Protein Interaction Screening for the Ankyrin Repeats and Suppressor of Cytokine Signaling (SOCS) Box (ASB) Family Identify Asb11 as a Novel Endoplasmic Reticulum Resident Ubiquitin Ligase

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The ankyrin and SOCS (suppressor of cytokine signaling) box (ASB) family of proteins function as the substrate recognition subunit in a subset of Elongin-Cullin-SOCS (ECS) E3 ubiquitin ligases. Despite counting 18 members in humans, the identity of the physiological targets of the ASB proteins remains largely unexplored. To increase our understanding of the function of ASB proteins, we conducted a family-wide SILAC (stable isotope labeling by amino acids in cell culture)-based protein/protein interaction analysis. This investigation led to the identification of novel as well as known ASB-associated proteins like Cullin 5 and Elongins B/C. We observed that several proteins can be bound by more than one ASB protein. The additional exploration of this phenomenon demonstrated that ASB-Cullin 5 complexes can oligomerize and provides evidence that Cullin 5 forms heterodimeric complexes with the Cullin 4a-DBD1 complex. We also demonstrated that Asb11 is a novel endoplasmic reticulum-associated ubiquitin ligase with the ability to interact and promote the ubiquitination of Ribophorin 1, an integral protein of the oligosaccharyltransferase (OST) glycosylation complex. Moreover, expression of Asb11 can increase Ribophorin 1 protein turnover in vivo. In summary, we provide a comprehensive protein/protein interaction data resource that can aid the biological and functional characterization of ASB ubiquitin ligases.

Ubiquitination is a fundamental post-translational modification that regulates a variety of biological processes. The addition of the ubiquitin modifier to proteins is mediated by a sophisticated enzymatic cascade. In an ATP-dependent first step, the E1 enzyme is activated with ubiquitin, which is later transferred to an E2-conjugating enzyme. The final covalent attachment of ubiquitin to an ε-lysine residue on the targeted protein is facilitated by E3 ligases. Sequential reloading of E2s, occasionally with the help of an accessory factor (E4), can elongate the ubiquitin chain (1). The human genome encodes for >600 E3 ubiquitin ligases categorized into two major classes characterized by the presence of either a RING (really interesting new gene) or a HECT (homologous with E6-associated protein C terminus) domain.

The Cullin-RING-ligases (CRLs)2 are based on a crescent-shaped Cullin scaffold, where the C-terminal domain binds a RING protein (RBX1 or 2) while the N terminus binds the target recognition subunit of the complex. More than 300 different CRL complexes are estimated to exist in humans, but only a handful of them are well characterized. A subtype of CRLs is the Elongin-Cullin-SOCS box family of E3 ligases (ECSs). The hallmark of the ECSs is the existence of a target recognition subunit containing a C-terminally located motif called the SOCS box that has the ability to bind to an Elongin B/C heterodimer. A neighboring motif forms a bridge to the Cullin 2 or Cullin 5 scaffold (2). The different SOCS box-containing proteins have variable N-terminal sequences encoding for domains that mediate protein/protein interactions and determine substrate recognition by ECS ligases. Depending on the nature of such domains, ECSs can be divided in subfamilies such as the SOCS, which exhibit an SH2 domain and the larger ASB, which has 18 members that display ankyrin repeats in their N terminus (3).

Although they are all thought to act as ubiquitin ligases, specific protein ubiquitination targets have only been identified for a few ECS. We have demonstrated that SOCS2 targets the growth hormone receptor for ubiquitination whereas SOCS6

2 The abbreviations used are: CRL, Cullin-RING-ligase; ASB, ankyrin repeats and SOCS box; CHX, cycloheximide; ECS, Elongin-Cullin-SOCS; ER, endoplasmic reticulum; MS/MS, tandem MS; SFS, S-FLAG-STREP; SILAC, stable isotope labeling by amino acids in cell culture; SOCS, suppressor of cytokine signaling; PDI, protein disulfide isomerase.

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Background: The biological function of the ASB family of ubiquitin ligases is poorly characterized.

Results: A family-wide analysis of ASB-associated proteins by mass spectrometry identified novel interactors.

Conclusion: ASB proteins can associate in multimeric complexes. Asb11 ubiquitinates Ribophorin 1 and regulates its turnover.

Significance: This data resource will facilitate the characterization of the biological function of ASB proteins.
targets both the c-Kit and Flt3 tyrosine kinase receptors (4, 5). SOCS1 has been shown to promote the ubiquitination of JAK2, and VHL is responsible for the ubiquitination and degradation of HIF1α (6, 7). In contrast, we have very little information about the biological and molecular function of the ASBs. Only a few ASB family members have been tested for E3 ligase activity (8, 9), and just a small subset of substrates have been identified to date. For example, ASB2 is up-regulated by retinoic acid in acute promyelocytic cells where it targets filament A and B for proteasomal degradation, thereby regulating hematopoietic cell differentiation (10). ASB3 negatively regulates the TNF-R2 pathway leading to the ablation of JNK activation (11), whereas Asb9 is a regulator of creatine kinase B as well as the mitochondrial form of creatine kinase (12, 13). Furthermore, the zebrafish counterpart of the mammalian ASB11 (d-ASB11) is a positive regulator of Notch signaling in vivo and influences the neural progenitor compartment of the embryos. This effect is mediated by degradation of the Notch ligand Delta A and is dependent on the SOCS box of d-ASB11 (14). Zebrafish d-ASB11 was recently found to regulate regenerative myogenesis (15).

