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The UBX Protein SAKS1 Negatively Regulates Endoplasmic Reticulum-associated Degradation and p97-dependent Degradation*[S]

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Endoplasmic reticulum-associated degradation (ERAD) is an essential quality control process whereby misfolded proteins are exported from the endoplasmic reticulum and degraded by the proteasome in the cytosol. The ATPase p97 acts as an essential component of this process by providing the force needed for retrotranslocation and by serving as a processing station for the substrate once in the cytosol. Proteins containing the ubiquitin regulatory X (UBX) ubiquitin-like domain function as adaptors for p97 through their direct binding with the amino terminus of the ATPase. We demonstrate that the UBX protein SAKS1 is able to act as an adaptor for p97 that negatively modulates ERAD. This requires the ability of SAKS1 to bind both polyubiquitin and p97. Moreover, the association between SAKS1 and p97 is positively regulated by polyubiquitin binding of the UBX protein. SAKS1 also negatively impacts the p97-dependent processing required for degradation of a cytosolic, non-ERAD, substrate. We find SAKS1 is able to protect polyubiquitin from the activity of deubiquitinases, such as ataxin-3, that are necessary for efficient ERAD. Thus, SAKS1 inhibits protein degradation mediated by p97 complexes in the cytosol with a component of the mechanism being the ability to shield polyubiquitin chains from ubiquitin-processing factors.

The endoplasmic reticulum (ER)[2] is primarily responsible for the synthesis, folding, and *N*-glycosylation of proteins destined for the secretory pathway (1). To ensure proper protein quality control, there is a system of chaperones and lectins in the ER meant to monitor the appropriate folding and glycosylation of molecules. Proteins that do not pass these checkpoints are subjected to endoplasmic reticulum-associated degradation (ERAD) whereby misfolded proteins are identified in the ER and retrotranslocated into the cytosol, where they are then degraded by the proteasome (2). The core cytoplasmic component of retrotranslocation is the homohexameric ATPase p97, also known as valosin-containing protein (VCP), which provides the force needed to extract the substrate from the ER (3, 4). This is an evolutionarily conserved molecule that is among the most abundant cytosolic proteins. Mutations in p97 create disease phenotypes in multiple tissues, and mutations are associated with the autosomal dominant disorder inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (5). These are aggregate disorders associated with loss of appropriate protein clearance (6).

Retrotranslocation via p97 generally occurs concomitant with substrate ubiquitination on the cytosolic side of the ER membrane via ER resident ubiquitin E3 ligases (7). Recently, it has been appreciated that p97-associated deubiquitinases are also necessary for this process, although it is not yet certain what function they perform (8). Cytosolic p97 forms multimolecular complexes that contain ubiquitin ligases, chain elongation factors (E4 ubiquitin ligases), and deubiquitinases that process substrates once they have exited the retrotranslocon, modifying their ubiquitin chains (9, 10). Once extracted from the ER and properly ubiquitinated, substrates are sent to the proteasome either through interactions of p97 with the proteasome or via cytosolic proteasomal targeting factors such as ubiquilin/plic1 or hHR23A/B (11). p97 therefore acts as both the driving force for retrotranslocation from the ER and as a cytosolic processing center for substrates on their way to the proteasome. Notably, p97 is also required for the processing of certain cytosolic substrates for proteasomal degradation that do not originate in the ERAD pathway, and this occurs at least in part through an unfoldase activity of the hexamer (12, 13).

p97 utilizes multiple accessory proteins during this process with two of the best characterized being the heterodimeric adaptor proteins Ufd1 and Npl4, which link p97 to polyubiquitin chains and participate in the processes of ERAD and nuclear envelope formation (3, 14, 15). Among the adaptor proteins that regulate the interactions of p97 with various pathways are the ubiquitin-like ubiquitin regulatory X (UBX) domain-containing proteins. UBX domains are evolutionarily conserved and are found in multiple human proteins that interact directly with p97 to serve as adaptors, although their roles are largely uncharacterized (16–18). The ER transmembrane UBX protein erasin acts as a positive regulator of ERAD (19, 20). The cytosolic UBX protein SAKS1 is known to form

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[2] The abbreviations used are: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; UBA, ubiquitin-associated; UBX, ubiquitin regulatory X; VCP, valosin-containing protein.

complexes with components of the cellular degradation machinery, but a role in ERAD has not been established (21, 22).

Here, we show that SAKS1 is a negative regulator of endoplasmic reticulum-associated degradation. This requires the SAKS1 UBX domain to bind p97 and its UBA domain to bind substrates through polyubiquitin. SAKS1 is regulated by polyubiquitin binding, which unmasks the UBX domain for interaction with p97. We also find that SAKS1 is able to inhibit the degradation of a cytosolic p97-dependent substrate indicating the importance of this UBX protein in multiple pathways. SAKS1 protects polyubiquitin chains from deubiquitinases *in vitro,* and its substrate stabilization is partially reversed by overexpression of ataxin-3, providing mechanistic insight into the regulatory effect of SAKS1 toward p97-dependent protein degradation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—VCP/p97 and calnexin polyclonal antibodies were purchased from Cell Signaling Technology. Monoclonal antibodies *versus* ubiquitin, protein-disulfide isomerase, and VCP/p97 were purchased from BD Biosciences. Polyclonal antibody *versus* SAKS1 was obtained from Millipore. FLAG-M2 antibody and FLAG peptide were purchased from Sigma. The monoclonal antibody HA.11 *versus* the HA epitope was obtained from Covance. Polyclonal antibody Y-11 *versus* the HA epitope was purchased from Santa Cruz Biotechnology. Infrared-labeled secondary antibodies and IRDye blue protein stain were purchased from LI-COR Biosciences. Lys-48-linked polyubiquitin chains and His_6-tagged Ataxin-3 were purchased from Boston Biochem.

Cell Culture and Transfections—HEK-293A and U2OS cells were obtained from the American Type Culture Collection and maintained in a 5% CO_2 environment at 37 °C in DMEM containing 10% fetal bovine serum. DNA transfections were performed with FuGENE HD (Roche Applied Science) according to the manufacturer's specifications. siRNA was purchased from Integrated DNA Technologies, and transfections were performed with Lipofectamine RNAiMAX (Invitrogen) using the manufacturer's specifications.

DNA Constructs—Human SAKS1 and UBXD3 cDNAs were purchased from Open Biosystems. The vectors pFLAG-CMV-4 (Sigma) and pGEX-6P-1 (GE Biosciences) were used for mammalian expression of FLAG-tagged proteins and the bacterial expression of GST-tagged proteins, respectively. All point-mutated constructs were generated using the QuikChange site-directed mutagenesis kit (Stratagene). pQE9-His-p97 (wild type), ubiquitin-G76V-GFP, and pGEX hHR23A were obtained from Addgene (Addgene plasmids 14666, 11941, and 10864) (23–25). Null Hong Kong variant α_1-antitrypsin (referred to herein as NHK-HA) and the T-cell receptor α-subunit (TCRα-HA) in pcDNA3.1(−) tagged with the HA epitope at the carboxyl terminus were provided by Ron Kopito (Stanford University) (26). His_6-tagged VCP/p97 and FLAG-tagged ataxin-3 constructs for mammalian expression were provided by Yihong Ye (National Institutes of Health) (9, 27).

