

## ASB2 Is an Elongin BC-interacting Protein That Can Assemble with Cullin 5 and Rbx1 to Reconstitute an E3 Ubiquitin Ligase Complex\*

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**The ankyrin repeat-containing protein with a suppressor of cytokine signaling box-2 (ASB2) gene is identified as a retinoic acid-response gene and a target of the promyelocytic leukemia-retinoic acid receptor- $\alpha$  oncogenic protein characteristic of acute promyelocytic leukemia. Expression of ASB2 in myeloid leukemia cells inhibits growth and promotes commitment, recapitulating an early step known to be critical for differentiation. Here we show that ASB2, by interacting with the Elongin BC complex, can assemble with Cullin5-Rbx1 to form an E3 ubiquitin ligase complex that stimulates polyubiquitination by the E2 ubiquitin-conjugating enzyme Ubc5. This is a first indication that a member of the ASB protein family, ASB2, is a subunit of an ECS (Elongin C-Cullin-SOCS box)-type E3 ubiquitin ligase complex. Altogether, our results strongly suggest that ASB2 targets specific proteins to destruction by the proteasome in leukemia cells that have been induced to differentiate.**

Identification of genes controlling proliferation and differentiation of myeloid cells is critical for understanding how myelopoiesis is dysregulated by chromosomal abnormalities in leukemia cells. Because committed hematopoietic progenitors are likely targets for leukemic transformation, genes involved in myeloid differentiation might be repressed in leukemia cells and activated when these cells are exposed to agents that induce differentiation. Acute promyelocytic leukemia (APL)<sup>1</sup> is associated with five reciprocal translocations always involving

the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) (1, 2).

RAR $\alpha$  is a retinoic acid (RA)-dependent transcription factor (3) involved in hematopoietic differentiation (4). Although RARs are dispensable for granulopoiesis (5), a role for RA in the commitment of hematopoietic progenitors into the granulocytic lineage has been proposed (6–8). Furthermore, overexpression of wild-type RAR $\alpha$  or dominant-negative forms of RAR $\alpha$  including PML-RAR $\alpha$  blocks granulocytic differentiation at the promyelocytic stage (9, 10). These findings suggest that RAR $\alpha$  may inhibit neutrophil differentiation when unbound and promote differentiation when bound to its specific ligand. In more than 95% of APL, the specific translocation t(15;17) produces the PML-RAR $\alpha$  fusion proteins. Although most patients express the reciprocal RAR $\alpha$ -PML protein, some do not, suggesting that RAR $\alpha$ -PML may be dispensable for leukemogenesis. The association of histone deacetylases with PML-RAR $\alpha$  has been described in APL cells (11–13), suggesting that PML-RAR $\alpha$  may recruit a histone deacetylase complex leading to the repression of RA target genes critical to myeloid differentiation. It has been shown that the histone deacetylase complex dissociates from the PML-RAR $\alpha$  fusion protein in the presence of pharmacological concentrations of RA, suggesting a mechanism by which APL cells are sensitive to RA treatment (11–13). Indeed, when treated with RA, these cells withdraw from the cell cycle and undergo terminal maturation both *in vitro* (14) and *in vivo* (15–17). This suggests a molecular mechanism by which RA-responsive genes critical to myeloid differentiation are repressed in leukemia cells and derepressed when these cells are treated with RA. A few PML-RAR $\alpha$  target genes have been identified including (i) *PRAM-1*, a novel molecular adaptor of myeloid cells (18), (ii) *ASB2*, a member of the suppressors of cytokine signaling (SOCS) protein family (19), (iii) *p21<sup>WAF1/CIP1</sup>*, a mediator of growth inhibition that plays a role during commitment to differentiation of RA-treated APL cell (20), (iv) ubiquitin-activating enzyme E1-like (*UBE1L*), which mediates PML-RAR $\alpha$  ubiquitination and subsequent degradation (21), and (v) the CCAAT/enhancer binding proteins (*C/EBP*)  $\beta$  and  $\epsilon$ , which are myeloid transcription factors (22, 23). *C/EBP $\epsilon$*  is the only example of a gene that is up-regulated by RA in APL cells and dominantly repressed by PML-RAR $\alpha$  and whose expression is required for the reactivation of the differentiation program.

The *ASB2* gene was isolated by applying a subtractive hybridization strategy to identify novel genes activated during RA-induced maturation of APL cells (24). *ASB2* is both a PML-RAR $\alpha$  target and a RA-response gene (19). Expression of the *ASB2* protein in myeloid leukemia cells inhibits growth and promotes chromatin condensation, which are characteristics of commitment known as critical to differentiation of myeloid

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<sup>1</sup> The abbreviations used are: APL, acute promyelocytic leukemia; RAR, retinoic acid (RA) receptor; SOCS, suppressors of cytokine signaling; ECS, Elongin C-Cullin-SOCS box; VHL, von Hippel Lindau; Cul, cullin; GST, glutathione S-transferase; HA, hemagglutinin; DTT, dithiothreitol; PML, promyelocytic leukemia.

leukemia cells (19). The *ASB2* gene encodes a protein harboring ankyrin repeats, a protein-protein interaction domain, and a BC motif located within a SOCS box. By interacting with the Elongin BC complex through the BC motif, several proteins containing a BC motif are able to assemble into an ECS (Elongin C-Cullin-SOCS box)-type E3 ubiquitin ligase complex that functions with the E1 ubiquitin-activating enzyme and an E2 ubiquitin-conjugating enzyme to ubiquitinate its specific partners (25). These targets are then degraded by the proteasome machinery. The mammalian Elongin BC complex, which is a heterodimer composed of the Elongin B and Elongin C proteins, was initially identified as a positive regulator of RNA polymerase II elongation factor Elongin A (26, 27) and subsequently as a component of the von Hippel Lindau (VHL) tumor suppressor complex (28, 29). Both Elongin A and VHL protein have been shown to bind to Elongin C via interaction with the highly conserved leucine residue at position 2 of the BC box (30, 31). Elongin B binds to Elongin C and does not interact directly with the BC box. The Elongin BC complex functions as an adaptor that links the BC box-containing protein to cullin (Cul) 2 or 5, which in turn binds to the ring finger protein Rbx1 to reconstitute a multisubunit complex with ubiquitin ligase activity (32–35). Within this complex, the BC-box protein is also involved in the recruitment of specific targets, suggesting that BC-box proteins regulate turnover of proteins by targeting them for proteasomal degradation. Indeed, the VHL protein can be considered as the receptor subunit of an E3 ubiquitin ligase complex. Consequently, the VHL-ubiquitination machinery controls the stability of hypoxia-inducible transcription factor HIF1 $\alpha$  by promoting its ubiquitination and proteasome-dependent degradation (36–38).