Considering their role in bridging protein substrates with E2 ligases, a better understanding of ASB interaction partners can potentially shed light on the enigmatic physiological actions of this family and unveil elusive facets of the ubiquitination machinery as a whole. Several recent publications show the power of the application of mass spectrometry in interactome studies for ubiquitin-related protein families such as deubiquitinating enzymes and Cullins (16). In this study we set out to map the interactome of the entire ASB family of putative E3 ligases using stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomic profiling. For this purpose, we immunoprecipitated Asb, isolated putative interactors, and subsequently identified them in a comprehensive mass spectrometry analysis. For the first time, a protein/protein interaction data set for the entire family of ASB proteins is presented.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and SILAC Labeling**—Human U2OS osteosarcoma cells, the human hepatoma cell HuH7, human epithelioid cervical carcinoma cells HeLa, human embryonic kidney cells HEK293T were grown in Dulbecco’s modified Eagle’s medium (DMEM +4500 mg/liter Glutamax™ and pyruvate; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cells were maintained at 37°C and 5% CO2. Inducible Abs U2OS cell lines were generated using the T-Rex system (Invitrogen) according to the manufacturer's instructions.

SILAC labeling of cells was performed by culturing four different cell lines (U2OS, HuH7, HEK293T, and HeLa) in DMEM without arginine, lysine, and leucine (Sigma-Aldrich) supplemented with glucose (4.5 g/liter), leucine (0.1 mg/ml; Sigma-Aldrich), and 10% dialyzed serum (Sigma-Aldrich). To the medium was added either light/natural (Arg6/Lys5), medium (Arg7/Lys4), or heavy (Arg10/Lys2) isotopes. Media supplementation was performed with 27.9 µg/ml arginine and 48.5 µg/ml lysine (Cambridge Isotope Laboratories). Labeled cells were transfected with Asb-expressing constructs. In all cases, Elongins B/C were co-transfected.

**Construction of Expression Vectors and Cell Transfection**—To generate ASB expression vectors, the open reading frame was cloned from an in-house library and inserted into expression vectors: pcMV-MyC (Clontech), pcDNA4/TO (Invitrogen) modified with an N-terminal S-FLAG-STREP (SFS) epitope or pCEP4 HisG, GFP tobacco etch virus. All cell lines were transfected with FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions. The cells were harvested 24–48 h after transfection.

**Lysate Preparation, Immunoblotting, and Antibodies**—Cell extracts were prepared using a lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40 with the addition of protease and phosphatase inhibitors. Western blotting was performed using standard protocols. The following antibodies were used in this work: FLAG (M2, F1804; Sigma-Aldrich, 1:1000), FLAG HRP-conjugated (M2, A8592; Sigma-Aldrich, 1:1500), Myc (9E10, sc-40; Santa Cruz Biotechnology, 1:1000), Myc (9E10, sc-40; Santa Cruz Biotechnology, 1:500), DDB1 (ab-21080; Abcam, 1:1000), HisG, (ab18184; Abcam, 1:1000), ubiquitin (6C1, sc-47721; Santa Cruz Biotechnology, 1:1000), GAPDH-HRP-conjugated (V-18, sc-2354; Santa Cruz, 1:1000), Ribophorin 1 (C-15, sc-12164; Santa Cruz Biotechnology, 1:500), Cullin 5 (sc-13014; Santa Cruz, 1:500), Cullin 4a (ab72548; Abcam, 1:500), and β-actin (AC-74; Sigma-Aldrich, 1:4000).

**Immunoprecipitations**—Small scale immunoprecipitation experiments were performed using 4–5 mg of whole cell protein extracts with either BSA-blocked (10 mg/ml) protein G-Sepharose beads, STREP-Tactin-Sepharose beads (IBA-Go), Myc (9E10) monoclonal antibody affinity matrix (Covance), anti-FLAG M2 (Sigma-Aldrich)-covered magnetic beads, or magnetic protein G-Dynal beads (Invitrogen). FLAG fusion proteins were eluted with 3× FLAG peptide (150 ng/µl; Sigma-Aldrich) and STREP-tagged proteins were eluted with biotin (biotin elution buffer; IBA-Go). For affinity purification of proteins on NHS beads, recombinant proteins (His-Asb3 and His-Asb7, recombinantly co-expressed with Elongins B and C in Escherichia coli) were immobilized on NHS-activated G-Sepharose beads, STREP-Tactin-Sepharose beads (IBA-Go). For affinity purification of proteins on NHS beads, recombinant proteins (His-Asb3 and His-Asb7, recombinantly co-expressed with Elongins B and C in Escherichia coli) were immobilized on NHS-activated agrose beads ( Pierce) according to the manufacturer’s instructions. Immunoprecipitation samples were analyzed by SDS-PAGE.