Cycloheximide Chase Experiments—Cycloheximide was added at a concentration of 100 μM to HEK-293A cells 24 h post-transfection. Cell lysates were then collected at 0, 2, 4, and 6 h and analyzed by quantitative Western blotting. Total levels of NHK-HA were quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences), and all time points were compared with time 0 and expressed as a percentage thereof. Statistical differences were determined using analysis of variance tests followed by pairwise comparisons. For knockdown experiments, siRNA was transfected 1 day post-plating, followed 24 h later by transfection with DNA for the model substrate. The cycloheximide chase experiment was performed the following day.

Protein Purification, FPLC, and Binding Experiments—All His_6- or GST-tagged proteins were purified according to standard protocols. Nontagged soluble proteins were generated from GST-tagged versions using C3-protease-mediated cleavage overnight at 4 °C. His_6-tagged soluble proteins were eluted from TALON metal affinity resin (Clontech) using 300 mM imidazole and then desalted using PD-10 Sephadex columns (GE Healthcare). Characterization of proteins via FPLC was performed with an AKTA FPLC using a Superose 6 10/300 gel filtration column (GE Healthcare) in FPLC running buffer (150 mM KCl, 25 mM Tris, pH 7.4, 2.5 mM $MgCl_2$, 1 mM ATP, and 5% glycerol) (28). When proteins were run in combination, they were premixed in FPLC buffer for 1 h before the run. FLAG immunoprecipitations were performed essentially as published previously (18). Co-immunoprecipitation of endogenous proteins was performed with the Trueblot system (eBiosciences). For *in vitro* binding experiments, the recombinant proteins were mixed in binding buffer (25 mM Tris, pH 7.4, 0.1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, and 5% glycerol) and nutated at 4 °C. Samples were then washed in binding buffer and boiled in SDS sample buffer.

Microsome Preparation—Microsomes were isolated as described previously (20). Cells were resuspended in cold hypotonic lysis buffer (10 mM Hepes, pH 7.4, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, Complete protease inhibitor mixture (Roche Applied Science)). The cell suspension was passed multiple times through a 25-gauge needle and then spun at 1000 × *g* for 5 min. The supernatant was then spun at 100,000 × *g* for 1 h at 4 °C with the resulting pellet representing microsomes and the supernatant considered cytosol.

Fluorescence Microscopy—Cells were processed for fluorescence microscopy according to standard protocols. Images were acquired with an Intelligent Imaging Innovations CSU-X spinning disc microscope with a ×40 1.25 NA objective and Photometrics HQ2 CCD camera. Acquired images were processed with Slidebook 5.0. For MG132 treatment, the cells received the proteasome inhibitor at a concentration of 10 μM for 2 h before processing for fluorescence microscopy.

Deubiquitinase Experiments—For deubiquitinase assays from cells, HEK-293A cells were transfected to express His_6-p97 and allowed to express the protein for 48 h. The p97 was then precipitated using Talon beads (Clontech), and the resulting complexes were then incubated with either excess BSA or recombinant SAKS1 in deubiquitination buffer (50 mM Tris, pH 7.4, 20 mM potassium chloride, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% bovine serum albumin) at 37 °C

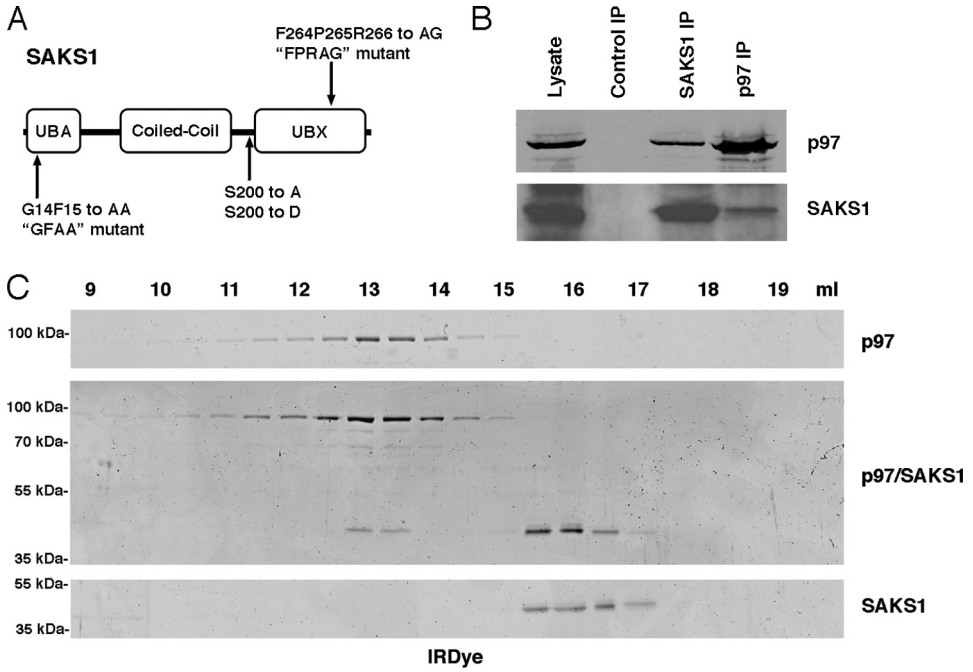

FIGURE 1. **SAKS1 interacts with hexameric p97.** *A,* schematic of SAKS1 domain structure and mutants. *B,* immunoprecipitations (*IP*) were performed from HEK-293A cells with antibodies *versus* SAKS1, p97, or a control antibody. The lysate represents 5% input. Endogenous SAKS1 and p97 co-immunoprecipitate. *C,* SAKS1 and His_6-p97 were purified from bacteria and analyzed by gel filtration alone and together. SAKS1 co-migrates with hexameric p97.

(9). Samples were taken at the indicated time points and processed for Western blotting. For completely *in vitro* assays, 2.5 μg of Lys-48-linked ubiquitin chains were preincubated with 10 μg of either bovine serum albumin or recombinant SAKS1 in buffer (50 mM Tris, pH 7.4, and 1 mM dithiothreitol) before addition of 2.5 μg of His_6-tagged ataxin-3 (Boston Biochem) (29). The reactions were incubated at 37 °C, and samples were taken at both time 0 and after overnight incubation and then processed for Western blotting.

RESULTS

SAKS1 Interacts with Hexameric p97 and Inhibits ERAD—The SAKS1 domain structure (Fig. 1*A*) and *in vitro* interactions with components of the cellular protein degradation pathway led us to investigate a role for SAKS1 in p97-dependent ERAD (21, 22). An interaction between endogenous SAKS1 and p97 was confirmed *in vivo* using co-immunoprecipitations from HEK-293A cells (Fig. 1*B*). Recombinant soluble, nontagged full-length SAKS1 and His_6-tagged p97 were analyzed by gel filtration singly and together and confirm that SAKS1 interacts directly with hexameric p97 (Fig. 1*C*). A role for SAKS1 in ERAD was then tested using the soluble model ERAD substrate NHK-HA (null Hong Kong variant of α_1-antitrypsin carboxyl-terminally tagged with the HA epitope), which is synthesized in the ER and subjected to retrotranslocation into the cytosol for proteasomal degradation (26). siRNA treatment to knock down endogenous SAKS1 led to a significant reduction of NHK-HA during a 6-h cycloheximide chase (Fig. 2, *A* and *B*). Overexpression of FLAG-tagged SAKS1 with NHK-HA significantly stabilized the ERAD substrate over a similar time course (Fig. 2, *C* and *D*). The alternative ERAD substrate TCRα was similarly stabilized by SAKS1 overexpression (data not shown) (26). As overexpression of tagged-SAKS1 was able to potently regulate ERAD, an overexpression approach was used in the subsequent experiments to determine which domains are essential for this effect. The results establish SAKS1 as an inhibitor of the degradation of ERAD substrates.

