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The Deubiquitinating Enzyme BAP1 Regulates Cell Growth via Interaction with HCF-1*[S]

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The deubiquitinating enzyme BRCA1-associated protein 1 (BAP1) possesses growth inhibitory activity and functions as a tumor suppressor. In this study we report that BAP1 also plays positive roles in cell proliferation. BAP1 depletion by RNAi inhibits cell proliferation as does overexpression of a dominant negative mutant of BAP1. Mass spectrometry analyses of copurified proteins revealed that BAP1 is associated with factors involved in chromatin modulation and transcriptional regulation. We show that the interaction with host cell factor-1 (HCF-1), a cell-cycle regulator composed of HCF-1N and HCF-1C, is critical for the BAP1-mediated growth regulation. We found that HCF-1N is modified with Lys-48-linked polyubiquitin chains on its Kelch domain. The HCF-1 binding motif of BAP1 is required for interaction with HCF-1N and mediates deubiquitination of HCF-1N by BAP1. The importance of the BAP1-HCF-1 interaction is underscored by the fact that growth suppression by the dominant negative BAP1 mutant is entirely dependent on the HCF-1 binding motif. These results suggest that BAP1 regulates cell proliferation by deubiquitinating HCF-1.

Post-translational modification of proteins with ubiquitin is a crucial regulatory mechanism in many cellular processes. Like other post-translational modifications, ubiquitination is a reversible process. The human genome encodes nearly 100 potential deubiquitinating enzymes that are classified into 5 different families (1). These deubiquitinating enzymes are highly specialized and are involved in diverse cellular processes such as cell-cycle regulation, gene expression, DNA repair, signal transduction, and protein trafficking (2–6). Importantly, deubiquitinating enzymes appear to be involved in disease-related pathways as exemplified by the genetic alterations of deubiquitinating enzyme genes in various types of cancer (7). Although it is becoming clear that deubiquitinating enzymes play critical roles in various cellular pathways, physiological roles for most of the human deubiquitinating enzymes remain unknown.

BRCA1-associated protein 1 (BAP1)[2] was originally discovered as an interactor of a tumor suppressor protein, BRCA1 (8). BAP1 is a deubiquitinating enzyme in the ubiquitin C-terminal hydrolase (UCH) family, and accumulating evidence suggests that BAP1 functions as a tumor suppressor; first, BAP1 enhances BRCA1-mediated growth suppression (8), second, mutations and deletions in the *BAP1* gene have been reported in breast and lung cancers (8–10), and third, restoration of BAP1 in a BAP1-null lung cancer cell line suppresses cell growth and tumorigenicity (11). In addition to its growth-suppressive function in cancer cells, BAP1 may play a role in the normal cell cycle. BAP1 depletion by RNA interference (RNAi) has been shown to cause cell-cycle defects such as slow S-phase progression or accumulation of S and G_2/M phase cells (12, 13). Although all these facts suggest that BAP1 is important to ensure proper cell proliferation, the underlying molecular mechanism for BAP1-mediated growth control is still unclear.

Host cell factor-1 (HCF-1) is an important regulator of cell proliferation (14, 15). It is a heterodimer of HCF-1N and HCF-1C, which are produced by proteolytic processing of a large precursor protein (16–18). HCF-1 plays critical roles at the multiple stages of the cell cycle; HCF-1N is essential for G_1 phase progression, whereas HCF-1C is important for proper cytokinesis (15). HCF-1 is a chromatin-bound protein, and association with chromatin is dependent on the presence of the intact Kelch domain at the N terminus of HCF-1N (19). The Kelch domain binds to target proteins by recognizing a conserved peptide sequence, (D/E)H*X*Y, known as the HCF-1-binding motif (HBM) (20, 21). A number of transcription factors contain an HBM and recruit HCF-1 to promoters to regulate transcription (22–25). HCF-1 is also known to associate with histone-modifying complexes such as the Set1/Ash2 histone methyltransferase complex, the Sin3-histone deacetylase complex, and MOF (males absent on the first) histone acetyltransferase (26–29). By functioning as a scaffold to recruit histone-modifying enzymes to promoters, HCF-1 regulates gene expression through selective modulation of chromatin structure (22, 30).

