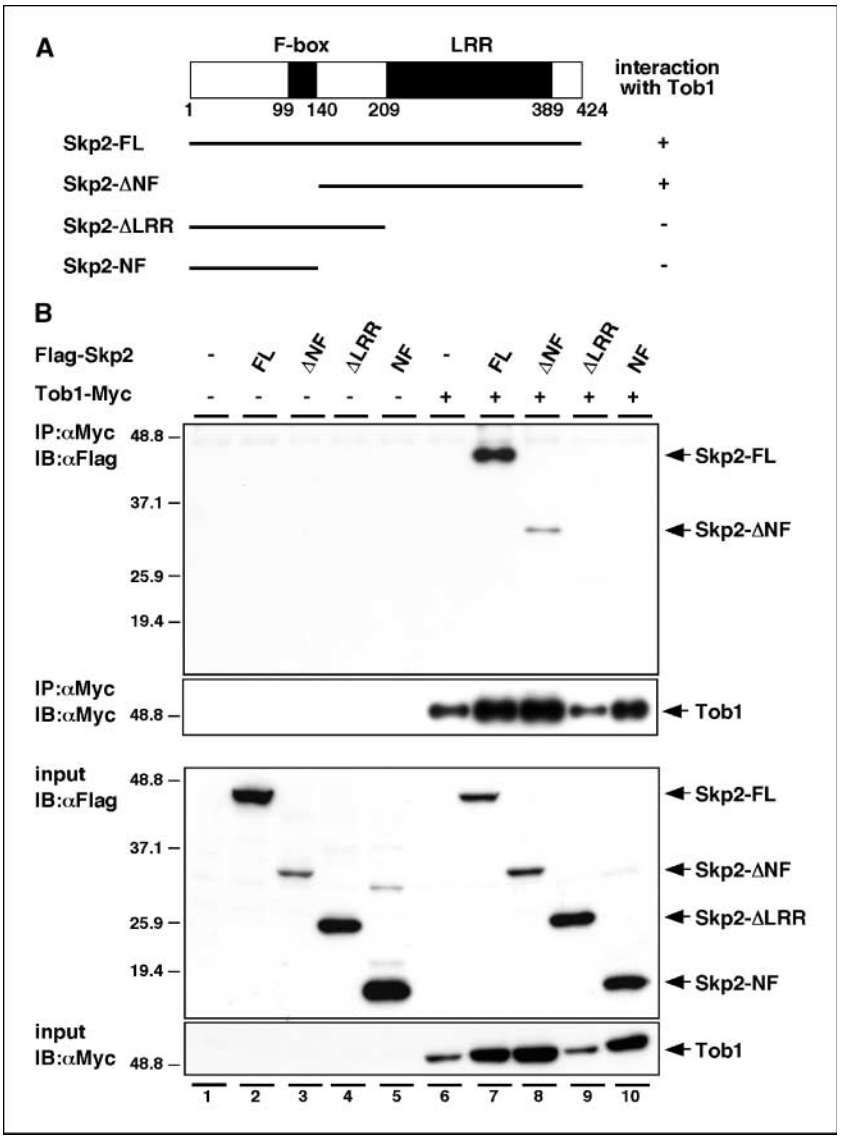

Figure 3. Binding region of Skp2 to Tob1. *A,* schematic representation of Flag-tagged Skp2 deletion mutants. *B,* FL or deletion mutants of Flag-tagged Skp2 were cotransfected with or without Tob1-Myc into HEK293 cells. Lysates were subjected to immunoprecipitation with anti-Myc antibody following immunoblotting with anti-Flag or anti-Myc antibodies. Results of interactions between Skp2 mutants and Tob1 are summarized in (*A*).

mutants formed complexes with Skp2 as well as Tob1-FL. These results suggested that the 166 to 237 amino acid region of Tob1 contained the Skp2 interaction domain. Skp2-FL and Skp2-ΔNF coprecipitated with Tob1, whereas mutants without the LRR were not found in the immune complexes (Fig. 3). Thus, the LRR was regarded as the essential domain for interaction with Tob1. These results suggested that Tob1 and Skp2 proteins interact with each other through a specific region. We further investigated whether the binding is phosphorylation dependent. As shown in Supplementary Fig. S2, phosphatase treatment did not affect the binding between Tob1 and Skp2, and phosphorylation of Tob1 by Erk1 did not increase the binding. These data suggest that Skp2 binds to Tob1 in a phosphorylation-independent manner. Moreover, we found that Cks1 was not required for the binding as shown in Supplementary Fig. S3.