SAKS1 Requires Its Interactions with p97 and Polyubiquitin to Inhibit ERAD—SAKS1 mutants defective for binding either polyubiquitin or p97 were then designed and tested for their impact on ERAD to help define the mechanism whereby SAKS1 is affecting this process. First, point mutations were made in the UBX domain to inhibit binding to the ATPase p97. UBX domains are related to ubiquitin but have an extended loop between their third and fourth β-strands that is responsible for their interaction with the amino-terminal domain of p97 (17, 30). A variant of SAKS1 was constructed in which residues 264–266 encoding amino acids FPR of the extended loop were mutated to AG (the FPRAG mutant, Fig. 1*A*) (30). The FPRAG mutant was found to have greatly reduced binding to p97 both *in vitro* and *in vivo* (Fig. 3, *A* and *B*).

Next, point mutations were made in the SAKS1 UBA domain to inhibit polyubiquitin binding (the GFAA mutant, Fig. 1*A*) (31, 32). Recombinant SAKS1 GFAA was found to have greatly reduced binding to Lys-48-linked ubiquitin chains *in vitro* as compared with wild-type SAKS1 (Fig. 4*A*). This assay also shows that SAKS1 binds primarily to polyubiquitin, with little affinity for monomeric ubiquitin (Fig. 4*A*). Similarly, the GFAA mutant was unable to co-precipitate polyubiquitinated proteins *in vivo*, although the FPRAG mutant was able to co-precipitate (Fig. 4*B*). Importantly, overexpression of either the p97 or polyubiquitin binding SAKS1 mutants could not stabilize NHK-HA (Fig. 5, *A* and *B*). Thus, the ability of SAKS1 to

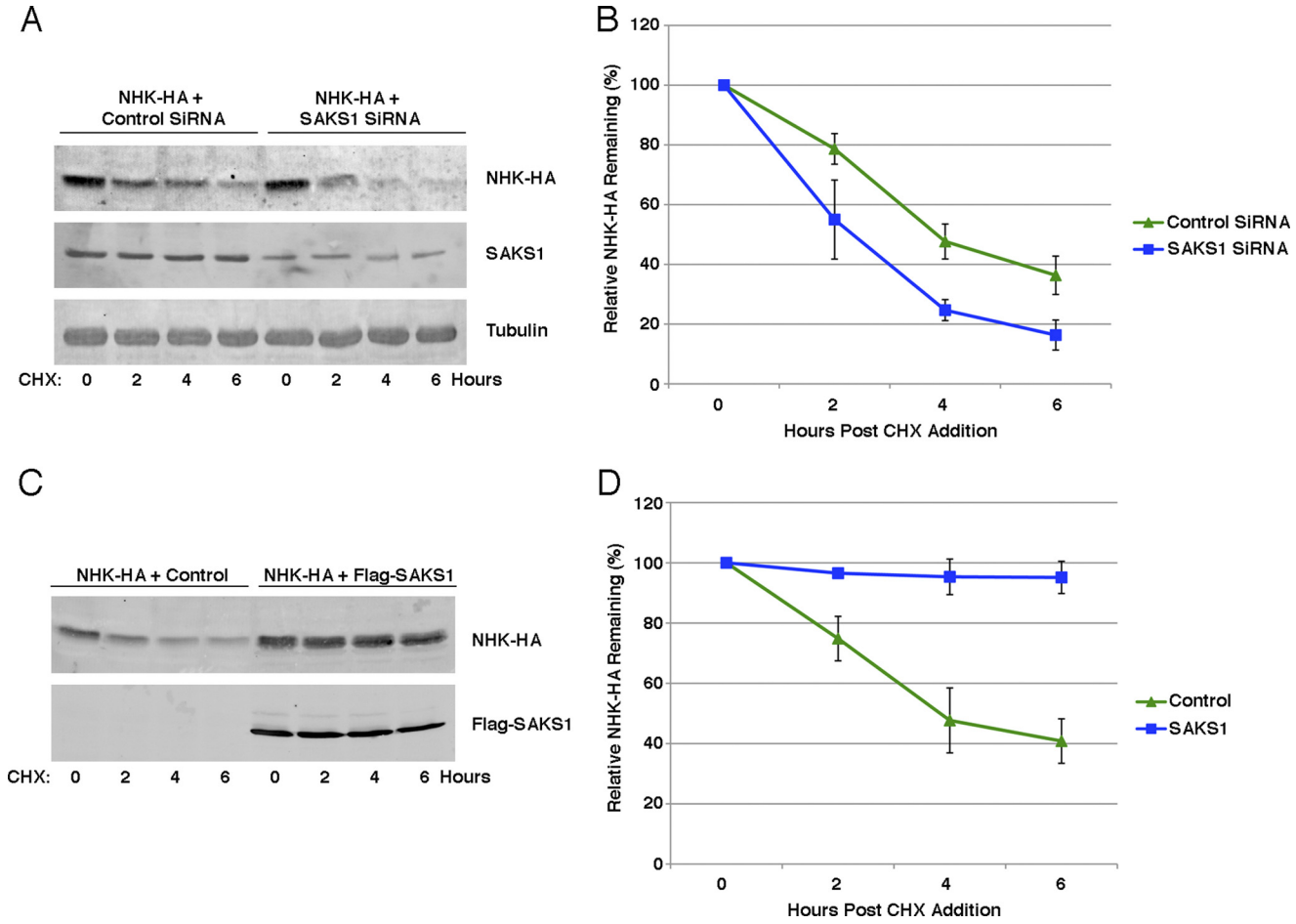

FIGURE 2. **SAKS1 inhibits degradation of the model ERAD substrate NHK-HA.** *A,* HEK-293A cells transfected with siRNA toward either luciferase, as a control, or SAKS1 and expressing NHK-HA were subjected to a cycloheximide (*CHX*) chase experiment and lysed at the indicated time points. The lysates were analyzed by Western blotting. *B,* multiple experiments performed as in *A* were quantified and graphed. SAKS1 knockdown significantly decreased NHK-HA levels during the chase, $p < 0.05$. *C,* HEK-293A cells were co-transfected with NHK-HA and either FLAG-SAKS1 or control DNA. The following day, a cycloheximide chase experiment was performed, and lysates were collected at the indicated times. The lysates were analyzed by Western blotting. *D,* results of several replicates of experiments performed as in *C* were quantified and graphed. SAKS1 overexpression significantly stabilized NHK-HA levels during the chase, $p < 0.01$.