We previously developed RNAi-based screening for genes important for cell proliferation (31). We applied this RNAi screening strategy to the subfamily of deubiquitinating enzymes to identify those important for cell proliferation. In

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[2] The abbreviations used are: BAP1, BRCA1-associated protein 1; HBM, HCF-1-binding motif; HCF-1, Host cell factor-1; UCH, ubiquitin C-terminal hydrolase; shRNA, short hairpin RNA; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HA, hemagglutinin.

this study, we investigated molecular mechanisms of growth control by one of the identified genes, BAP1. We found that BAP1 is associated with chromatin-related proteins including HCF-1. BAP1 binds HCF-1 via its HBM and negatively regulates the ubiquitination levels of HCF-1N. We propose that deubiquitination of HCF-1 is a crucial step in BAP1-mediated growth regulation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human breast epithelial cell line MCF10A was cultured in Dulbecco's modified Eagle's medium/F12 containing 5% donor calf serum, 0.02 μg/ml epidermal growth factor (BD Biosciences), 1 μg/ml insulin (Sigma), 1.4 μM hydrocortisone (Sigma), and 0.1 μg/ml cholera toxin. Human embryonic kidney cell line 293T was cultured in RPMI1640 supplemented with 10% fetal bovine serum. For transient expression of proteins, 293T cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen). Retroviruses were produced in a packaging cell line, 293GPG, or by transfecting retroviral and packaging plasmids to 293T cells. Cells were infected with supernatants containing retroviruses in the presence of 4 μg/ml Polybrene (Sigma) and selected with 1 μg/ml puromycin (Sigma) for 48–72 h. Infected cells were cultured in the absence of puromycin at least for 24 h for recovery from drug selection.

RNA Interference—Short hairpin RNAs (shRNAs) were expressed as a part of a mir30 precursor (32). The target sequences of shRNAs are: shControl, CGUACGCGGAAUACUUCGA; shBAP1.214, CGACCUUCAGAGCAAAUGU; shBAP1.321, CGUCCGUGAUUGAUGAUGA; shBAP1.2132, GAGUUCAUCUGCACCUUUA.

Plasmids—Full-length BAP1 cDNA was amplified by reverse transcription-PCR using LA Taq (Takara) from MCF10A mRNAs and cloned into the following mammalian expression vectors: pcDNA3.1/Zeo, pEG2-FLAG, pEG2-FLAG-2NLS, pEF4H (for 4xHA tag), pMMP-puro, pBABE-puro/3xFLAG-His_8. HCF-1N fragments and HCF-1C were amplified from pAPN-HA-HCF-1 (a gift from Winship Herr) and cloned into pEG2-FLAG-2NLS and pEG2-FLAG, respectively. For construction of retroviral vectors expressing shRNAs, hairpin sequences were cloned into pENTRmir, which contains a mir30 precursor sequence between *att*L1 and *att*L2. mir30 cassettes harboring an shRNA were transferred by LR recombination into pMMP-puro-DEST, which was created by inserting a Gateway destination cassette (Invitrogen) in the multicloning site of pMMP-puro. Site-directed mutagenesis was performed by *pfu*Turbo (Stratagene), and all introduced mutations were confirmed by sequencing. Plasmids for ubiquitin mutants were reported previously (33).

GST Pulldown Assays—GST fusion proteins were produced in *Escherichia coli* BL21(DE3). *In vitro* translation of HCF-1N was performed in the presence of [^{35}S]methionine (Perkin-Elmer Life Sciences) using the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instruction. For GST pulldown assays, GST fusion proteins (5 μg) were incubated with glutathione beads (Sigma) in GST binding buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% Nonidet P-40, 25 μg/ml bovine serum albumin) for 1 h at 4 °C. Beads were then incubated with 15 μl of *in vitro* translation products in GST binding buffer for 2 h at 4 °C followed by five washes with GST binding buffer. Proteins bound to the beads were eluted by boiling in 2× SDS-PAGE sample buffer. After SDS-PAGE, GST proteins were visualized by Coomassie Brilliant Blue staining, and ^{35}S-labeled proteins were detected by autoradiography.

Western Blotting and Immunoprecipitation—Cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 10% glycerol) supplemented with protease inhibitor mix (Sigma). Cell fractionation was performed essentially as described previously (34). For Western blotting, 30 μg of proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies. Antibodies used for Western blotting: anti-BAP1 (Santa Cruz), anti-β-actin (Sigma), anti-ERK (Cell Signaling), anti-FLAG (Sigma), anti-HA (Santa Cruz), anti-HCF-1C (a gift from Winship Herr), anti-ORC2 (BD Biosciences). Anti-HCF-1N was raised by immunizing rabbits with GST-HCF-1 (961–1011). For immunoprecipitation, cell lysates containing ~2 mg of proteins were mixed with antibodies for 2 h to overnight, and immunocomplexes were precipitated with protein A or protein G-Sepharose beads (GE Healthcare). Anti-FLAG M2 affinity agarose gel (Sigma) was used for precipitation of FLAG-tagged proteins. *In vivo* ubiquitination assays were performed essentially as described previously (35).