In an attempt to decipher the *ASB2* mechanism of action, we carried out a yeast two-hybrid screen and identified Elongin B as specifically associated with *ASB2*. By interacting with the Elongin BC complex through the BC motif, *ASB2* can assemble with the Cul5-Rbx1 module to reconstitute an active E3 ubiquitin ligase complex.

#### MATERIALS AND METHODS

**Cell Lines, Culture, and Differentiation**—The COS-7 and HEK293 cell lines were grown in Petri dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). To inhibit *de novo* protein synthesis, 100  $\mu$ g/ml cycloheximide (Sigma) was added to HEK293 cells 48 hours post-transfection. Cells were harvested after an additional incubation of 5, 10, and 24 h. NB4 cells were cultured in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum (PAA Laboratories), 2 mM glutamine, and 1% penicillin-streptomycin. Exponentially growing NB4 cells were seeded at  $2 \times 10^5$  cells/ml 16 h before all-*trans* RA treatment (Sigma). Cell viability was estimated using standard trypan blue dye exclusion assay. Differentiation was assessed by (i) the percentage of cells with nitro blue tetrazolium (Sigma) deposits and (ii) cell morphology under light microscopy on May-Grünwald-Giemsa-stained cytospin slides. Sf21 cells were cultured at 27 °C in Sf-900 II medium (Invitrogen) with 10% fetal bovine serum and 1% penicillin-streptomycin.

**Plasmid Constructs**—The *ASB2* coding sequence was subcloned into (i) the pGBKT7 vector (Clontech), (ii) the pGST, a derivative of pGEX-3X (Amersham Biosciences), in-frame with the glutathione *S*-transferase (GST) sequence, (iii) pSG5- (Stratagene)- and pBacPAK8 (Clontech)-derived vectors to direct the expression of *ASB2* fused to the FLAG epitope at its N terminus (pSG5FN-*ASB2* and pBacPAK8FN-*ASB2*, respectively), and (iv) the pAT4 vector (39) to direct the expression of *ASB2* tagged with the F domain of the human estrogen receptor at its N terminus. Deletion of the SOCS box (amino acids 545–587) was generated by PCR amplification. Construction of the *ASB2*LPCF-mutated vectors was achieved using the QuikChange site-directed mutagenesis kit (Stratagene) and the mutated oligonucleotide sequence, as indicated in boldface, 5'-CTCCAAGACTCCGGCTCACCTTTTC-CGACTGCGGGTT-3'. The human Elongin B was subcloned into the pAT4 vector. The human Elongin C open reading frame was obtained by PCR amplification using bone marrow cDNA and subcloned into pSG5-derived vectors to direct the expression of Elongin C tagged with the

hemagglutinin (HA) or the FLAG epitope at its N terminus. Human Ubc5 containing an N-terminal FLAG tag and a C-terminal His<sub>6</sub>, *Saccharomyces cerevisiae* Uba1 containing an N-terminal Myc tag and a C-terminal His<sub>6</sub>, and mammalian GST-ubiquitin expression vectors were as described (38). All constructs were verified by sequencing.

**Yeast Two-hybrid Screen**—A human bone marrow library was screened using the Matchmaker Two-Hybrid System-3 protocol (Clontech). Briefly, a Gal4-DNA binding domain fusion of *ASB2* (pGBKT7-*ASB2*) was employed as bait in the screen carried out by mating AH109-MAT $\alpha$  pre-transformed with pGBKT7-*ASB2* to Y187-MAT $\alpha$  library-pre-transformed cells. The AH109 transformation was carried out by standard lithium acetate protocol, and the library was maintained in the Y187 strain according to manufacturer's recommendations. Recombinant pGBKT7-*ASB2* clones were tested for protein expression by Western blotting (positive), autonomous lacZ reporter activation by the bait alone (negative), cell proliferation effects through the bait (negative), and mating efficiency with Y187-MAT $\alpha$  and were constantly maintained under selection of the auxotrophy marker Trp<sup>-</sup>. Once AH109-MAT $\alpha$ -pGBKT7-*ASB2* was confirmed through the above protocol, mating of a sufficient large pool of these cells to the pre-transformed Y187-MAT $\alpha$  library-carrying strain (titer,  $5 \times 10^7$  colony-forming units/ml) was conducted according to the manufacturer's recommendations. Mating efficiency was determined in this case at 79%, with a total of  $1.9 \times 10^8$  independent clones, thus covering the library ( $3.5 \times 10^6$  independent inserts) approximately 53 times. Mated cells were spread onto selective media (SD, Clontech) for Trp (bait), Leu (prey), His (reporter 1), Ala (reporter 2), and lacZ (reporter 3) (SD-HALTX). After plating, positive clones were collected from days 8–20 after plating and re-streaked on SD-HALTX, SD-LTX, and SD-LX plates for verification of growth phenotype. Clones confirmed through this secondary screening were amplified in SD-L media, selecting only for the "prey" library insert. Plasmid DNA was finally isolated for sequencing according to standard procedures.