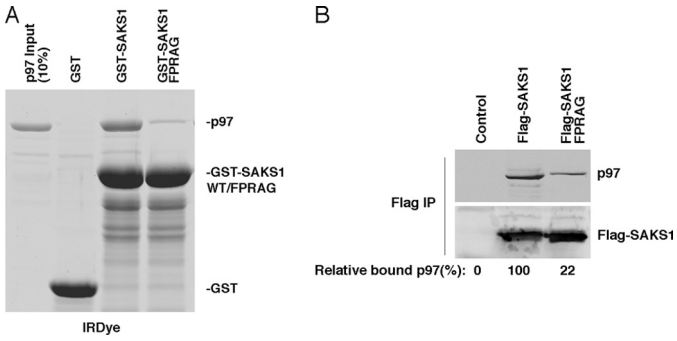

FIGURE 3. **Generation of a SAKS1 mutant deficient in p97 binding.** *A,* indicated resin-bound GST constructs were tested for binding to recombinant p97 *in vitro*. The resulting complexes were run on SDS-PAGE and stained with the protein stain IRDye. *B,* HEK-293A cells transfected with the indicated constructs were subjected to FLAG immunoprecipitations (*IP*) and blotted for the FLAG construct as well as endogenous p97. The amount of p97 precipitated relative to the wild-type construct (*middle lane*) is shown.

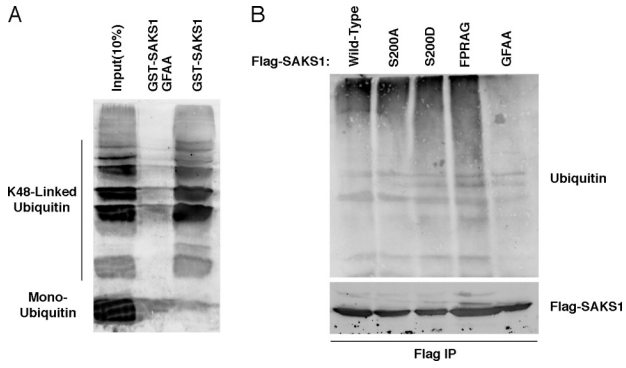

FIGURE 4. **Generation of a SAKS1 mutant deficient in polyubiquitin binding.** *A,* resin-bound full-length GST-SAKS1 constructs were tested for binding to Lys-48 (*K48*)-linked ubiquitin chains alongside mono-ubiquitin *in vitro*. The resulting complexes were analyzed by Western blotting for ubiquitin. *B,* indicated FLAG-SAKS1 constructs were precipitated from HEK-293A cells. The immune complexes were analyzed by Western blotting for the presence of FLAG-SAKS1 and ubiquitin. *IP*, immunoprecipitation.

modulate ERAD is a specific result of the localization of the SAKS1 UBA domain to p97 complexes in cells.

SAKS1 is phosphorylated on serine 200 in response to stress (21). Phosphomimetic (S200D) and -deficient (S200A) mutants bound polyubiquitin (Fig. 4*B*) and were tested in the ERAD assay, and both exhibited an intermediate suppression of NHK-HA degradation (supplemental Fig. S1), indicating that cycling of this phosphorylation may regulate ERAD in stressed cells.

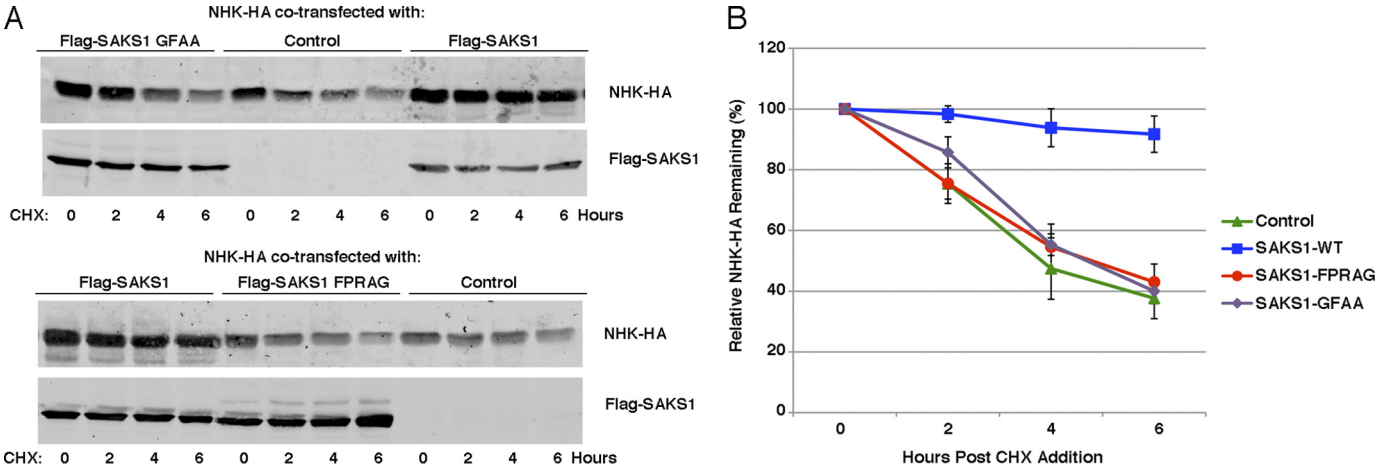

FIGURE 5. **SAKS1 p97 and polyubiquitin binding mutants do not impact ERAD.** *A,* HEK-293A cells were co-transfected with NHK-HA and either FLAG-SAKS1 constructs or control DNA. Lysates from a cycloheximide (*CHX*) chase were analyzed by Western blotting. *B,* results of several replicates of experiments performed as in *A* were quantified and graphed. The mutants do not significantly differ from the control at any time point, $p < 0.01$.

Polyubiquitin Binding to SAKS1 Enhances the SAKS1/p97 Interaction—Unexpectedly, it was found that the ubiquitin-binding deficient GFAA mutant exhibited reduced co-precipitation of p97 from HEK-293A cells as compared with wild-type SAKS1 and that this loss of binding was not found when the UBA domain was removed (FLAG-SAKS1ΔUBA) (Fig. 6*A*). This result suggests that binding of SAKS1 to polyubiquitin enhances its interaction with p97 through relief of an inhibitory effect of the UBA domain. To test this in a defined system, *in vitro* binding experiments between purified soluble SAKS1 and His_6-tagged p97 revealed that there is a significant increase in wild-type SAKS1 precipitated with p97 when polyubiquitin is added (Fig. 6*B*). Note that polyubiquitin only precipitates with p97 in the presence of SAKS1, thereby confirming the ability of SAKS1 to effectively link polyubiquitin to p97 complexes (Fig. 6*B*). The GFAA mutant could be seen to co-precipitate with p97 *in vitro*, although the interaction was not increased by the addition of Lys-48-linked polyubiquitin (Fig. 6*B*). Taken together, SAKS1 ubiquitin binding enhances the SAKS1 association with p97, which increases it to a point that is detectable via immunoprecipitation from cell lysates, with a similar level of binding seen with removal of the UBA domain. We could not demonstrate an interaction between the SAKS1 UBA and UBX domains, and thus the inhibitory effect of the presence of the unbound UBA is more likely conformational than via a direct intra- or inter-molecular association (data not shown). Thus, SAKS1 undergoes conformational inhibition of its full capacity to bind p97 that is relieved through binding to polyubiquitin, likely involving steric hindrance of its UBX domain by the unbound UBA domain, with a somewhat similar possibility having been suggested for UBXD7 (18).