The immunoprecipitation experiments intended for mass spectrometry analysis were performed with 70–80-mg protein extracts. Extracts were filtered through a 0.22-µm filter (Millipore) before they were incubated with anti-FLAG M2 magnetic beads. After washing, bound proteins were eluted from the beads with a FLAG peptide and concentrated on a 10 K spin centrifugal filter (Millipore). Eluates from the three SILAC labeling conditions were pooled and resolved on 4–20% SDS-PAGE. The gel was stained with a Coomassie colloidal blue staining kit (Invitrogen) according to instructions, and the gel lane was excised in 10 fragments and subjected to in-gel proteolytic digestion (17). The excised gel fragments were reduced...
using 1 mM DTT, alkylated with 5 mM CAA, and trypsin-digested overnight (18). The peptides were extracted from the gel pieces and loaded onto StageTips prior to liquid chromatography MS/MS analysis.

**Mass Spectrometric Analysis and Identification of Asb Interactors**—All mass spectrometric experiments were performed on a nanoscale HPLC system (EASY-nLC; Thermo Fisher Scientific) connected to a hybrid linear ion-trap Orbitrap (LTQ-Orbitrap Velos; Thermo Fisher Scientific) equipped with a nanospray source (Thermo Fisher Scientific) (19). Each peptide sample was separated by online reverse phase C18 liquid chromatography on a 15-cm × 75-μm column, in-house packed with 3-μm C18 beads (ReproSil Pur-AQ, Dr. Maisch) with a 60-min gradient from 5 to 40% acetonitrile in 0.5% acetic acid. The effluent from the HPLC was directly electrospayed into the mass spectrometer. The mass spectrometer was operated in a data-dependent mode automatically switching between single and tandem mass spectrometry acquisitions. Survey full-scan mass spectra were acquired in the Orbitrap, and the most intense peptides were sequentially isolated and fragmented by higher energy C-trap dissociation. The data analysis was performed with the MaxQuant software as described (20) supported by Andromeda as the database search engine for peptide identifications (21). Parent ion and tandem mass spectra were searched by Andromeda against a concatenated target/decoy (forward and reversed) version of the IPI human database version 3.68 with a false discovery rate of 1%. Spectra were searched with a mass tolerance of 6 ppm in the single-mass spectrometry mode, 20 ppm in the higher energy C-trap dissociation tandem mass spectrometry mode, strict trypsin specificity, and allowing up to two missed cleavage sites.

**Cell Fractionation**—The isolation of rough endoplasmic reticulum (ER) was performed by differential centrifugation using the Endoplasmic Reticulum Isolation kit (Sigma-Aldrich) in cells transfected with a FLAG-Asb11 expression vector. Whole cell extracts, nucleus-depleted whole cell extracts, mitochondria-depleted microsomal fractions, and the rough ER fraction were analyzed by Western blotting for the presence of Asb11, the nuclear protein laminin A/C, the mitochondrial protein prohibitin, the cytosolic protein β-actin, and the ER resident protein PDI.

**Immunofluorescence Analysis**—Cell immunofluorescence was performed as described (22). Confocal images were captured using a Zeiss 710 (Carl Zeiss Microimaging Inc.) laser-scanning microscope with a 40× oil-immersion objective. Fluorescent images were also captured using an upright Axio Imager A2 microscope (Carl Zeiss). Images were processed using Adobe Photoshop CS4.

**Duolink Assay**—To verify the in situ co-localization of exogenous Asb11a and endogenous Ribophorin 1, a Duolink assay (Olink Bioscience) was performed according to the manufacturer’s instructions. The assay is based on antibody binding to the proteins of interest. Secondary antibodies are conjugated to small oligonucleotides (a plus and a minus probe), which when situated in close proximity can prime a subsequent DNA amplification reaction performed directly on the adherent cells on the coverslip. Fluorescently labeled nucleotides are incorporated into the amplified DNA and are easily visible as distinct dots under a microscope. In brief, U2OS cells grown on glass coverslips were transiently transfected with an expression vector encoding a FLAG-tagged version of Asb11a or left untreated. Anti-FLAG and anti-Ribophorin 1 antibodies were applied to the FLAG Asb11-transfected cells or to untransfected cells as a negative control. Fluorescent images were captured using an upright Axio Imager A2 microscope (Carl Zeiss).

**Cycloheximide Chase Assays**—To monitor protein turnover, cycloheximide (CHX) (Sigma) was added to cells in a final concentration of 100 μg/ml to inhibit protein translation. CHX was added at t = 0, and cells were harvested at various time points for Western blot analysis.

**In Vitro Ubiquitination Assays**—The assays were performed as described previously (5). Briefly, U2OS cells were transiently transfected with indicated constructs and immunoprecipitated with magnetic FLAG-M2 beads. UBE2D1 was used as standard E2 enzyme. Aliquots of the reactions were stopped with 2X Laemmli sample buffer and boiled prior to SDS-PAGE analysis. For isolation of Myc-Ribophorin 1, the immobilized Asb complexes were eluted from the M2 beads using a 5×FLAG peptide (Sigma) and recovered by a brief centrifugation. The eluates were incubated overnight with anti-Myc antibody and 50 μl of BSA-blocked protein G-Sepharose beads. The immunoprecipitates were washed four times in TBS buffer and boiled in SDS-PAGE loading buffer before analysis on SDS-PAGE followed by Western blotting.