Skp2 promotes ubiquitination of Tob1 protein. To determine whether expression of Skp2 facilitates ubiquitination of Tob1, we did an *in vivo* ubiquitination assay in HEK293 cells using double immunoprecipitation following immunoblotting, as described in Materials and Methods. Ubiquitination of Tob1 was detected in HEK293 cells transfected with an empty vector and was markedly enhanced by ectopic expression of Skp2 (Fig. 4*A*). Importantly, ubiquitination of Tob1 was not facilitated by mutation of Skp2, as mutants lack the functional F-box domain or the Tob1 interaction LRR domain (Fig. 4*B, lanes 7* and *8*). Moreover, we did an *in vitro* ubiquitination assay to confirm the ability. SCF-Skp2 complex was purified by immunoprecipitation with anti-Myc antibody from HEK293 cells transfected with Myc-Cul1, HA-Skp1, HA-Roc1/Rbx1, Myc-Skp2 (FL or ΔLRR), and HA-Nedd8. The immunoprecipitated SCF-Skp2 was then incubated with immunopurified Flag-Tob1 protein in the reaction mixture for ubiquitination. After the reaction mixtures were denatured with SDS-PAGE sample buffer, Tob1 protein was immunoblotted with anti-Tob1 antibody or reimmunoprecipitated with anti-Flag antibody following immunoblotting with anti-ubiquitin antibody or anti-Tob1 antibody. Immunoprecipitated Tob1 migrated with a high molecular weight smear that was detected with anti-ubiquitin antibody by