In light of these results, we examined the effect of overexpression of SAKS1 amino-terminal truncation mutants in NHK-HA degradation assays (Fig. 6*C*). The SAKS1ΔUBA mutant that is able to immunoprecipitate p97 but has lost polyubiquitin binding did not affect ERAD (Fig. 6*C*). Similarly, NHK-HA stability was unaffected in cells overexpressing SAKS1 lacking both the UBA domain and coiled-coil section (SAKS1 UBX) or UBXD3, an alternative UBX domain-containing protein that lacks a UBA domain (Fig. 6*C*). Overall, these results reinforce that SAKS1 must be competent to bind both polyubiquitin chains and p97 to effectively inhibit ERAD. Importantly, it is not simply due to SAKS1 competing off other factors from p97, as the mutant lacking the UBA domain still retains p97 binding activity yet has no effect on ERAD.

SAKS1 Inhibits the Degradation of Cytosolic p97-dependent Proteasomal Substrates—Cell fractionation experiments in cells overexpressing NHK-HA with SAKS1 or bacterial alkaline phosphatase, as a control, were performed to determine where the stabilized pool of NHK-HA was localized. When SAKS1 is overexpressed, there is a significant enhancement of the ERAD substrate at the ER (microsome fraction) and also a minor increase in the cytoplasm, where levels of NHK-HA are generally exceedingly low and hard to detect (Fig. 7*A*). Tagged-SAKS1 was found both associated with the ER and in the cytosolic pool, and there is a banding pattern indicative of a post-translational modification, possibly ubiquitination, that is most prevalent in the microsomal fraction (Fig. 7*A*). Furthermore, there is no detectable change in association between p97 and NHK-HA or polyubiquitin in the presence of overexpressed SAKS1 (Fig. 7*B*). Thus, SAKS1 does not lock substrates into an ineffective complex with p97 but instead they are released from the ATPase.

The strong cytoplasmic pools of p97 and SAKS1, as well as the enhanced level of cytosolic NHK-HA, indicated that SAKS1 might also be exerting some effect on p97-related degradation in the cytosol. The degradation of Ub-G76V-GFP, which is a cytosolic protein dependent on p97-mediated unfolding for efficient proteasomal degradation, was therefore tested (12, 13). First, the requirement for p97 in Ub-G76V-GFP degradation was confirmed in HEK-293A cells using overexpression of an ATPase-defective p97 variant.[3] Cycloheximide chase experiments were then performed in HEK-

[3] D. P. LaLonde and A. Bretscher, unpublished observations.

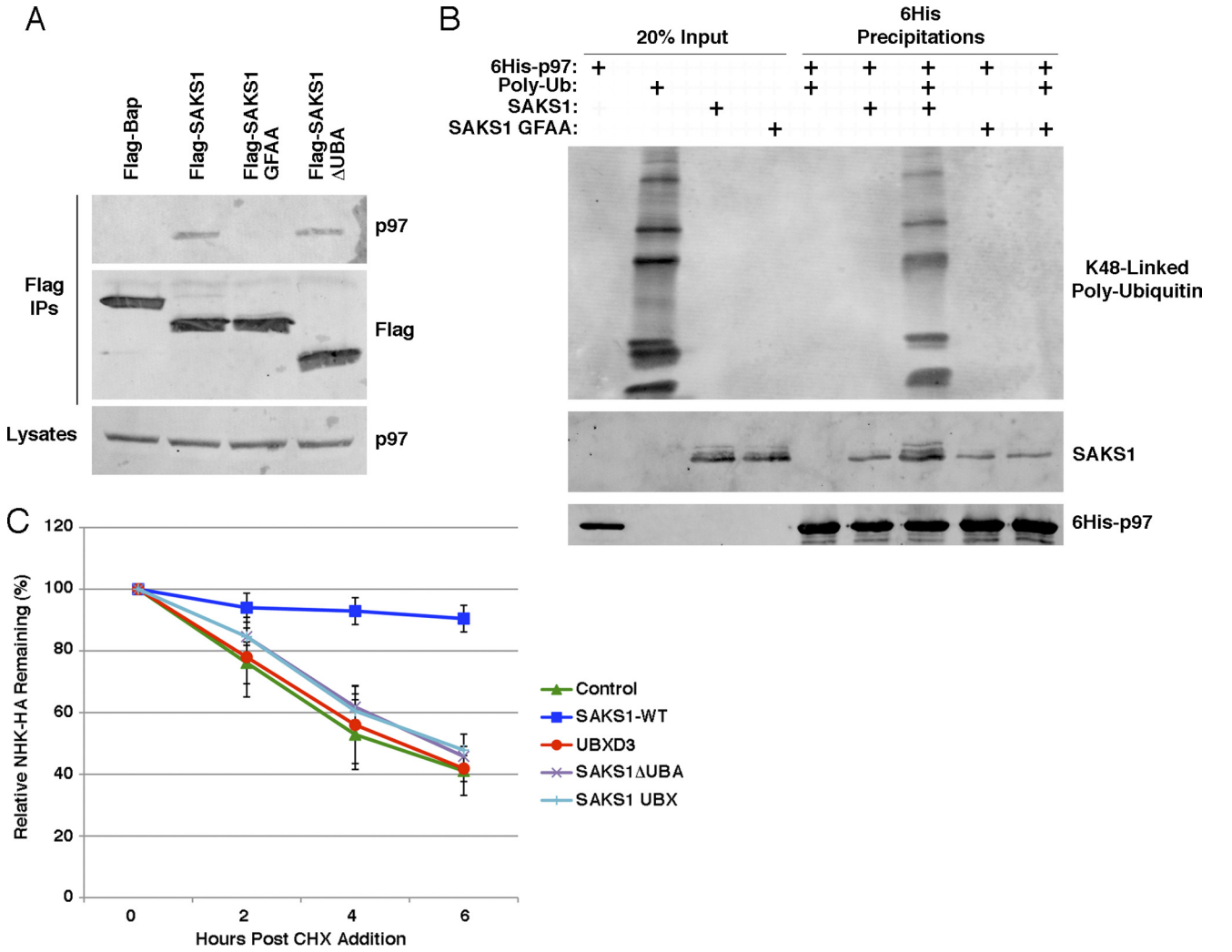

FIGURE 6. **Polyubiquitin increases the association between SAKS1 and p97.** *A,* HEK-293A cells transfected with the indicated constructs were subjected to FLAG immunoprecipitations (*IP*), and the resulting complexes were blotted for the FLAG construct as well as endogenous p97. FLAG-Bap is tagged bacterial alkaline phosphatase and acts as a control. *B,* His_6-tagged recombinant soluble p97 was incubated with the indicated proteins then precipitated and analyzed by Western blotting. *C,* ERAD assays were performed multiple times in HEK-293A cells for the indicated constructs and then quantified and graphed. Both SAKS1 truncations and UBXD3 are equivalent to the control at all time points but are statistically different from wild-type SAKS1 at the two later time points, $p < 0.01$. *CHX*, cycloheximide.