**Expression and Purification of Recombinant Proteins in Escherichia coli**—Overnight starter cultures (20 ml) of *E. coli* XL1-Blue (Stratagene) transformed with pGST, pGST-*ASB2*, pGST-Elongin B, pGST-Elongin C, and pGEX4T-2-ubiquitin were inoculated into 500 ml of culture medium and grown at 30 °C to an optical density of about 0.6 at 600 nm. After isopropyl 1-thio- $\beta$ -D-galactopyranoside induction (0.5 mM, 2 h at 30 °C), bacteria were collected and sonicated in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 10  $\mu$ M ZnCl<sub>2</sub>, 10% glycerol, 100  $\mu$ g/ml lysozyme freshly supplemented with 0.1 mM dithiothreitol (DTT) and the protease inhibitor mixture for use with mammalian cell extracts (0.1%, Sigma). Uba1 and Ubc5 were expressed in *E. coli* BL21 (DE3). After isopropyl 1-thio- $\beta$ -D-galactopyranoside induction (0.5 mM, 2 h at 30 °C), bacteria were collected and sonicated in lysis buffer containing 20 mM Tris-HCl, pH 7.9, 150 mM NaCl, 20% glycerol, 0.1% Nonidet P-40, 100  $\mu$ g/ml lysozyme, 5 mM  $\beta$ -mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% protease inhibitor mixture for use with mammalian cell extracts. Cellular extracts were clarified by centrifugation (10,000  $\times g$ , 45 min at 4 °C).

To immobilize GST, GST-*ASB2*, GST-Elongin B, and GST-Elongin C proteins, 32 mg of extracts were incubated with 300  $\mu$ l of glutathione-Sepharose beads (Amersham Biosciences) for 2.5 h at 4 °C. Beads were washed 3 times with lysis buffer and resuspended in 300  $\mu$ l of the same buffer. Uba1 and Ubc5 were purified by Ni<sup>2+</sup>-agarose chromatography as recommended by the manufacturer (Qiagen). Uba1 and Ubc5 were eluted with 100 mM imidazole. GST-ubiquitin was purified by glutathione-Sepharose chromatography as recommended by the manufacturer (Amersham Biosciences). Purified Uba1, Ubc5, and GST-ubiquitin proteins were dialyzed against 40 mM Hepes-NaOH, pH 7.9, 60 mM potassium acetate, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 10% glycerol, 2 mM DTT.

**In Vitro Binding Assays**—One  $\mu$ g of T7 promoter-fused cDNA was incubated according to the manufacturer's recommendations (Promega) in the presence of [<sup>35</sup>S]methionine for 2 h at 30 °C. *In vitro* binding assays were performed using  $\sim 1 \mu$ g of GST fusion proteins and 5  $\mu$ l of translation products of a coupled *in vitro* transcription/translation of cDNAs for 30 min at room temperature. After several washes to remove unbound material in washing buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.3 mM DTT, 5% glycerol, 0.1% Nonidet P-40), proteins were recovered in Laemmli buffer, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by Coomassie staining and autoradiography of dried gels. Alternatively, *ASB2* and the Elongin BC complex were produced using a coupled *in vitro* transcription/translation reaction, and immunoprecipitation experiments were carried out with anti-*ASB2* antibodies as described below.

**In Vivo Expression and Protein Extracts**—COS-7 and HEK293 cells were transfected using calcium phosphate coprecipitation of the ap-

appropriate amount of DNA vectors adjusted to 14  $\mu$ g per 9-cm Petri dish with pBluescript carrier DNA. Medium was changed after 16 h. After an additional 20-h culture, cells were washed in phosphate-buffered saline (1 $\times$ ), collected, and resuspended in whole cell extract buffer (50 mM Tris-HCl, pH 7.9, 0.15 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 0.1% protease inhibitor mixture for use with mammalian cell extracts). After 2 freeze-thaw cycles in liquid nitrogen, the resulting cell lysates were cleared by a 30-min 10,000  $\times$  *g* centrifugation at 4 °C. PLB-985 cells were transfected and selected with G418 as described (19).

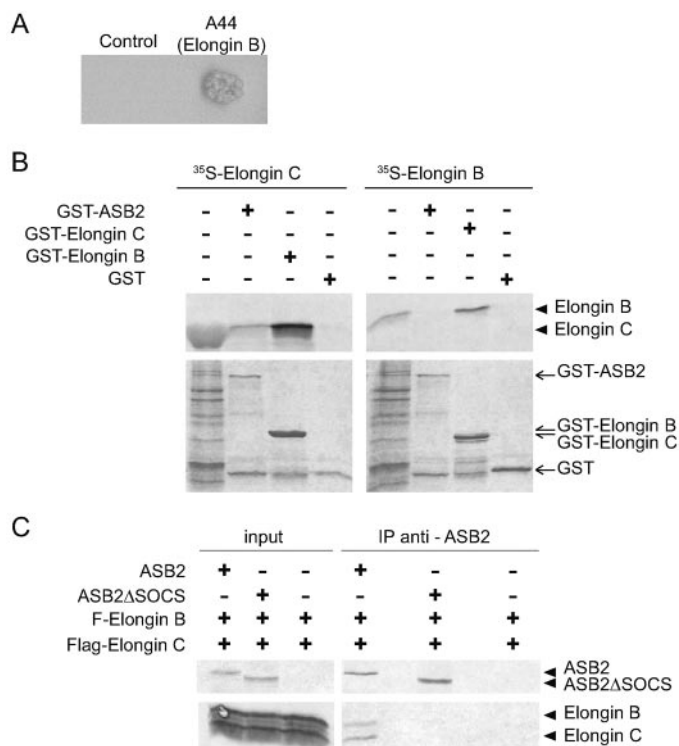
**Antibodies, Western Blots, Immunoprecipitations**—The rabbit serum raised against ASB2 (1PNA) has been described previously (19). Anti-FLAG (2EL-1B11), anti-F (4ERb-2D7), anti-HA, and anti-GST (1D10) monoclonal antibodies were purchased from Euromedex. Anti-herpes simplex virus tag was from Novagen. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20, proteins were probed with the corresponding rabbit antiserum or mouse monoclonal antibodies. For detection of proteins, appropriate peroxidase-coupled secondary antibodies (Jackson Laboratories Research) were used with a standard chemiluminescence system (PerkinElmer Life Sciences). After one-step preclearing (30 min at 4 °C) with protein A-Sepharose fast flow (Amersham Biosciences), antibodies were added to the cell protein extract in a binding buffer adjusted to 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.1% Nonidet P-40. After 2 h of incubation, immunocomplexes were recovered with protein A-Sepharose. After three washes with binding buffer, proteins were eluted in sample buffer and resolved by SDS-PAGE electrophoresis.