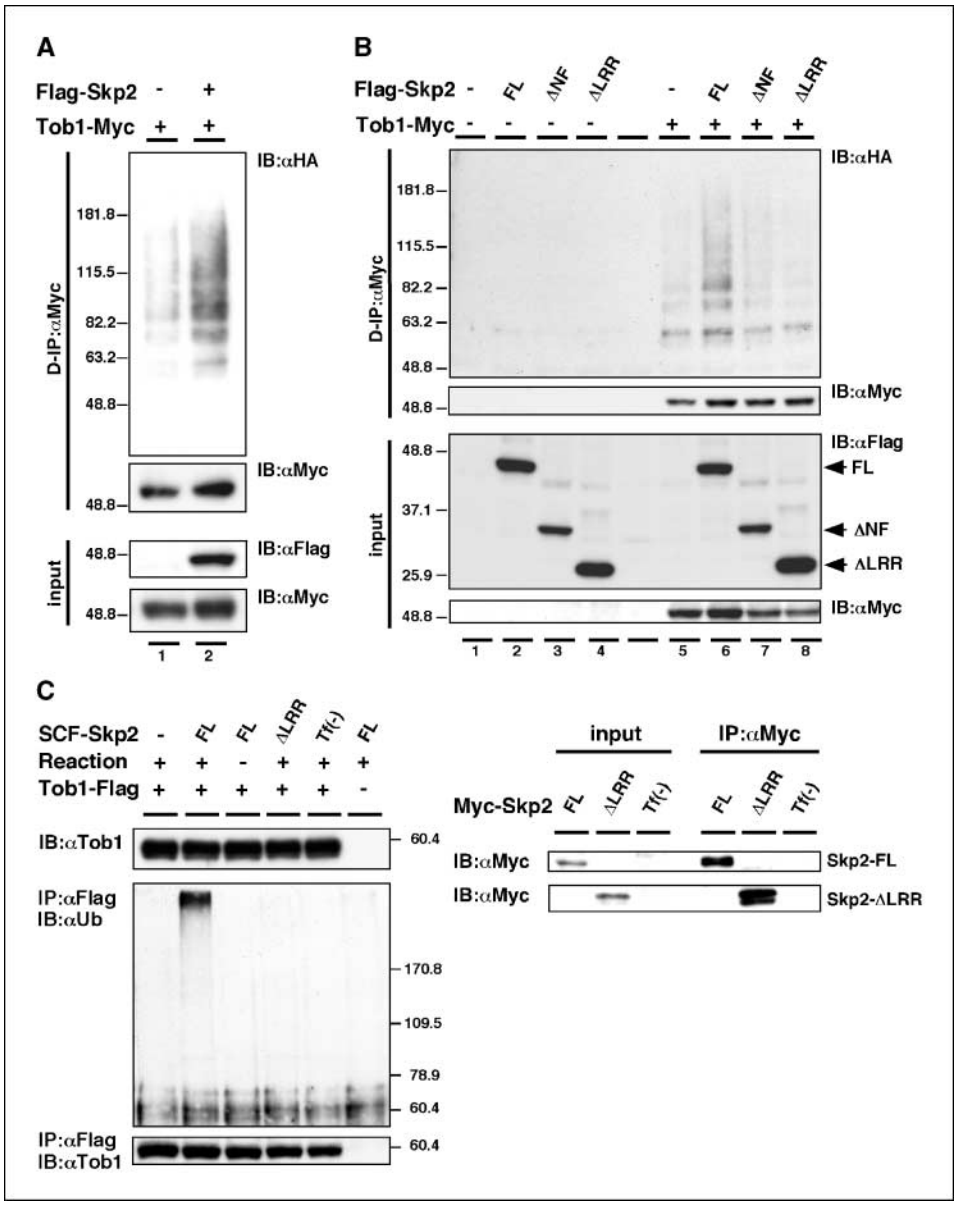

Figure 4. Enhanced ubiquitination of Tob1 by Skp2 *in vivo* and *in vitro*. *A,* Tob1-Myc and HA-ubiquitin were cotransfected with or without Flag-Skp2 into HEK293 cells. To detect ubiquitination of Tob1 protein, lysates were subjected to double immunoprecipitation (*D-IP*) with anti-Myc antibody following immunoblotting with anti-HA (*IB:αHA*) antibody. *B,* FL or deletion mutants of Flag-tagged Skp2 were cotransfected with or without Tob1-Myc into HEK293 cells. Lysates were subjected to double immunoprecipitation with anti-Myc antibody following immunoblotting with anti-HA antibodies. *C,* SCF-Skp2 complex was purified by immunoprecipitation with anti-Myc antibody from HEK293 cells transfected with Myc-Cul1, HA-Skp1, HA-Roc1/Rbx1, Myc-Skp2 (FL or ΔLRR), and HA-Nedd8. The immunoprecipitated SCF-Skp2 was then incubated with immunopurified Flag-Tob1 protein in the reaction mixture for ubiquitination. After the reaction mixtures were denatured with SDS-PAGE sample buffer, immunoblot analyses with anti-Tob1 antibody were done for half of the samples. The remaining samples were reimmunoprecipitated with anti-Flag antibody following immunoblotting with the indicated antibodies. *SCF-Skp2,* purified SCF-Skp2 complex. *Tf(-),* without transfection of SCF-Skp2 components. *Reaction, in vitro* ubiquitination reaction.

incubation with wild-type (WT) SCF-Skp2 as shown in Fig. 4*C*. These findings suggest that Skp2 promotes ubiquitination of Tob1.

Skp2 facilitates Tob1 degradation. Next, we investigated the stability of Tob1 in Skp2$^{-/-}$ MEFs. As shown in Fig. 5*A*, Tob1 protein accumulated in Skp2$^{-/-}$ MEFs compared with WT MEFs. Moreover, we did a monitoring experiment using cycloheximide to measure turnover rates of Tob1 protein in MEFs. We found that turnover rates of Tob1 protein and p27^{Kip1} were decreased in Skp2$^{-/-}$ MEFs compared with WT MEFs (Fig. 5*B*). We next carried out a monitoring experiment with cycloheximide in HeLa cells transfected with siRNA oligos for Skp2 or nonspecific control siRNA (Fig. 6*A*). Depletion of Skp2 resulted in a remarkable stabilization of Tob1 protein (Fig. 6*A, bottom*). Moreover, accumulation of endogenous Tob1 protein was observed in the Skp2-deleted cells (Figs. 6*A, lane 1* versus *lane 5* and Fig. 6*C*). These findings strongly suggest that Skp2 promotes degradation of endogenous Tob1.