Tandem Affinity Purification—293T cells expressing 3× FLAG/His_8-BAP1 were cultured in ten 15-cm plates and lysed in Nonidet P-40 lysis buffer. Cell lysates were incubated with anti-FLAG affinity agarose beads (Sigma) for 4 h, and beads were washed 5 times with Nonidet P-40 lysis buffer. Purified BAP1 complexes were eluted with 1 mg/ml 3× FLAG peptide (Sigma) and subjected to His_8 tag purification using TALON polyhistidine affinity resin (Clontech) followed by elution with 150 mM imidazole. A small portion of the purified BAP1 complexes was separated by SDS-PAGE and visualized by silver staining. The rest of the samples were precipitated with trichloroacetic acid and analyzed by mass spectrometry.

Mass Spectrometry—Trichloroacetic acid precipitates were resuspended in digestion buffer (100 mM Tris-HCl, pH 8.5, 8 M urea), digested by the sequential addition of Lys-C and trypsin proteases, and analyzed using shotgun proteomics methods on a LTQ-Orbitrap mass spectrometer (Thermofisher) as previously described (36–39). SEQUEST and DTASelect algorithms were used to identify peptides sequences from tandem mass spectra (40, 41). Proteins were considered present in a sample if at least two peptides were identified per protein using a peptide level false positive rate of 5% as determined using a decoy data base strategy (42). Proteins were considered to associate with BAP1 if they were present in the BAP1 IP but absent from other control purifications. We excluded common contaminants like ribosomal proteins and keratins. For ubiquitination site mapping, differential modification searches that considered a mass shift of +114.04292 Da on lysines were utilized. Additionally, cysteine alkylation was performed using chloroacetamide instead of iodoacetamide to prevent the generation of ubiquitin-mimicking adducts chemical adducts as previously described (43).

RESULTS

BAP1 Depletion Results in Growth Retardation—To identify deubiquitinating enzymes important for cell proliferation, we screened all known members of two families of deubiquitinating enzymes, UCH and ubiquitin-specific protease, by RNAi using BrdUrd incorporation assays as a measure of proliferative activity (31).[3] BAP1 was among the hit genes that affected incorporation of BrdUrd after small interfering RNA-mediated depletion in a breast epithelial cell line MCF10A. BAP1 depletion by RNAi also affected proliferation of another breast epithelial cell line MCF12A and breast cancer cell lines MCF7 and T-47D (supplemental Fig. S1). Furthermore, the negative effect of BAP1 depletion on cell proliferation was recapitulated in cell growth assays using MCF10A and MCF12A cells infected with retroviruses expressing BAP1 shRNAs (Fig. 1*A* and supplemental Fig. S2). Three shRNAs targeting different portions of the BAP1 mRNA efficiently depleted BAP1 proteins and caused growth retardation.

BAP1 is the largest member of the human UCH family. It contains an extended C terminus, which may be involved in interaction with other proteins. Therefore, overexpression of BAP1 with defective deubiquitinating enzyme activity may have a dominant negative effect by sequestering interactors from endogenous BAP1. Substitution of cysteine 91 to serine (C91S) was previously reported to diminish ubiquitin hydrolase activity *in vitro* (8, 44). We overexpressed wild type and the C91S mutant of BAP1 in MCF10A and MCF12A using high titer retroviruses (Fig. 1*B*). Expression of the C91S mutant inhibited cell growth, whereas wild-type BAP1 did not. From these experiments, we concluded that deubiquitinating activity of BAP1 is required for efficient cell proliferation.