**Expression of Recombinant Proteins in Sf21 Insect Cells**—Recombinant baculoviruses encoding ASB2 and ASB2LPCF containing an N-terminal FLAG tag were generated with the BacPAK baculovirus expression system (Clontech). Baculoviruses encoding human Elongin B, human Elongin C containing an N-terminal herpes simplex virus tag, human Cul2 and Cul5 containing an N-terminal HA tag, and mouse Rbx1 containing an N-terminal Myc tag were as described (38). Sf21 cells were coinfectd with the recombinant baculoviruses indicated in the figure legends. 48 h after infection, cells were collected and lysed in ice-cold buffer containing 40 mM Hepes-NaOH, pH 7.9, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM DTT. Cell lysates were cleared by centrifugation at 10,000  $\times$  *g* for 20 min at 4 °C.

**Ubiquitination Assays**—Cell extracts were immunoprecipitated with anti-FLAG or anti-ASB2 antibodies and protein A-Sepharose fast flow. After 3 washes with binding buffer and 2 washes with a buffer containing 40 mM Hepes-NaOH, pH 7.9, 60 mM potassium acetate, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 10% glycerol, 2 mM DTT, the beads were mixed with 500 ng of Uba1, 1.4  $\mu$ g of Ubc5a, 2.5  $\mu$ g of GST-ubiquitin in a 15- $\mu$ l reaction containing 4 mM Hepes NaOH, pH 7.9, 6 mM potassium acetate, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 1.5 mM ATP. Reaction mixtures were incubated for 1 h at 30 °C. Reaction products were fractionated by SDS/PAGE and analyzed by immunoblotting with anti-GST or anti-ASB2 antibodies.

## RESULTS

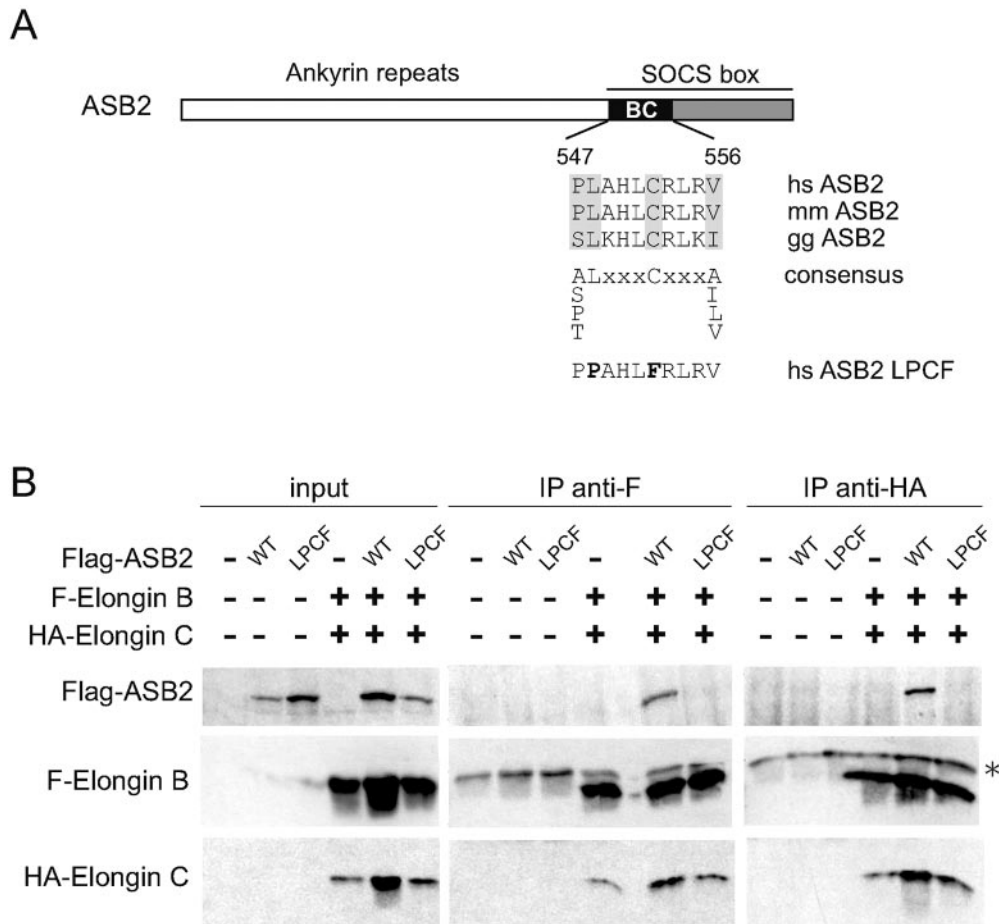
**ASB2 Bound to the Elongin BC Complex through Its BC Box**—To identify binding partners for ASB2, a yeast two-hybrid screen was used with full-length ASB2 as bait together with a cDNA library from human bone marrow cells. One cDNA identified in this screen was found to encode Elongin B. The yeast strain AH109 was then transformed with Elongin B or the pACT2 empty plasmid together with the ASB2 bait plasmid and grown onto a selective media. As shown in Fig. 1A, Elongin B was found to associate specifically with ASB2 in yeast. Because Elongin B has not been shown to bind directly to BC box-containing proteins, interaction of ASB2 with Elongin B in yeast is likely to involve yeast Elongin C. To confirm the binding results from the yeast two-hybrid screen, we examined *in vitro* binding of Elongins B and C to ASB2. Affinity-purified GST, GST-ASB2, GST-Elongin C, and GST-Elongin B proteins expressed in *E. coli* were incubated with *in vitro* translated Elongin B and C proteins. As shown in Fig. 1B, GST-ASB2 did not bind Elongin B, whereas it bound Elongin C. Association between ASB2 and the Elongin BC complex was further assessed in coimmunoprecipitation experiments using a transcription/translation system. Both Elongins B and C coprecipi-