Skp2 inhibits transactivation of Tob1. It has been reported that Tob1 regulates progression of the cell cycle by suppressing cyclin D1 expression. To investigate whether Skp2-dependent degradation of Tob1 affected its transactivational activity, we measured the effects of Skp2 depletion on cyclin D1 expression. We found that mRNA expression of cyclin D1 was significantly decreased in Skp2-depleted HeLa cells, whereas Tob1 and β-actin mRNA expressions were unaffected (Fig. 6*B*). Consequently, cyclin D1 protein was also reduced in the Skp2-depleted cells (Fig. 6*C*). Thus, the results suggested that Skp2 negatively regulates Tob1-mediated transcriptional suppression via ubiquitin-dependent degradation of Tob1.

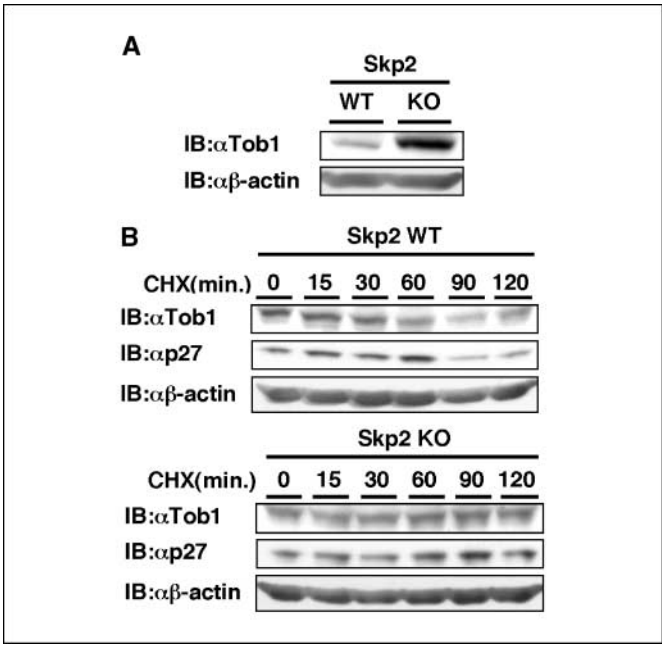

Figure 5. Stabilization of Tob1 in Skp2$^{-/-}$ cells. *A,* cell lysates prepared from Skp2$^{+/+}$ (WT) or Skp2$^{-/-}$ (KO) MEFs were subjected to Western blot analysis using appropriate antibodies. *B,* Skp2 KO or WT MEFs were treated with cycloheximide (*CHX*; 12.5 μg/mL) for indicated times, and protein levels of Tob1, p27, and β-actin were analyzed by Western blot analysis.

Discussion

We showed here that Skp2 bound to Tob1 and promoted ubiquitination of Tob1 in a LRR-dependent manner. Gene disruption and depletion of Skp2 caused accumulation of endogenous Tob1 protein as a result of the specific impairment of its degradation. These results strongly suggest that Skp2 is involved in ubiquitin-dependent degradation of Tob1.

Tob1 negatively regulates cell cycle progression from G_1 to S phases (1, 2). The protein level of Tob1 was greatly decreased in the early G_1 phase as shown in Fig. 1. Skp2 level was higher in HeLa than in normal cells; moreover, expression of Skp2 began from the G_1 phase in HeLa cells but started from the S phase in normal ones. However, degradation of Tob1 began earlier than induction of Skp2 even in HeLa cells. We cannot ignore the possibility that Tob1 might also be targeted by another E3 ligase. In general, one substrate protein is ubiquitinated by multiple ubiquitin ligases. p27 is targeted not only by SCF-Skp2 but also by the KPC complex (21, 22). KPC-mediated proteolysis of p27 begins in the G_0-G_1 phase earlier than Skp2-mediated proteolysis of p27.

The function of Tob1 is mainly regulated via phosphorylation by extracellular signal-regulated kinase (Erk) 1 and Erk2. It is rapidly phosphorylated at Ser^{152}, Ser^{154}, and Ser^{164} by Erk1/Erk2 on growth factor stimulation, and Erk1/Erk2-mediated phosphorylation cancels the antiproliferative function of Tob1 that inhibits cell growth by suppressing cyclin D1 expression (2). Because F-box proteins in SCF ubiquitin ligases, including SCF-Skp2, frequently recognize phosphorylated substrates as specific targets, we assessed whether substitution of the Erk phosphorylation sites of Ser^{152}, Ser^{154}, and Ser^{164} to alanine or asparate affected the interaction of Tob1 with Skp2, along with its abundance. The results showed that phosphorylation of these sites had no effect (data not shown). We also showed that the 166 to 237 region was necessary for binding to Skp2 because this region does not contain the Erk phosphorylation sites of Ser^{152}, Ser^{154}, and Ser^{164}. Additionally, phosphorylation of Tob1 by Erk1 did not increase the binding (Supplementary Fig. S2). Skp2-mediated degradation of Tob1 may be independent from phosphorylation by Erk, which is suggested to be involved in the functional regulation of Tob1 but not in its stability. As shown in Supplementary Fig. S2, phosphatase treatment did not affect binding between Tob1 and Skp2. Moreover, Cks1 was not required for the binding (Supplementary Fig. S3). Although, further investigations are required, Skp2 may target Tob1 in a different manner from the case between Skp2 and p27 for which the interaction requires both Cks1 and phosphorylation of p27.