293A cells, and the presence of FLAG-SAKS1 was found to significantly stabilize the cellular levels of Ub-G76V-GFP (Fig. 8, *A* and *B*), a finding that was confirmed in the osteosarcoma cell line U2OS (data not shown). Notably, there was an increase in higher molecular weight bands that are possibly indicative of either increased ubiquitination or decreased deubiquitination of the Ub-G76V-GFP substrate (Fig. 8*A*). This result was also evaluated by fluorescence microscopy, wherein cells were examined for the presence of Ub-G76V-GFP. Similarly to published results, there is a low percentage of cells clearly expressing the construct in untreated cells, but this percentage is greatly increased when the proteasome inhibitor MG132 is added for 2 h (Fig. 8*D*) (13). Co-transfection of wild-type FLAG-SAKS1 also increased the number of cells expressing strong levels of the GFP reporter, although not to the level seen with MG132, and similar to their effect on NHK-HA degradation, this was not apparent for cells expressing the FPRAG, GFAA, or ΔUBA mutants (Fig. 8, *C* and *D*). This was also tested by SAKS1 knockdown, wherein the number of cells expressing Ub-G76V-GFP, as evaluated by fluorescence microscopy, was less than 2%, which is significantly less than the 7% of control cells. Thus, SAKS1 is a negative regulator of the p97-dependent processing of cytosolic proteins destined for proteasomal degradation, and this appears to be mechanistically similar to the effect that SAKS1 has on ERAD substrates, which also depend on p97 for their degradation.

Mechanisms of SAKS1-dependent Negative Regulation of ERAD—The results thus far indicate that the inhibition of degradation of p97 substrates is dependent on the targeting of the SAKS1 ubiquitin-binding domain to p97 complexes. Additionally, because Ub-G76V-GFP co-expressed with SAKS1 displayed higher molecular weight bands indicative of increased ubiquitination, we hypothesized that SAKS1 may inhibit the ubiquitin chain processing by deubiquitinases that is required for modulating retrotranslocation and ERAD (8, 9). To test this possibility, His_6-p97 complexes were precipitated from cells, and the level of polyubiquitin in the recovered

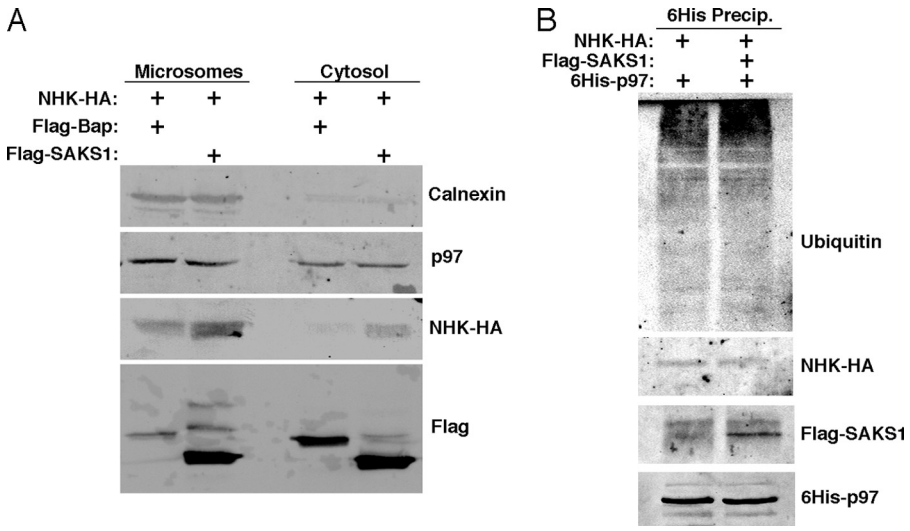

FIGURE 7. **SAKS1 stabilizes NHK-HA at the ER and in the cytosol.** *A,* HEK-293A cells were transfected with the indicated constructs and then separated into microsomal and cytosolic fractions 24 h later. Calnexin is an ER resident protein used as a marker. *B,* HEK-293A cells were co-transfected with His_6-p97, NHK-HA, and either FLAG-SAKS1 or control DNA. The following day, the His_6-p97 was precipitated, and the resulting complexes were analyzed by Western blotting for the indicated proteins.

complexes was monitored over time, as has been done to evaluate the role of ataxin-3 in this process (9). However, we modified the protocol by adding recombinant SAKS1 after collecting the His_6-p97 complexes. SAKS1 was found to stabilize polyubiquitin chains in these complexes over the time course examined, with the strongest protection found for the highest molecular weight targets (Fig. 9*A*, observe the high molecular weight band retained in the *last lane* of the gel). Note that ubiquitin chains only decrease due to deubiquitination in such assays and do not increase as ubiquitin activating enzymes (E1s) are not expected to be present in significant quantities in these complexes.

The deubiquitinase ataxin-3 has been shown to act on substrates at p97 complexes to process them for effective ERAD and to act on Lys-48-linked chains (9, 29). Thus, the ability of SAKS1 to protect Lys-48-linked polyubiquitin from ataxin-3-mediated deubiquitination was explored. *In vitro* deubiquitinase assays show that SAKS1 protects polyubiquitin chains from processing by ataxin-3, with notable protection observed for chains containing 6–7 ubiquitins, which are strong ataxin-3 substrates (Fig. 9*B*) (29). This was next evaluated *in vivo* using cycloheximide chase experiments of NHK-HA-expressing cells with either FLAG-tagged wild type or catalytically inactive C14A ataxin-3 either with or without FLAG-SAKS1 co-transfection. Ataxin-3 appeared to enhance degradation of this substrate, but the effect was not significant, although the C14A mutant did significantly stabilize NHK-HA cellular levels (Fig. 9*C*). However, co-expression of ataxin-3 with SAKS1 did significantly block the stabilization caused by SAKS1 overexpression at later time points, thereby reinforcing the finding that SAKS1 works by opposing deubiquitinase functioning (Fig. 9*C*). Thus, SAKS1 is able to protect substrates from the effects of the deubiquitinase ataxin-3 both *in vitro* and *in vivo*. This ability to shield polyubiquitin chains is not all encompassing, as SAKS1 is not able to inhibit the association of polyubiquitin with GST resin-bound proteasomal targeting factor hHR23A either from cells or *in vitro* (supplemental Fig. S2, *A* and *B*). The ability of SAKS1 to protect polyubiquitin chains from deubiquitination is not unique, with examples including the aggresome targeting factor HDAC6 and the fission yeast ubiquitin-binding proteins Rhp23, Dph1, and Pus1 (33, 34).

DISCUSSION

Misfolded proteins in the ER are retrotranslocated into the cytoplasm where they are degraded by the proteasome in a process known as ERAD. The homohexameric ATPase p97 is a well characterized component of this process that provides the driving force for the extraction of substrates from the ER. The amino terminus of p97 interacts with UBX domains, which are ubiquitin-like motifs found in over a dozen human proteins, and it is thought that proteins containing this motif act as p97 adaptors. One of these proteins, SAKS1, has been demonstrated to interact with polyubiquitin and p97 and enter into a multimolecular complex *in vitro* containing other molecules associated with protein degradation, such as the autocrine motility factor receptor (also known as gp78), peptide *N*-glycanase, and HR23B (RAD23) (21, 22, 35). Furthermore, this UBX protein has been implicated in the proteasomal degradation of the postsynaptic density scaffolding protein Homer2 and has been biochemically isolated with the proteasome (36, 37). Thus, we examined a role for SAKS1 in protein degradation to gain more mechanistic insight into its functioning.