**FIG. 1. ASB2 associated with the Elongin BC complex.** **A**, ASB2 associated with Elongin B in yeast. Clone A044 encoding Elongin B and the pACT2 empty vector (*Control*) were transformed into yeast strain AH109 together with the pGBKT7-ASB2 vector and grown on SD/-Ade/-His/-Leu/-Trp medium. **B**, ASB2 interacted with Elongin C. GST protein alone, GST-Elongin B, GST-Elongin C, or GST-ASB2 fusion proteins were first immobilized on glutathione-agarose beads. *In vitro* produced and [<sup>35</sup>S]methionine-labeled Elongin B and Elongin C were incubated with the immobilized GST proteins. The interacting complexes were resolved by SDS-PAGE and examined by autoradiography (*upper panel*) and Coomassie staining (*lower panel*). **C**, the cDNAs encoding wild-type ASB2, deletion mutant ASB2ΔSOCS, and Elongins B and C were expressed in a coupled transcription/translation system in the presence of [<sup>35</sup>S]methionine. 15- $\mu$ l aliquots of the translation products were immunoprecipitated with anti-ASB2 antibodies. Immunoprecipitated proteins (*IP anti-ASB2*, *right panel*) as well as a 3- $\mu$ l aliquot of the transcription/translation reaction (*input*, *left panel*) were fractionated by SDS-PAGE and detected by autoradiography.

tated with the wild-type ASB2 (Fig. 1C). In contrast, the ASB2ΔSOCS mutant lacking the entire SOCS box did not associate with the Elongin BC complex (Fig. 1D). To confirm that ASB2 was capable of interacting with Elongin BC complex *in vivo* and to determine whether ASB2 associated with the Elongin BC complex through the BC motif located between amino acids 547 and 557, COS-7 cells were transfected with constructs expressing epitope-tagged elongins or ASB2. As shown in Fig. 2B, the expressed wild-type ASB2 could be coprecipitated specifically with tagged Elongins B and C. The N-terminal region of the SOCS box of ASB2, which is highly conserved in human, mouse, and chicken, includes a consensus Elongin BC binding site or BC box (Fig. 2A). The association between ASB2 and the Elongin BC complex was strongly dependent on the presence of an intact BC box since mutation of conserved residues within this motif abolished the binding of the Elongin BC complex to ASB2 (Fig. 2B).

**Elongin BC Binding Prevented Degradation of ASB2**—We obtained high levels of wild-type ASB2 expression when Elongins B and C were co-expressed. Therefore, we assessed the effect of the Elongin BC complex binding on ASB2 stability. Cells expressing wild-type or BC-box mutant ASB2 proteins were treated with cycloheximide to block further protein synthesis for various times (Fig. 3). In the absence of cotransfected

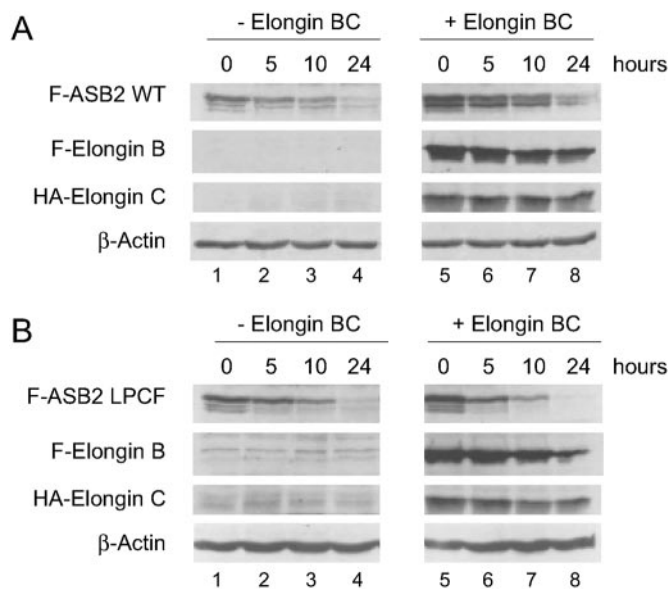


**FIG. 2. ASB2 associated with the Elongin BC complex through the BC motif.** *A*, alignments of BC boxes of *Homo sapiens* (*hs*), *Mus musculus*, (*mm*) and *Gallus gallus* (*gg*) ASB2 protein with the consensus sequence. Shaded regions represent residues identical to the consensus sequence. Point mutations within the BC box of ASB2 are indicated in *bold* (*hsASB2LPCF*). *B*, COS-7 cells were transfected in 10-cm dishes with 0.2  $\mu$ g of the F-Elongin B expression vector, 2  $\mu$ g of the expression vector HA-Elongin C, and 1  $\mu$ g of the wild-type FLAG-ASB2 expression vector or 4  $\mu$ g of the FLAG-ASB2LPCF expression vector as indicated. 100- $\mu$ g aliquots of each protein extract were immunoprecipitated with monoclonal anti-F or anti-HA antibodies. Immunoprecipitated proteins (IP) as well as a 10- $\mu$ g aliquot of the protein extracts (*input*) were separated by SDS-PAGE and analyzed for ASB2, Elongin B, and Elongin C as indicated (Western blot). The asterisk indicates the light chain of immunoglobulins. WT, wild type.