It has been reported that Skp2 often shows increased expression in human cancers and is related to poor prognosis (18, 19, 23). Enhanced expression of Skp2 accelerates degradation not only of p27 but also of other several tumor suppressor proteins, including p21, p57, p130, and FOXO1. (11–17). Here, we show that another tumor suppressor protein, Tob1, is a target for Skp2. It has been reported that development of spontaneous tumors is observed in *Tob1*$^{-/-}$ mice (3). Moreover, ablation of Tob1 accelerates the formation of carcinogen-induced liver cancer, and deficiencies in both p53 and Tob1 cooperatively enhance tumor formation. Furthermore, *TOB1* expression is often decreased in human lung cancers (3). Therefore, Tob1 can act as a tumor suppressor protein. Our results suggest that Skp2 acts as an oncogenic protein to negatively regulate multiple tumor suppressors, including Tob1 and so is associated with malignancy and accelerated growth of tumors.

Oncogenic signals mediated by the receptor tyrosine kinase/Ras/mitogen-activated protein kinase pathway stimulate phosphorylation of Tob1 by activating Erk kinase (2). Whereas active Tob1 suppresses the expression of cyclin D1, Erk-mediated phosphorylation of Tob1 restores cyclin D1 expression. On the other hand, Ras signaling induces expression of Skp2 through the binding of the