**In Vitro Neddylation**—The in vitro neddylation step was included before ubiquitination when indicated. Proteins were left in 10 μl of HEB buffer after immunoprecipitation, and the NEDD8 system from Boston Biochem was added: 0.55 μg of NEDD8 E1 (APPBP1/UBA3), 0.93 μg of NEDD8 E2 (His6-UBE2F), 4.4 μg of NEDD8, and 2 μl of NEDD8 reaction buffer (500 mM HEPES, pH 8, 500 mM NaCl), and 1 mM ATP. The reaction was incubated for 40 min at 37 °C with shaking. After incubation the reaction was used directly in ubiquitination assays.

**In Vivo Ubiquitination Analysis**—U2OS cells were transfected with the indicated constructs. MG132 (25 μm final concentration) was added 24 h after transfection, and cells were lysed after 5 h of proteasome inhibition and analyzed by SDS-PAGE and Western blotting.

**RESULTS**

A Family-wide Proteomic Screen to Identify the ASB-interacting Proteins—To increase our understanding of the possible functions of the members of the ASB family, we set out to identify interaction partners. A strategy was developed based on the expression of tagged ASBs in mammalian cells followed by immunoprecipitation with epitope-specific antibodies and the identification of ASB-associated proteins by liquid chromatography coupled to tandem MS (LC-MS/MS) analysis. In brief, the open reading frames encoding ASB1–18 were inserted into a pcDNA4TO backbone (Fig. 1). A triple epitope, SFS, was inserted upstream and in-frame of these genes. A control vector and constructs encoding the baits were transiently transfected into U2OS cells SILAC labeled with either light/natural (Arg0/Lys5), medium (Arg2/Lys4), or heavy (Arg10/Lys8) isotopes (Fig.
The ASB Family Interactome

1A). Cell lysates were prepared and mixed with SILAC-labeled extracts from HuH7, HEK293T, and HeLa cells to expand the pool of potential interactors. Immunoprecipitates were pooled, fractionated by SDS-PAGE, trypsin-digested, and analyzed by LC-MS/MS, a method that in the past has proven to be successful in quantitative proteomics (23).

The ASB family members are putative E3 ligases, and a subset of tested ASBs (Asb 1, 2, 6, 7, and 12) (8, 9) have proven to bind...
to Cullin 5. Therefore, as a positive control to validate our vectors and pulldown strategy, we measured the Cullin 5 presence in immunoprecipitated material obtained for the entire Asb family. Cullin 5 was efficiently immunoprecipitated with all Asb constructs tested (data not shown). Given these results, we utilized Cullin 5 association as a quality control of the immunoprecipitation assay prior to the MS analysis for all ASBs. Quantitative mass spectrometry analysis was performed using SILAC-labeled cells.

The MaxQuant software was used to analyze the LC-MS/MS data, identify SILAC triplets, quantify relative abundance changes, and assign MS/MS spectra for protein identification. The peptides originated from ASB-specific pulldowns were recognized by their isotope composition, and the ratios between peptides originated from control immunoprecipitations (Arg<sup>0</sup>, Lys<sup>0</sup>), and those specifically bound to two different Asb family members were calculated. The ASB-specific interacting protein candidates were defined as those specifically enriched in Asb versus control immunoprecipitate, defined as protein with a -fold ratio to controls within the upper 5% percentile of the distribution and with a minimal -fold change of 50%. The list of candidate interactors is shown in Table 1, whereas the full dataset is shown in supplemental Data Sets S1 and S2. Noticeably, with the defined threshold, we detected Cullin 5 as an ASB-interacting protein in the vast majority of cases, but also Elongins B/C complex in 68 and 59% of cases, respectively, and RBX2 in 23% of all pulldown experiments (Fig. 1B). This suggests that the threshold used is able to distinguish expected interacting proteins but is sufficiently strict to exclude false positives. Furthermore, the presence of proteins like ubiquitin, NEDD8, and CAND1 underscores the notion that the expressed tagged forms of ASBs form active and dynamic E3 complexes (24). Importantly, we were able to confirm the association of ASB9 to the creatine Kinase b as well as the association of ASB9 to the ubiquitous mitochondrial creatine kinase, as reported previously (12, 13).

**Asb Proteins Assemble in Noncanonical E3 Complexes**—An overview of the interactome data for the ASB family (Table 1) shows that although most of the 366 proteins identified as putative ASB interactors are unique to specific ASB proteins, there are several interactors that are found in more than one of the individual pulldowns. As expected, these groups include Cullin 5, and Elongins B and C, but also the co-chaperone protein BAG2 (25) was identified as associated with ASB 1, 3, 4, 5, 8, 10, 14, 15, 16, 17, and 18. This is probably explained by BAG2 role as a cofactor for the cystolic chaperone HSP70, which likely participates in the folding of overexpressed ASB proteins. Indeed, other putative interactors such as BAG3, DNAJA1, DNAJA2, STIP1, CANX, and CRYAB also have protein folding functions and may not be of relevance for ASBs ubiquitin ligase activity. Likewise, we also identified a number of secreted proteins such as annexin 2 and fibrinogen subunits among our candidate interactors whose cellular localization does not match that of Asbs, all of which are intracellular proteins. Therefore, proteins with chaperone-related functions as well as extracellular proteins should not be considered as biologically significant interactors until further evidence is collected.