Elongins B and C, both the wild-type and mutated ASB2 proteins were unstable (Fig. 3, *A* and *B*; lanes 1–4). Coexpression of Elongins B and C stabilized wild-type ASB2 protein in the presence of cycloheximide (Fig. 3, *A* and *B*; lanes 6–9). In contrast to the wild-type ASB2, elongin-mediated stabilization of ASB2 did not occur when the ASB2 elongin binding mutant was used (Fig. 3*B*, lanes 6–9). These results indicated that the ASB2 protein was directly stabilized by complexing with both Elongins B and C.

*The ASB2-Elongin BC Complex Interacted with a Cul5-Rbx1 Module to Reconstitute an Active E3 Ubiquitin Ligase Complex*—To determine whether the Elongin BC complex could function as an adaptor linking ASB2 to a Cullin-Rbx1 module and reconstituting an E3 ubiquitin ligase complex, anti-FLAG immunoprecipitations were carried out on lysates of Sf21 cells co-infected with baculoviruses encoding FLAG-ASB2, Elongin B, herpes simplex virus-Elongin C, HM-Rbx1, and HA-Cul2 or -5 (Fig. 4). The ASB2-Elongin BC complex did not associate with the Cul2-Rbx1 module. However, the ASB2-Elongin BC complex did assemble with the Cul5-Rbx1 module, reconstituting a multiprotein complex containing ASB2, Elongins B and C, Rbx1, and Cul5 (Fig. 4*A*). The ASB2 BC-box mutant, which did not bind the Elongin BC complex, also did not assemble with the Cul5-Rbx1 module (Fig. 4*B*). To investigate whether the ASB2-Elongin BC-Cul5-Rbx1 complex possesses ubiquitin li-

gase activity, the complex was immunoaffinity-purified and assayed for its ability to activate formation of polyubiquitin chains by the E2 ubiquitin-conjugating enzyme Ubc5 in the presence of ATP, the E1 ubiquitin-activating enzyme Uba1, and GST-ubiquitin. As shown in Fig. 4*C*, the ASB2-Elongin BC-Cul5-Rbx1 complex stimulated formation of a ladder of GST-ubiquitin conjugates by E2 ubiquitin-conjugating enzyme, whereas the ASB2 BC-box mutant did not. As expected, formation of polyubiquitin conjugates depended on the presence of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and ATP (Fig. 4*C*). The fact that the ASB2-Elongin BC complex associated with the Cul5-Rbx1 module and not with the Cul2-Rbx1 module suggested a direct contact between ASB2 and Cul5. To address this possibility, either the Cul5-Rbx1 or the Cul2-Rbx1 module was expressed in Sf21 insect cells alone or together with ASB2. As shown in Fig. 5*A*, the Cul5-Rbx1 module associated with ASB2, whereas the Cul2-Rbx1 module did not. Although ASB2 associated with the Cul5-Rbx1 module, the resulting ASB2-Cul5-Rbx1 complex, in contrast to its ASB2-Elongin BC-Cul5-Rbx1 counterpart, only weakly supported polyubiquitination by the E2 ubiquitin-conjugating enzyme Ubc5 (Fig. 5*B*). The presence of an ASB2-Cul5-Rbx1 complex is unlikely to be due to an association of the insect Elongin BC complex with mammalian ASB2 and the Cul5-Rbx1 module given (i) the similar levels of immuno-



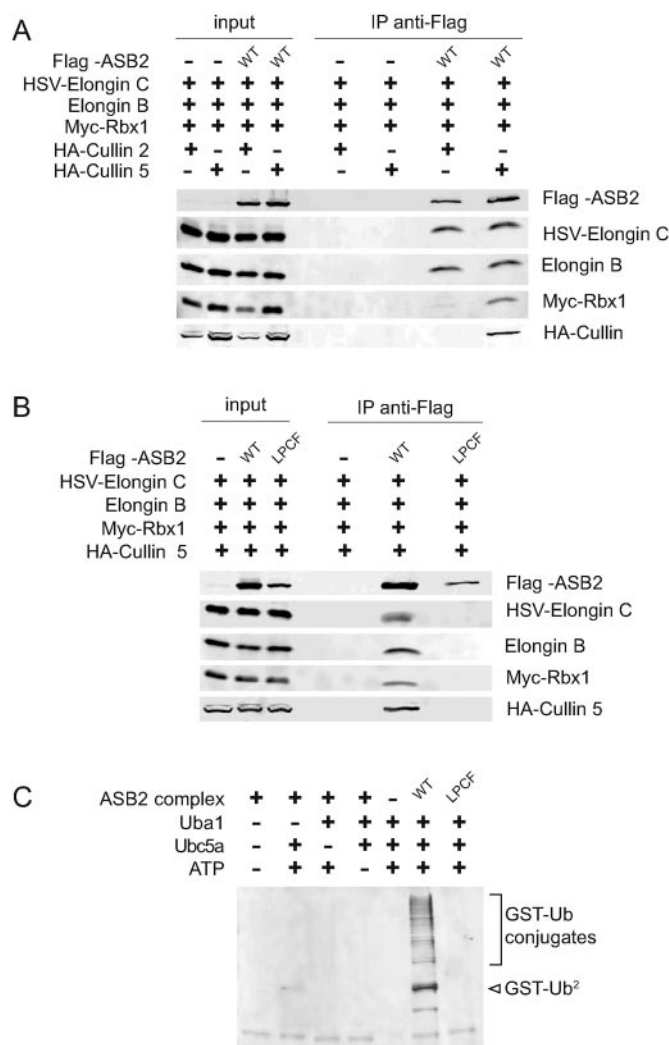
**FIG. 3. Elongin BC complex binding stabilized ASB2 protein.** HEK293 cells were transfected in 10-cm dishes with 0.2  $\mu$ g of the F-ASB2 (A) or the F-ASB2LPCF (B) expression vector either alone (*left panels*, -Elongin BC) or in cotransfection with 0.2  $\mu$ g of F-Elongin B and 2  $\mu$ g of HA-Elongin C expression vectors (*right panels*, +Elongin BC) as indicated. Forty-eight hours after transfection cells were treated with 100  $\mu$ g/ml cycloheximide (CHX) for various times (5, 10, and 24 h). 40- $\mu$ g aliquots of each whole cell extracts was immunoblotted with the indicated antibodies (Western blot). WT, wild type.