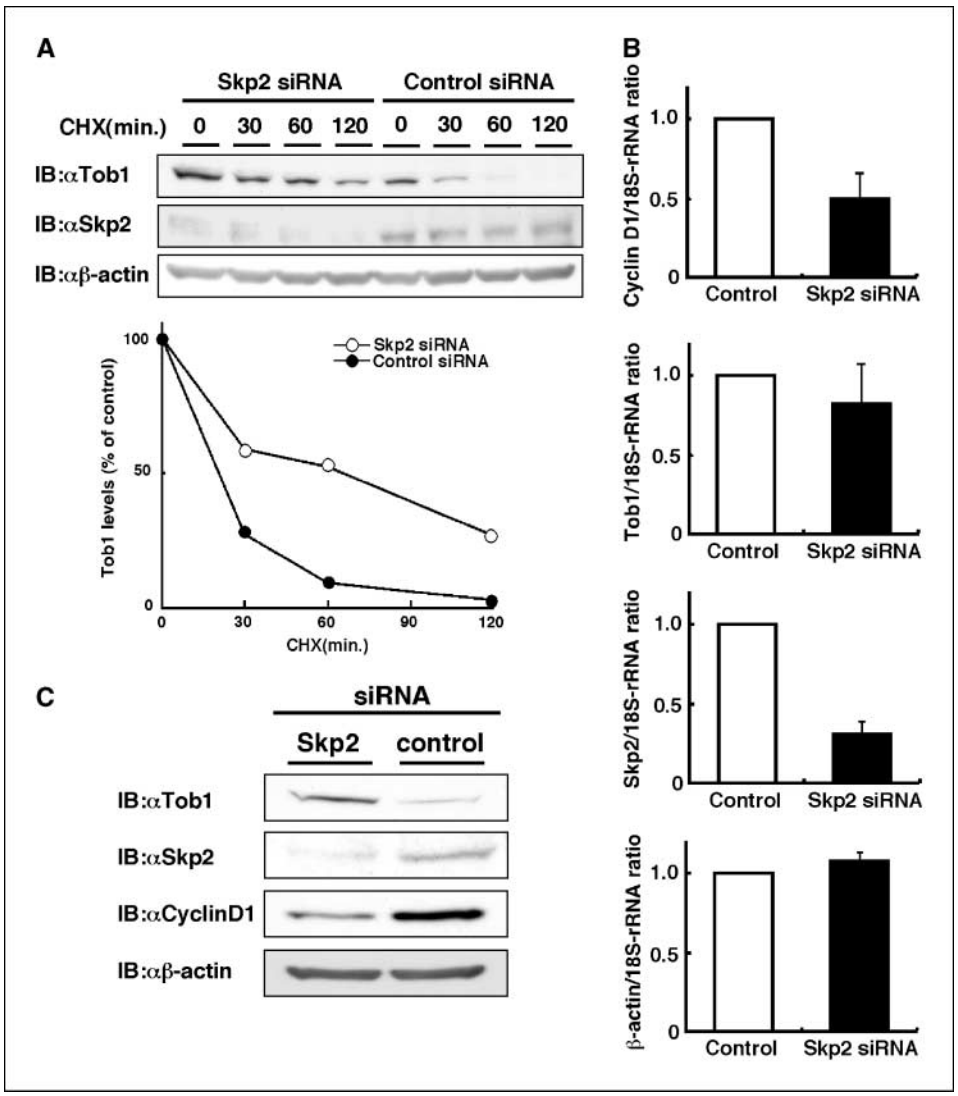

Figure 6. Effects of Skp2 depletion on Tob1 stability and cyclin D1 mRNA expression. *A,* HeLa cells were treated with siRNA oligos (200 nmol/L) specific for Skp2 or with nonspecific control for 48 hours and then with cycloheximide (12.5 μg/mL) for appropriate times. *Top,* protein turnovers of Tob1, Skp2, and β-actin were analyzed by Western blot analysis using the indicated antibodies. Band intensities were quantitated using Image Gauge 4.21 image analysis software. *Bottom,* relative ratios of Tob1 protein levels at each time point. *B,* relative mRNA levels of Skp2, Tob1, cyclin D1, and β-actin in HeLa cells with (Skp2 siRNA) or without (control) Skp2 depletion were measured by quantitative reverse transcription-PCR analysis. The abundance of the mRNA of interest was normalized against that of 18S rRNA as an internal standard. *Columns,* mean of triplicate analyses of three different samples; *bars,* SD. *C,* protein levels of Tob1, Skp2, cyclin D1, and β-actin in HeLa cells with (Skp2 siRNA) or without (control) Skp2 depletion was analyzed by immunoblotting with indicated antibodies.

GA-binding protein, an Ets family transcription factor, to Skp2 promoter (24). It has been reported recently that Skp2 expression was positively regulated by E2F1 (25). In human cancers, the RB pathway is frequently abrogated by various causes, such as mutation/deletion of the *RB* gene, overexpression of cyclins, down-regulation of CDK inhibitors, and missense mutations of *CDK* genes (26). It is presumed that inactivation of the RB pathway results in increased activity of E2F1 inducing Skp2 gene transcription. Enhanced expression of *Skp2* may facilitate degradation of Tob1 to induce cyclin D1 expression. Therefore, oncogenic signaling negatively regulates antiproliferative activity of Tob1 via inactivation of Tob1 not only by Erk-mediated phosphorylation but also by ubiquitin-dependent degradation of Tob1 mediated by SCF-Skp2. Therefore, our study provides a foundation for future investigations to understand the biochemical events underlying cell cycle regulation by Skp2 and Tob1 and/or the malignant potential of tumors.

Acknowledgments

Received 5/2/2006; revised 6/30/2006; accepted 7/10/2006.

Grant support: Ministry of Education, Science, Sports, Culture, and Technology of Japan grants-in-aid for Science Research 16021220 (M. Kitagawa), 16591307 (H. Konno), 17590056 (K. Kitagawa), and 17790911 (Y. Hiramatsu); Ministry of Health, Labor, and Welfare grant-in-Aid for Third-Term Comprehensive Control Research for Cancer (M. Kitagawa); and Ministry of Education, Science, Sports, Culture, and Technology Center of Excellence Program of Hamamatsu University School of Medicine (M. Kitagawa).

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We thank Takumi Kamura for baculoviruses; Sayuri Suzuki, Tomomi Abe, Daizo Ueno, and Daisuke Hiraoka for their technical assistance; and Satoshi Nakamura, Hayato Ihara, and the staff members of our laboratories for their helpful discussions and support.

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Cancer Res 2006;66:8477-8483.