After filtering the data to exclude candidate interacting proteins involved in protein folding or belonging to the class of highly abundant cellular proteins such as the ribosome or cytoskeleton components, we drew a protein/protein interaction network map (Fig. 1B). The analysis of the network shows that most candidate interacting proteins bind only one ASB, but some interact with several ASB proteins. As expected, this category includes core components of the ECS ubiquitin ligase complexes such as Cul5, TCEB2, TCEB1, and RBX2, which display the highest degree of centrality in the network. In addition, several proteins with functions not related to ubiquitination also interact with more than one ASB, suggesting some degree of promiscuity within the family for target recognition. This is the case with Ribophorin 1, which is listed as an interactor for both ASB5 and ASB11. Alternatively, ASB proteins could form oligomers when bound to targets to promote ubiquitination. This concept is supported by the presence of ASB family members identified as interactors of other ASBs, e.g. ASB3 co-purified in ASB6 pulldown, ASB4 co-purified in ASB7 pulldown and ASB1 in ASB5 pulldown (Table 1).

In recent years, E3 oligomerization has emerged as an important modus operandi of some CRL to regulate their ubiquitination activity in vivo (26, 27), but whether Cullin 5 ECS ligases oligomerize is not known. To test this, we set to confirm the ASB3/ASB6 and the ASB4/ASB7 interactions previously identified by mass spectrometry analysis. Recombinant His-tagged ASB3 (Fig. 2A) and His-tagged ASB7 (Fig. 2B) were immobilized on a NHS-agarose and used in pulldown experiments using extracts from cells expressing Myc-ASB6 and FLAG-ASB4, respectively. We observed a clear retention of the epitope-tagged ASB proteins. These interactions were confirmed in U2OS cells co-transfected with SFS-ASB3 and Myc-ASB6 and SFS-ASB4 and Myc-ASB7, respectively. In these experiments Myc-tagged proteins clearly co-purified with immunoprecipitated FLAG-fusion proteins (data not shown). Although the most likely explanation for the co-purification of ASBs is their ability to recognize the same target, we cannot exclude the possibility that the ASBs interact directly to regulate their turnover. We tested this hypothesis by studying whether co-expression of interacting ASBs result in altered protein turnover using CHX chase experiments. We found no
effects on ASB3 turnover upon overexpression of ASB6 (Fig. 2C). Therefore, it appears that the association between the two ASB proteins serves a scaffolding nature instead of a direct catalytic function.

### TABLE 1

| Bait  | Putative interacting proteins                                      |
|-------|-------------------------------------------------------------------|
| Asb1  | TCEB2; SSRP1; ERAB; HMG1B1; RSG; CAND1; ATP2A2; PTS; TCEB1; MRPP1; CPS1; SSF4; EFTUD2; ARL1; CUL5; SNRPD1; PNFI |
| Asb2  | H3.3A; HMG1B1; HNRNPAP1; RAN; SSRP1; NT5B; GIG3; VIM; ALP11; RPS2; WDR61; PNFI; HNRNP1; HNRNPX; HNRNPAP2B1; SNRPD1; EF4B; C11orf14 |
| Asb3  | CUL5; TCEB1; TCEB2; ASB3; PAGM5; SRP14; ERH; THRAP3; ASPEP; BCLAF1; RPL38; HLC3; AG3; CLASP2; RCN2; SLC2A5; NONO; DAL1; ATP5F1; AOP2; ATP5C; PSF; RPS2T |
| Asb4  | CUL4A; TFCR; HSPB8; ASB4; TUBB8; CUL5; TUBB3; DNAJB5; FARS3; STA1; STUB1; DNAJB4; TUBB4; HSPB1; HSPD1; RPS27L; RBX2; TCEB1; ASB12 |
| Asb5  | CUL5; TCEB2; HIF1AN; SLIRP; ASB5; ASB1; LRP130; HYRC; CRYA2; TCEB1; ATP5C; ATP2A2; RSG; PNFI; RAB1; PPZC8; MDU1; PTS; ENO2; ATP5B; AKR1B1; DNAJ2; GCNL1 |
| Asb6  | HIF1AN; ASB6; RBX2; ASB3; CUL5; SLC2A5; FG5; HSPB1; TCEB2; FGR; FGA; CAPDS2 |
| Asb7  | CUL5; RBX2; TCEB1; ASB7; TCEB2; HIF1AN; ASB4; VCL; FGR; FGA; GAPA; ANAB; CCT7 |
| Asb8  | ZCH6; CUL5; HIF1AN; NEDD8; TCEB1; TCEB2; PCNA; TMED10; ASB8; TUBB3; AMBRA1; KIF26B; SLC2A13; SLC2A6 |
| Asb9  | TCEB2; ASB9; H3.3A; CKB; CKMT1; H2BFD; H4A; TCEB1; H2AFZ; VIM; ENO2; HIF2; RAB1A; PGAM1; ACR3; CUL5; TLNI; ANXS5; AS1; AC02; AKR1B1; LDHA; ANX2; PKG1; PRPSAP1 |
| Asb10 | CUL5; RBX2; STUB1; AP1G1; TCEB1; TCEB2; ASB10; EMD; PGAM4; SLC2A5; SLC2A5; TUFM; SLC2A3; CPV1; HSPB1; HSPA1; ARL1; HSPA1A; TUBB6; RPL38; UBC; SUPPLEMENTAL DATA SET S1; RPS10 |
| Asb11 | ATP5B; ASB11; RN1 |
| *Asb11| DDOST; ASB11; MDU1; PSM2;PSM2D1; ERLIN1; RN1; HC3; TBPI; PRPS1; G2AN |