BAP1 Associates with Chromatin-related Proteins and Transcription Factors in Vivo—To better understand the molecular mechanisms underlying BAP1-mediated growth control, we set out to identify new interactors of BAP1. We expressed BAP1 with a 3× FLAG/His_8 tag in 293T so that proteins associated with BAP1 would be captured by tandem affinity purification using anti-FLAG beads followed by metal affinity resins. SDS-PAGE of the purified proteins revealed specific bands along with 3× FLAG/His_8-BAP1 (Fig. 2*A*), suggesting that BAP1 is associated with other cellular proteins. Using MudPIT (multidimensional protein identification technology) mass spectrometry (36, 37), we identified peptides present in the protein mixture purified from cells expressing affinity-tagged BAP1 but not in a control sample containing native BAP1. Proteins represented by three or more unique peptides are listed in Table 1. The identified BAP1-associated proteins were enriched with proteins involved in transcription and chromatin regulation. For instance, HCF-1 has been shown to interact with histone-modifying enzymes and to assist in transcriptional regulation through interaction with DNA binding proteins (26–29). ASXL1 and ASXL2 are members of the polycomb protein family and contain a PHD domain, which is known to recognize histone methylation (45). Histone-modifying enzymes were also present in the BAP1 precipitates, including the histone

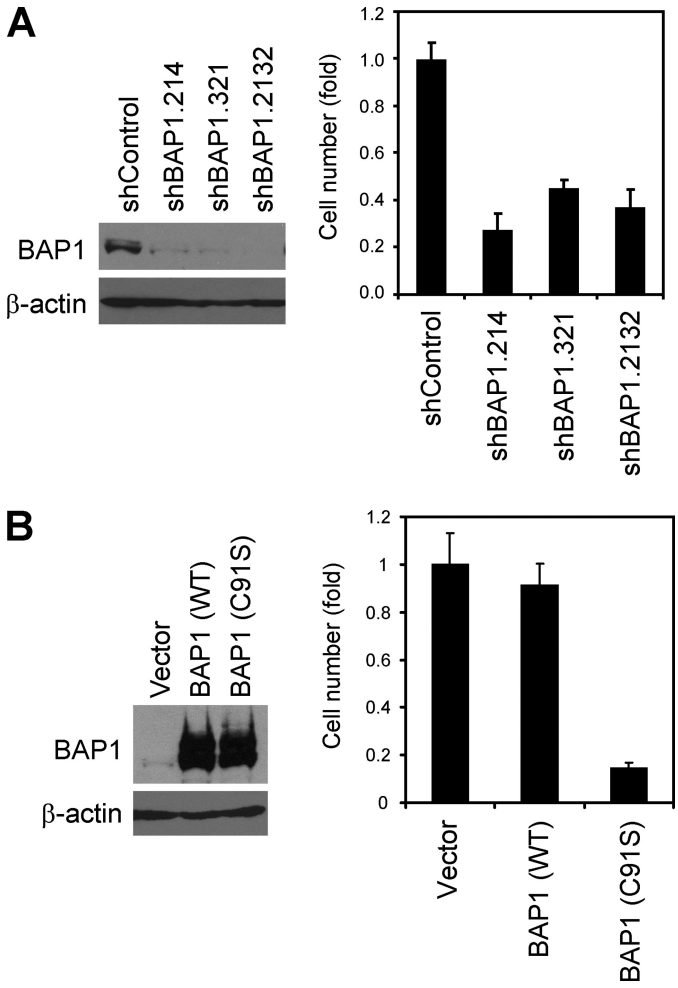

FIGURE 1. **Interference of BAP1 functions results in growth retardation.** *A*, shown is the effect of BAP1 depletion on cell proliferation. MCF10A cells were infected with retroviruses expressing the indicated shRNAs, and BAP1 protein levels were assessed by Western blotting (*left*). β-Actin is shown as a loading control. Proliferation of MCF10A cells expressing the indicated shRNAs were measured by counting cells after a 10-day culture. The ratio of cell count relative to that in shControl population is expressed as the mean ± S. D. ($n = 3$). *B*, shown is the effect of BAP1 overexpression on cell proliferation. MCF10A cells were infected with retroviruses expressing BAP1 or BAP1 C91S. BAP1 levels were examined by immunoblotting (*left*). β-Actin is shown as a loading control. Proliferation of MCF10A cells expressing the indicated proteins were measured by counting cells after a 10-day culture. Ratio of cell count relative to vector control is expressed as the mean ± S. D. ($n = 3$).

acetyltransferase HAT1 as well as AOF1, which was recently shown to possess H3-Lys-4 demethylase activity (46). In addition, several transcription factors including FoxK1 and FoxK2 were coprecipitated with BAP1. This result suggests that BAP1 may be involved in chromatin modulation and gene expression and prompted us to test whether BAP1 is a chromatin-bound protein. We fractionated cellular proteins into a cytosol (S2) fraction and a nuclear fraction, which was subsequently fractionated into nucleoplasmic (S3) and chromatin-enriched (P3) fractions (Fig. 2*B*). Treatment of the nuclei with micrococcal nuclease before the fractionation released chromatin-bound proteins such as ORC2 into the nucleosolic fraction (S3). BAP1 was most abundant in the chromatin-enriched fraction (P3) and was solubilized by micrococcal nuclease treatment like ORC2. Thus, consistent with the fact that BAP1 is associated

[3] Y. J. Machida, Y. Machida, and A. Dutta, unpublished data.

with chromatin-related proteins, the major fraction of BAP1 appears to be present on chromatin.