Overexpression of SAKS1 was able to almost completely abrogate the degradation of the soluble model ERAD substrate, NHK-HA. The transmembrane TCRα-HA substrate was also stabilized, providing no evidence for the role of SAKS1 being specific to different ERAD pathways based on substrate solubility (38). Interestingly, the ability of SAKS1 to modulate ERAD was lost when point mutations were introduced into either the UBA or UBX domains to inhibit associations with polyubiquitin or p97, respectively, indicating that SAKS1 must interact with both of these factors to exert its

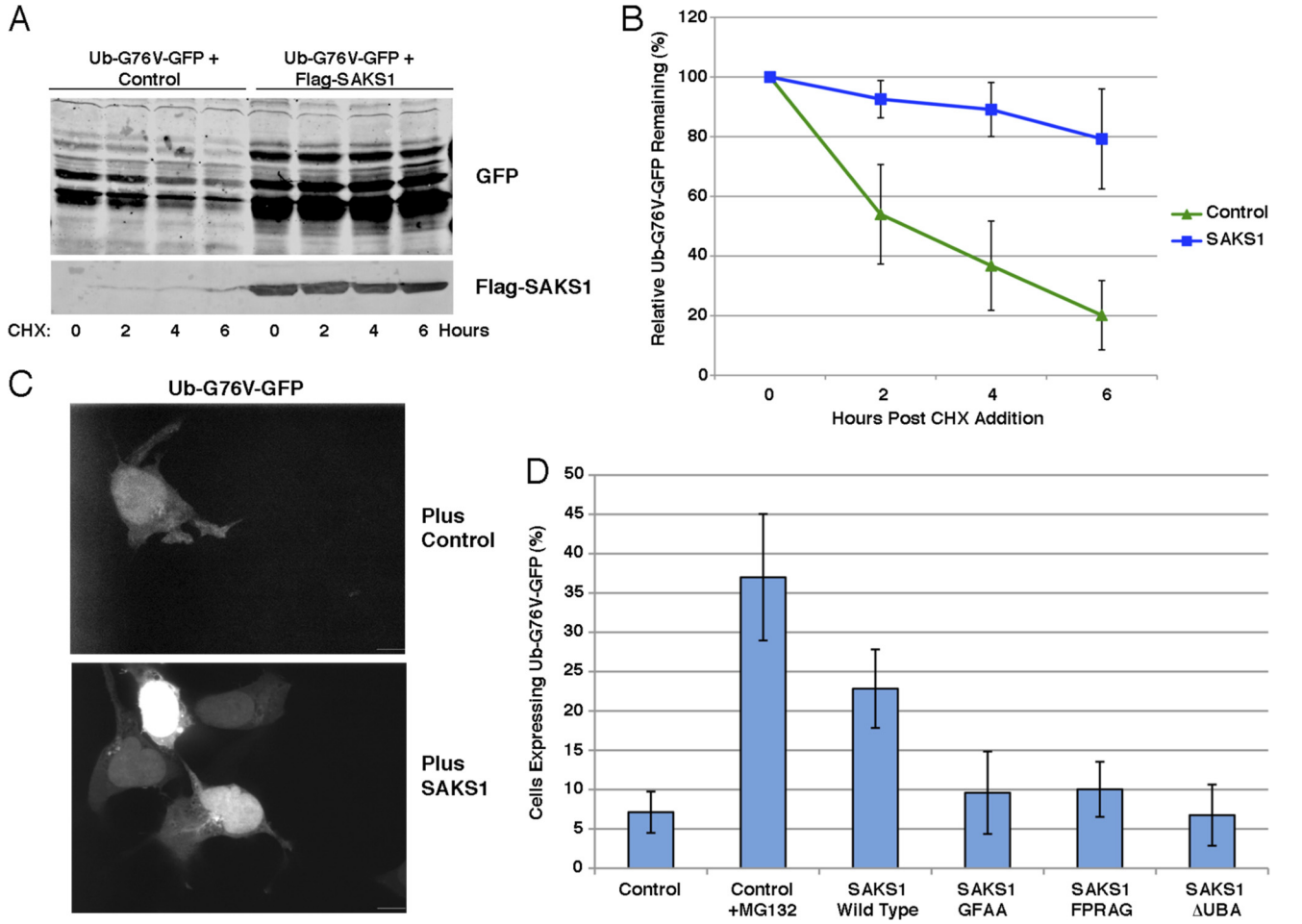

FIGURE 8. **SAKS1 negatively regulates the degradation of Ub-G76V-GFP.** *A,* HEK-293A cells were co-transfected with Ub-G76V-GFP and either FLAG-SAKS1 or control DNA. Cycloheximide (*CHX*) chase experiments were performed the following day and then analyzed by quantitative Western blotting. *B,* results of multiple experiments performed as in *A* were graphed. SAKS1 significantly stabilizes Ub-G76V-GFP at all time points, $p < 0.01$. *C,* HEK-293A cells were co-transfected with Ub-G76V-GFP and either FLAG-SAKS1 or control DNA and then processed for fluorescence microscopy 24 h later. Staining for filamentous actin confirmed equivalent cell confluency for each condition. *D,* cells were co-transfected with Ub-G76V-GFP and the indicated constructs and processed for fluorescence microscopy the following day either with or without MG132 pretreatment. The percentage of cells expressing Ub-G76V-GFP was then quantified, and the results of multiple experiments were graphed. The percentage of cells clearly expressing Ub-G76V-GFP in wild-type SAKS1-expressing cells is significantly less than cells treated with MG132 and significantly more than all other conditions, $p < 0.05$.

effect. SAKS1 negative regulation of ERAD is therefore dependent on the localization of the SAKS1 polyubiquitin binding domain to p97 complexes. We further find that SAKS1 is able to protect polyubiquitin chains from p97-associated deubiquitinases and, specifically, from ataxin-3. Efficient ERAD is dependent on deubiquitinases, perhaps because the removal of ubiquitin from retrotranslocating proteins is needed at the ER to allow threading into the p97 hexamer or it may be required for the cytosolic processing of substrates to change ubiquitin linkages and allow for more efficient targeting to the proteasome (8, 9). Thus, the SAKS1 UBA binds ubiquitin chains to protect them from deubiquitination in a similar manner to that seen with several other polyubiquitin-binding proteins, and this likely contributes to a depressed rate of ERAD (33, 34). We show that this effect has specificity, as SAKS1 does not protect ubiquitin chains from binding to the proteasome targeting factor hHR23A. However, we cannot exclude the possibility that SAKS1 may interfere with other ubiquitin processing factors found at p97 complexes, such as ubiquitin ligases (E3s) or chain elongation factors (E4s).

Our findings also demonstrate that SAKS1 is able to stabilize the cytosolic levels of Ub-G76V-GFP. It has been previously shown that this model proteasomal substrate requires p97 for efficient degradation and that this occurs through the ATPase unfolding the substrate enough to provide an initiation site for the proteasome (12, 13). The precise mechanism and components of this are not yet completely known. We find that the SAKS1 impact on this is similar to its effect on ERAD as it requires the ability of the UBX protein to bind both polyubiquitinated proteins and p97, and thus it may be shielding Ub-G76V-GFP from appropriate ubiquitin processing. However, SAKS1 is not required for degradation of this substrate in *Drosophila,* indicating some divergence in mechanism between flies and humans (13).