Our interactome analysis also identified enriched amounts of Cullin 4a and 5 from heterodimeric complexes in vivo. To verify the interaction biochemically, we performed a co-immunoprecipitation analysis. U2OS cells were transiently transfected with SFS-ASB4 or control vector. Anti-FLAG antibody was used to precipitate the SFS-tagged protein. As shown in Fig. 2D, the immunoprecipitation of SFS-ASB4 resulted in retention of endogenous Cullin 4a, and noteworthy, the Cullin 4a-specific adaptor protein DBD1 was also present in the complex. As anticipated, the analysis also shows that Cullin 5 is part of the complex (Fig. 2E). These results demonstrate the association between a Cullin 4a- and a Cullin 5-based E3 complex. To investigate whether ASB4 can target Cullin 4a and thereby affect its turnover, we performed a CHX chase experiment in U2OS cells transiently transfected with FLAG-ASB4 or control vector and a GFP-tagged version of Cullin 4a (Fig. 2F). CHX was added at t = 0, and the protein level of GFP-Cullin 4a was monitored at the indicated time points. We observed that ectopic levels of ASB4 do not destabilize Cullin 4a. Instead, the turnover of Cullin 4a is reduced in cells overexpressing ASB4, indicating that complex formation between the proteins may have functional relevance. Interestingly, our proteomic analysis confirms the presence of DDB1 with ASB8, 10, 13, and 17 immunoprecipitates, suggesting that additional ASB complexes may also interact with the Cullin 4a-DDB1 complex.

**Ribophilin 1 as a Bona Fide Interactor of ASB11**—Ribophilin 1, encoded by the rpn1 gene, was identified in the ASB11 pulldown experiment with a ratio of 3.62 relative to control (supplemental Data Set S1). Ribophilin 1 has an established function within the OST complex, which N-glycosylates newly synthesized proteins in the rough endoplasmic reticulum. To confirm the protein interaction data, we repeated the analysis using a FLAG-tagged ASB11 expression vector. In this new analysis we again identified Ribophilin 1 as a putative interactor of ASB11 (6.48-fold induction, compared with control; supplemental Data Set S1). We also used recombinant His-tagged ASB11 immobilized on agarose and incubated with U2OS extracts containing either exogenous or endogenous levels of Ribophilin 1. As shown in Fig. 3, A and B, Ribophilin 1 binds specifically to the immobilized ASB11 and not to control beads. Thus, ASB11 is able to form a complex with Ribophilin in vitro, confirming the LC-MS/MS protein interaction data. These results also suggest that ASB11 may locate to the rough ER were Ribophilin 1 resides. Protein secondary structure predictions identified a putative transmembrane region in ASB11 that is also associated with the other ASB, thus the other ASB found to interact with Ribophilin 1. Due to the hydrophobic motif found in the Ribophilin 1 N terminus, co-localization and thus interaction with Ribophilin 1 seemed plausible. Therefore, we next examined the subcellular localization of FLAG-ASB11 in U2OS cells (Fig. 3C) and found a typical ER distribution. Confocal immunofluorescence analysis demonstrated a co-localization with PDI, a bona fide ER marker. Furthermore, cellular fractionation experiments show that the rough ER subcellular fractions contain FLAG-ASB11 as well as PDI, a well known ER marker, but are negative for nuclear protein laminin A/C and prohibitin, a mitochondrial protein (Fig. 3D). Overall, these experiments demonstrate...
that Asb11 locates to the ER, the established localization of endogenous Ribophorin 1.

To confirm the ASB11 and Ribophorin 1 association, we also studied their cellular location by microscopy. The analysis of exogenously expressed EGFP-Asb11 and Cherry-ASB11 also revealed an overlap with endogenously expressed Ribophorin 1 (Fig. 3E). In addition, a Duo-link assay, monitoring close proximity of two proteins in situ by means of antibody binding, further corroborated the interaction between the two proteins. The center panel in Fig. 3F shows the generation of high intensity fluorescent dots indicative of close proximity of the antibodies bound to FLAG-ASB11 and Ribophorin 1 in U2OS cells. This is not seen in untransfected U2OS cells (left panel). The right panel shows the interaction between FLAG-ASB11 and endogenous Cullin 5 that serves as a positive control. Overall, these findings support the protein interaction data and further substantiate that ASB11 and Ribophorin 1 form a complex within the ER compartment in human cells.