precipitated Cul5-Rbx1 observed in the absence of overexpressed Elongins B and C and (ii) the substantial decrease in polyubiquitination activity in the absence of Elongins B and C. Altogether, our results indicated that ASB2 could assemble with the Elongin BC complex and the Cul5-Rbx1 module to reconstitute an active E3 ubiquitin ligase complex.

**The Endogenous ASB2 Protein Copurified with Ubiquitin Ligase Activity**—We next explored whether endogenous ASB2 was involved in an E3 ubiquitin ligase complex. To this end, anti-ASB2 immunoprecipitations were carried out on untreated and RA-treated NB4 cells and assayed for their abilities to activate formation of polyubiquitin conjugates by the E2 ubiquitin-conjugating enzyme Ubc5 in the presence of the E1 ubiquitin-activating enzyme, GST-Ubiquitin, and ATP. As shown in Fig. 6, ASB2 copurified with ubiquitin ligase activity.

#### DISCUSSION

Targeting of regulators of hematopoiesis to proteasomal degradation is getting increasing attention. The ASB2 gene was originally identified as an RA-inducible gene whose expression recapitulates early differentiation events critical to induced differentiation of myeloid leukemia cells (19). In this article, we demonstrated that ASB2 interacts with the Elongin BC complex through the binding of Elongin C to the ASB2 BC box. Furthermore, we showed that Elongins B and C link ASB2 protein to a Cullin-Rbx1 module. Both Cullin 2 and 5 are known to be involved in ECS-type E3 ubiquitin ligase complexes. The fact that the ASB2-Elongin BC complex was capable of assembling with a Cul5-Rbx1 module but not with a Cul2-Rbx1 module to reconstitute a multiprotein complex with ubiquitin ligase activity suggests ASB2 may directly contact Cul5. Consistent with this idea, we observed an association of the Cul5-Rbx1 module with ASB2 in the absence of overexpressed Elongins B and C. Nevertheless, maximal ubiquitin ligase activity was achieved using the ASB2-Elongin BC-Cul5-Rbx1 E3 ubiquitin ligase complex. Interaction of the Elongin BC complex with the BC box markedly increased the stability of ASB2 protein, sug-

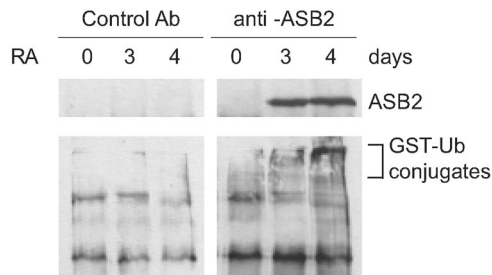
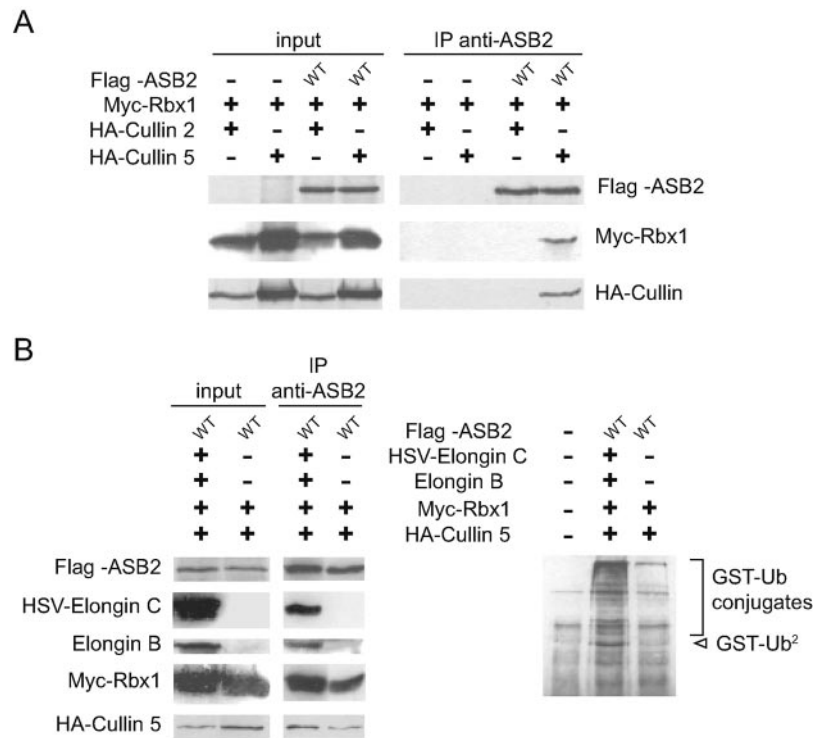


**FIG. 4. The ASB2-Elongin BC complex assembled with the Cul5-Rbx1 module to reconstitute a multiprotein complex with E3 ubiquitin ligase activity.** A, the ASB2-Elongin BC complex interacted with the Cul5-Rbx1 module. Sf21 cells were infected with baculoviruses encoding the proteins indicated. The lysates were immunoprecipitated (IP) using anti-FLAG antibodies. Crude extracts (*left panel*) and immune complexes (*right panel*) were separated by SDS-PAGE and immunoblotted with the indicated antibodies. B, the ASB2 BC box mutant that did not bind to the Elongin BC complex did not associate with the Cul5-Rbx1 module. C, the ASB2-Elongin BC-Cul5-Rbx1 complex had ubiquitin ligase activity. The cell lysates of A and B were subjected to anti-FLAG immunoprecipitation. The purified ASB2 complex was incubated with various combinations of Uba1, Ubc5a, GST-Ubiquitin (GST-Ub) in the absence or presence of ATP to assess its ability to stimulate ubiquitination by Ubc5 by Western blot using anti-GST antibodies.