An interesting aspect of this study is the ability of polyubiquitin to enhance the association between p97 and SAKS1 that we show is likely a consequence of an inhibitory effect of the SAKS1 UBA domain when not bound to polyubiquitin chains, possibly due to occlusion of the UBX that is relieved by a conformational change in the molecule upon binding of the UBA

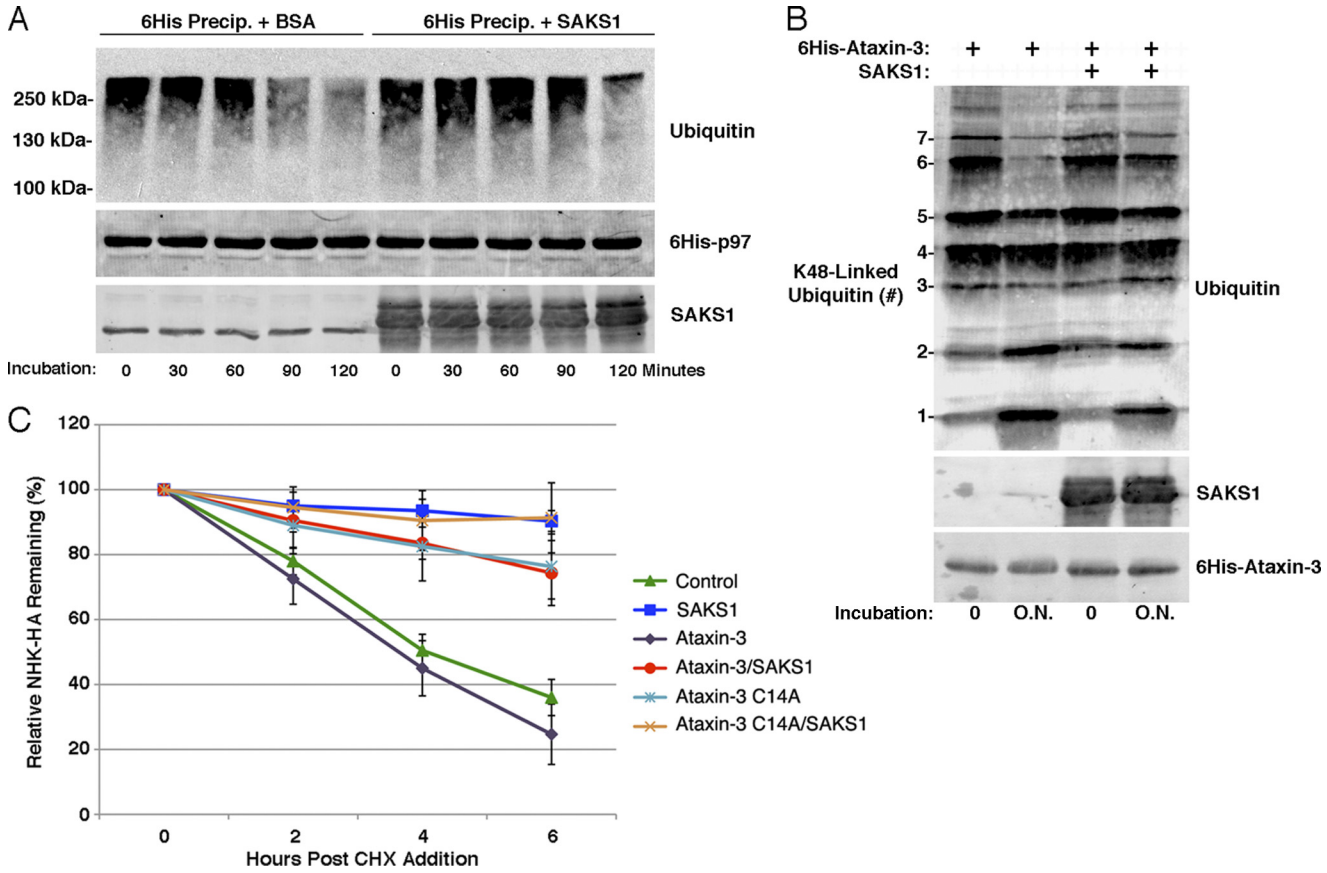

FIGURE 9. **SAKS1 protects polyubiquitin from deubiquitinases.** *A,* overexpressed His_6-p97 was precipitated from cells and then incubated with either BSA or recombinant SAKS1 at 37 °C. Samples were taken at the indicated time points and analyzed by Western blotting. *B,* Lys-48 (*K48*)-linked polyubiquitin chains were preincubated with either SAKS1 or BSA and then incubated overnight at 37 °C with recombinant ataxin-3. Samples were taken at time 0 and after overnight (*O.N.*) incubation and then blotted for the indicated proteins. *C,* cycloheximide (*CHX*) chase experiment was performed in HEK-293A cells co-transfected with the indicated tagged constructs, and lysates were collected at the indicated time points. The results of multiple experiments were quantified and graphed. The expression of ataxin-3 C14A significantly stabilizes substrate levels, although the wild-type ataxin-3 construct does not significantly decrease them. Co-transfection of wild-type ataxin-3 and SAKS1 does significantly decrease the stabilization of substrate otherwise seen with expression of SAKS1 at later time points, $p < 0.05$.

to ubiquitin. This is generally similar to the UBX protein UBXD7, which loses its association with ubiquitin *in vivo* when its p97 binding UBX domain is removed (18). Although the potential auto-regulation of SAKS1 and UBXD7 appear mechanistically different, it is suggestive of similar regulation paradigms for multiple UBX proteins. This p97-binding regulatory effect appears more pronounced *in vivo* than in our *in vitro* conditions, so there is likely to be some additional factor in cells that regulates the effect. This could include possibilities such as co-binding of a cytoplasmic factor beyond ubiquitin or a post-translational modification such as phosphorylation. Alternatively, our *in vitro* results were obtained with Lys-48-linked ubiquitin chains, although Lys-11-linked ubiquitin chains could be more effective, as this linkage has been shown to be strongly co-precipitated with SAKS1 from cells (18).

Our findings are that the cytosolic protein SAKS1 is a wide ranging repressor of protein degradation of substrates that are dependent on the p97 ATPase. Another UBX protein, erasin, which is an ER resident transmembrane protein, has an opposite, stimulatory effect on ERAD (19, 20). It is interesting that two UBX proteins would have opposite effects on similar pathways. This may involve a potential ability for them to compete for p97 and form alternative multimolecular complexes with the ATPase. The possibility of alternative complexes is interesting as erasin promotes a complex containing p97 and the proteasome targeting factor ubiquilin, whereas SAKS1 has an enhanced association with Ufd3 as compared with the other human UBX proteins, although this is likely indirect (18, 20). Although the ubiquilin association enhances the efficiency of ERAD, it would be expected that SAKS1-dependent enhanced Ufd3 recruitment to p97 would inhibit ERAD, although this remains to be tested (10, 18). Regardless, the possibility of alternative complexes and competition among adaptors for p97 is a fertile area for active research. It has recently been shown that p97 mutations of inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia alter the dynamics of co-binding of adaptors to the ATPase demonstrating a relevant role for this paradigm in human disease states (39).

In summary, we provide mechanistic insight into the ability of the UBX domain-containing adaptor protein SAKS1 to regulate two different pathways of proteolysis that are dependent on the ATPase p97. This adaptor is able to decrease degradation by inhibiting the processing of substrates at cytosolic p97 complexes that is otherwise required for efficient

degradation of proteins. Because of the recently reported role for SAKS1 in regulation of the tumor suppressor complex BRCA1/BARD1 as well as the regulation of SAKS1 by stress-induced phosphorylation, this adaptor protein may prove to be an important target of future research relevant to pathological cellular functioning (21, 40).

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