Mapping Ribophorin 1 Interaction with Asb11—Having confirmed the interaction between ASB11α and Ribophorin 1 in vivo, we next attempted to gain insight into the regions of Asb11 important for Ribophorin 1 binding. We generated a series of ASB11 deletion mutants (Fig. 4A) and transiently co-transfected U2OS cells with a Myc-tagged version of Ribophorin 1 and the indicated constructs. Fig. 4B shows a repre
sentative experiment demonstrating that complex formation between Ribophorin 1 and ASB11 is dependent on ankyrin repeat 4 (residues 159–191), as ASB11 mutants lacking this segment fail to co-immunoprecipitate with Ribophorin 1. It cannot be excluded that additional regions in Asb11 can also participate in the interaction with Ribophorin 1. To elucidate this, further mutational analyses are required. However, we established that the SOCS box is dispensable for binding to Ribophorin 1 as the ASB11/H9251/H9004 SOCS retains its interaction with Myc-Ribophorin 1.

Asb11α Has Intrinsic Ubiquitin Ligase Activity and Targets Ribophorin 1 for Ubiquitination—The ASB protein family is presumed to be part of E3 complexes. However, ubiquitin ligase activity has so far only been demonstrated for a small subset of ASB proteins. In Fig. 5A, we demonstrate that ASB11, immunoprecipitated from mammalian cells, exhibits ubiquitination activity in vitro as judged by its capacity to promote the appearance of high molecular mass conjugates of HA-ubiquitin. To investigate the ubiquitination activity of ASB11 in vivo, we transfected U2OS cells with either a control SFS vector or one of the two Asb11 isoforms. The proteasome inhibitor MG132 was added to cells 5 h before harvest to allow accumulation of ubiquitinated proteins. Fig. 5B shows a marked increase of ubiquitinated proteins in cells overexpressing both the ASB11α and β isoforms, suggesting that they are both active ubiquitin E3 ligases in vivo.
dependent on the presence on an intact SOCS box. Furthermore, we demonstrate that the enzymatic activity is consistent with the previous results, we found that both ASB11 isoforms can promote ubiquitination activity measured as the appearance of high molecular mass HA-ubiquitin conjugates.

We next set out to dissect whether ASB11 isoforms can ubiquitinate Ribophorin 1 in vitro. U2OS cells were transiently transfected with expression vector encoding Asb11α mutants deleted for the ankyrin repeats or the SOCS box as indicated in A. Cells were co-transfected with Myc-Ribophorin 1 or empty Myc vector, serving as control. Immunoprecipitates (IP) were resolved on SDS-PAGE and proteins visualized by Western blotting (WB) with the indicated antibodies.

**DISCUSSION**

To gain a better understanding of the function of the Asb proteins, we analyzed the interactome of all 18 Asb members using a combination of SILAC labeling with high end LC-MS/MS analysis. We found that the entire Asb family co-purifies with Cullin 5, confirming the notion that all 18 members are ubiquitin ligases. In addition, we identified putative protein interactors for all Asb family members. It is outside the scope of this work to validate all possible interactions identified by our analysis. We have instead focused on highlighting some of the more novel findings to illustrate the utility of our approach to unveil unique aspects of ASB biology. Specifically, we demonstrated that Asb proteins oligomerize in vivo, and we described Asb11 as a novel ER resident ECS ligase that targets Ribophorin 1 for ubiquitination.

Our mass spectrometry analysis as well as co-immunoprecipitation experiments demonstrated the capacity of several Asb proteins to associate in vivo. Deviation from the canonical CRL architecture is emerging as an additional layer of complexity in the regulation of CRL-mediated ubiquitination. The distance between acceptor lysines and E2 active site can vary considerably due to difference in substrates, conformations, and the number of ubiquitin added to the substrate. To accommodate this, the activity of CRLs is controlled by various means; i.e. neddylation, which introduces a flexible hinge in the Cullin scaffold (28). Interestingly, recent findings suggest that dimerization is also an important mode of controlling the CRL activity (26). The biological justification for dimerization may in part be to increase avidity for the target proteins or to increase local concentration of the reactants needed for ubiquitination. However, also optimal positioning of E2s and substrate may play a role. The geometry of dimeric complexes poses different possibilities for generating ubiquitin chains with different topologies, as demonstrated for the dimerization of SCF-Cdc4 complexes, which does not change the affinity for the substrate Sic1, but allows for optimal ubiquitin chain initiation and elongation. In some cases, the CRL dimerization has proven to be essential in the in vivo setting, emphasized by the finding that CRLs mutated in their substrate recognition subunits but which have retained their dimerization properties can functions as dominant negative in vivo (29, 30). As for the case of the ASB3/ASB6 and ASB4/ASB7 dimers here described, we have no evidence of target-induced dimerization, as the putative interactors identified do not contain overlapping proteins.
The ASB Family Interactome