gesting that it needed to be protected from degradation to assemble in an E3 ubiquitin ligase complex, as previously proposed for SOCS1 and VHL proteins (32, 40). Taken together, our results suggest that binding of the Elongin BC complex served to stabilize ASB2 and to provide a bridge to Cul5, which functions together with the RING finger protein Rbx1 to recruit and activate an E2 ubiquitin-conjugating enzyme. Although Elongin BC-binding proteins are thought to serve as the substrate recognition subunits of ECS ubiquitin ligases, few specific targets have been identified.

Cul5 has a role in the regulation of signal transduction (41). The Cul5-Elongin BC complex was shown to interact with a number of BC-box-containing proteins, such as MUF1, VHL, Elongin A, and SOCS1 (34). Interestingly, a role for Cul5 in different stages of the cell cycle has been suggested (42). Further-

**FIG. 5. The ASB2 protein associated with the Cul5-Rbx1 module.** A, the Cul5-Rbx1 module did interact with the ASB2 protein, whereas the Cul2-Rbx1 module did not. Sf21 cells were infected with baculoviruses encoding the indicated proteins. The lysates were immunoprecipitated (IP) using anti-ASB2 antibodies. Crude extracts (*left panel*) and immune complexes (*right panel*) were separated by SDS-PAGE and immunoblotted with the indicated antibodies. WT, wild type. B, ubiquitination activity is barely detected with the exogenous ASB2-Cul5-Rbx1 complex. The cell lysates were subjected to anti-ASB2 immunoaffinity purification (*left panel*). The purified ASB2 complex was incubated with Uba1, Ubc5a, GST-ubiquitin (GST-Ub) in the presence of ATP (*right panel*). The ability of this complex to stimulate ubiquitination by Ubc5 was assessed by Western blot using anti-GST antibodies.



**FIG. 6. ASB2 copurified with ubiquitin ligase activity in RA-treated NB4 cells.** Cells were treated for different times with  $10^{-6}$  M ATRA as indicated. 1-mg aliquots of each protein extract were immunoprecipitated with anti-ASB2 or control antibodies (*control Ab*). Immunoprecipitated proteins were fractionated by SDS-PAGE and analyzed for ASB2 by immunoblotting (*upper panel*) or assessed for their abilities to activate polyubiquitination by Ubc5 in the presence of Uba1 and ATP by immunoblotting with anti-GST antibodies (*lower panel*).

more, expression of Cul5 protein inhibits cell growth and retards cytokinesis by a mechanism that involves p53 (43). Although the mechanism by which Cul5 functions in these processes remains unclear, Cul5-containing ECS ubiquitin ligases have been shown to regulate the turnover of molecules involved in cell-cycle control. For example, a Cul5-containing ECS complex was shown to be involved in adenovirus-dependent ubiquitin-mediated degradation of p53 (44, 45) and in E2F1 ubiquitination (46). It is noteworthy that the ASB2 protein can inhibit cell growth and promote commitment in myeloid leukemia cells (19). The recombinant ASB2 protein can assemble into an active ECS-type E3 ubiquitin ligase complex. This together with the fact that endogenous ASB2 protein copurified with ubiquitin ligase activity in RA-treated NB4 cells suggests that, during induced differentiation of leukemic cells, the ASB2 protein may target specific proteins involved in blocking differentiation for ubiquitination and destruction by the proteasome machinery.

In this context, identification of key regulators of normal and leukemic hematopoiesis targeted for proteasomal degradation is of utmost importance. Indeed, the stability of the HOXA9 homeodomain protein was shown to be under the control of the Cul-4A ubiquitination machinery. The fact that

Cul-4A is an essential component of the SCF (Skp1-Cullin-F box)-type E3 ubiquitin ligase complex, which is analogous to the ECS-type ubiquitin ligase complex, emphasizes the proteolytic targeting of HOXA9 as a novel pathway to control normal and leukemia hematopoiesis (47). Furthermore, HOXA9 overexpression is leukemogenic in mice, arresting marrow progenitors at a promyelocytic stage of differentiation (48, 49). Another example is emphasized by the role of the ubiquitin proteolytic system in the regulation of the cell cycle through the degradation of the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> (50), which inhibits cycling of committed hematopoietic progenitors (51). Interaction of phosphorylated p27<sup>Kip1</sup> with Skp2, a member of the F-box family of proteins that associates into a SCF-type E3 ubiquitin ligase complex, leads to specific polyubiquitination and degradation of p27<sup>Kip1</sup> (52–54). Finally, it is remarkable that, through a functional recruitment of Elongin BC and the Cul2-Rbx1 complex to its SOCS box, SOCS1 accelerated proteasome-dependent degradation of the TEL-JAK2 fusion protein, which is the consequence of the t(9;12) translocation associated with human leukemia, resulting in abrogation of growth of TEL-JAK2-transformed cells (55, 56). Altogether, these and our results reinforce the view that protein degradation might be an important step in control of normal and leukemia hematopoiesis. These findings provide a basis for searching degradation pathways specific to leukemogenesis. This may lead to the development of new means to inhibit oncogenic mechanisms involving ubiquitination and degradation of specific proteins in the proteasome. Further work will investigate potential regulators of normal and leukemia myelopoiesis targeted by ASB2 for proteasomal degradation.

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**ASB2 Is an Elongin BC-interacting Protein That Can Assemble with Cullin 5 and Rbx1 to Reconstitute an E3 Ubiquitin Ligase Complex**

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