FIGURE 5. Asb11α has intrinsic ubiquitination activity in vitro and in vivo and ubiquitinates Ribophorin 1. A, U2OS cells were transiently transfected with SFS-Asb11α or left untreated. SFS-tagged proteins were immunoprecipitated with anti-FLAG antibody and subjected to in vitro ubiquitination with E1 and E2 enzymes, HA-ubiquitin and ATP (0.8 mM) alone or in combination with an ATP reconstitution system (0.8 mM ATP, 0.8 mM MgCl2, 25 mM creatine phosphate, and 0.03 ng of creatine kinase). The reactions were analyzed on SDS-PAGE and immunoblotted with anti-HA and anti-FLAG antibodies. B, U2OS cells were transiently transfected with constructs encoding the SFS-Asb11α and β isoforms or empty control vector. MG132 was added 5 h prior to harvesting the cells in lysis buffer. Proteins were separated on SDS-PAGE and immunoblotted with anti-ubiquitin antibody to examine the amount of high molecular mass ubiquitin-protein conjugates. GAPDH served as a loading control. C, U2OS cells were transiently transfected with expression constructs encoding Asb11α, β, or the Asb11α-ΔSOCS mutant proteins fused to a FLAG epitope. Aliquots from whole cell extracts were assayed by immunoblotting (WB) with anti-Myc and anti-β-actin antibodies. FLAG-tagged proteins were immunoprecipitated (IP) with anti-FLAG antibody and were subsequently neddylated in vitro. The reactions were used in an ubiquitination assay with the addition of E1, E2, HA-ubiquitin, and ATP. The proteins from the ubiquitination reaction were eluted from the beads with 3×FLAG peptide and immunoprecipitated with anti-Myc antibody (9E10). The α-Myc immunoprecipitates were analyzed with anti-Myc or anti-HA antibodies to assess Ribophorin 1 ubiquitination. D, U2OS cells were transiently transfected with SFS-Asb11α or empty vector control. Cells were harvested in lysis buffer, and endogenous Ribophorin 1 levels were analyzed by Western blotting. E, U2OS cells transformed with an SFS-Asb11α encoding tetracycline-inducible construct were grown with or without doxycycline to induce or repress the transgene, respectively. Cycloheximide was added at t = 0, and the amounts of Ribophorin 1, Asb11, and GAPDH were analyzed by Western blotting. GAPDH served as a loading control.

suggesting that if they bind to the same targets they will do it with different affinities.

CRLs have not only been demonstrated to homodimerize through their substrate recognition subunits or the attached Nedd8 moiety (31, 32), but it was recently shown that they can exist as heterodimeric E3 Cullin-RING complexes. For example, ubiquitination of both Jak2 and Jak3 involves the corporative action of a SCF complex and an ECS complex involving Skp2 and ASB2 (33, 34). We have extended these findings by demonstrating that Asb complexes can heterodimerize with DDB1-Cullin 4α RING ligases. Cullin 4 is preserved from yeast to mammals (35) where it has essential functions (36). Cullin 4α has been shown to interact with the DDB1 as the adaptor protein (37) and DCAF1, containing WD40-repeat proteins acting as substrate recognition subunits for the Cullin 4-DDB1 E3 ligases (35). Recently, a proteomic screen based on Cullin 5 has identified both Cullin 4a and Cullin 4b as specific interactors of Cullin 5 (38), providing independent validation of our findings and suggesting an in vivo function of Cullin 4 and Cullin 5 oligomers. Consistently, we found that Cullin 4α co-purifies with ASB4 (Table 1), and we were able to confirm this interaction biochemically (Fig. 2, D and E). DDB1 and Cullin 5 were also found to co-immunoprecipitate with ASB4 and Cullin 4a, further substantiating that Cullin 4a and Cullin 5 assemble active heterodimeric complexes in vivo. Importantly, we found that increased ectopic levels of ASB4 decrease Cullin 4a turn-over. Further experiments are warranted to determine the physiological consequences of these findings.

In this study, we described a novel ER resident EC5S ligase ASB11 and identified one of its targets in that cellular compart-
Asb11-mediated ubiquitination of Ribophorin 1 may contribute to proteasomal degradation. In vitro ubiquitination assays show that Ribophorin 1 can be ubiquitinated by the two Asb11 isoforms, but no evidence for polyubiquitination was observed. A chain of four or more Lys48-linked ubiquitins serves to target proteins for the 26S proteasome. The analysis of the ubiquitination reaction failed to show Asb11-induced formation of high molecular mass ubiquitin polymers of Ribophorin (Fig. 5C), in contrast with the ability of Asb11 to modulate Ribophorin 1 turnover in vivo (Fig. 5D). One possible interpretation of these results is that Cullin 5/Asb11 monoubiquitinate Ribophorin whereas the subsequent polyubiquitination may be driven by yet unidentified E3 ligases. Indeed, it has been demonstrated that formation of polyubiquitin polymers may involve two separate reactions, one priming event leading to protein monoubiquitination and independent elongation events (41), i.e. proliferating cell nuclear antigen, which is monoubiquitinated by Rad18/Rad6 and Lys63-linked polyubiquitinated by the heterodimer RAD5/MMS2-UBC13 (42, 43). In other instances a single E3 is capable of driving both events (44). Therefore, it is also possible that Asb11 can drive polyubiquitination of Ribophorin 1 in vivo but not in vitro, perhaps through the recruitment of different E2 enzymes to the complex. A detailed understanding of the mechanisms whereby ASB11 regulates Ribophorin 1 turnover and its functional consequences would require additional studies.

In summary, we provide for the first time protein/protein interaction data for the entire ASB family of ubiquitin ligases. This has led to the discovery of a novel ER-associated E3 ligase, Asb11 that targets the OST component Ribophorin 1 for ubiquitination and modulates its turnover in vivo. We also demonstrate that ASBs can form supramolecular complexes with other Cullin RING ligases in vivo, suggesting the existence of additional regulatory mechanisms for E3 ligases. This study will greatly facilitate the future biological and functional characterization of the ASB protein family.

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