Interaction of BAP1 and HCF-1—Among the BAP1-associated proteins identified by mass spectrometry, we focused on HCF-1 because the functions of HCF-1 have been linked with cell growth regulation (14, 15). We confirmed that HCF-1 depletion by RNAi resulted in growth retardation in MCF10A (data not shown). We first examined the association of endogenous BAP1 and HCF-1 by coimmunoprecipitation (Fig. 3*A*). HCF-1 was recovered in BAP1 immunoprecipitation, and BAP1 was coprecipitated with HCF-1 in the reciprocal experiment.

HCF-1 is a heterodimer of HCF-1N and HCF-1C, which are produced by processing of the full-length precursor (16–18) (Fig. 3*B*). Because each subunit contains distinct functional domains and play different roles in cell-cycle regulation, we first determined which subunit of HCF-1 interacts with BAP1. FLAG-HCF-1N or FLAG-HCF-1C was coexpressed with 4HA-BAP1 and immunoprecipitated. We found that 4HA-BAP1 was coprecipitated with HCF-1N but not with HCF-1C (Fig. 3*C*). Furthermore, HCF-1N contains an N-terminal Kelch domain and a basic region. To determine the BAP1-interacting domain in HCF-1N, we performed similar coimmunoprecipitation assays using various deletion fragments of HCF-1N. We found that the Kelch domain is sufficient to interact with BAP1 (Fig. 3*D*).

We next determined the HCF-1N-binding region of BAP1. Four GST fusion proteins containing different regions of BAP1 were used to perform pulldown assays with HCF-1N produced by *in vitro* translation. The fragment corresponding to amino acids 282–434 of human BAP1 is sufficient to associate with HCF-1N (Fig. 4*A*). This portion of BAP1 contains a short sequence (residues 356–385) that is highly conserved among species (Fig. 4*B* and supplemental Fig. S3). In examining this conserved region, we noted the NHNY sequence, which is similar to HBM ((D/E)HXY) (Fig. 4*B*). Prompted by this finding and the notion that the Kelch domain interacts with BAP1, we hypothesized that the NHNY sequence is required for interaction with HCF-1N. A mutant BAP1 in which the NHNY sequence was substituted with AAAA (BAP1 ΔHBM) was examined for its interaction with endogenous HCF-1. BAP1 ΔHBM as well as BAP1, with a deletion of the conserved sequence (amino acids 356–385), lacks the ability to interact with endogenous HCF-1N *in vivo* (Fig. 4*C*).

A point mutation in the Kelch domain of HCF-1 (P134S) has been shown to disrupt the interaction between the Kelch domain and HBM-containing proteins (14). We, therefore, speculated that interaction of BAP1 with the HCF-1 Kelch domain may also be affected by this mutation if the HBM-like sequence, NHNY, in BAP1 is recognized by the Kelch domain in the same fashion. Indeed, the mutant Kelch domain (P134S) pulled down substantially less BAP1 protein than wild-type Kelch domain in the coprecipitation assays (Fig. 4*D*). Therefore, we concluded that the amino acid sequence NHNY in BAP1 function as an HBM and that the Kelch domain of HCF-1 recognizes and binds this element.

The Kelch Domain of HCF-1 Is Ubiquitinated in Vivo at Lys-105, Lys-163, Lys-244, and Lys-363—Because BAP1 is a deubiquitinating enzyme, an interesting possibility is that HCF-1 is ubiquitinated *in vivo*, and the level of ubiquitination is regulated by BAP1. To address this possibility, we first tested whether HCF-1 is ubiquitinated *in vivo* by ubiquitination assays. FLAG-tagged HCF-1N and HA-ubiquitin were co-expressed, and FLAG-HCF-1N was immunoprecipitated under denaturing conditions. Ubiquitin molecules conjugated to HCF-1N were then

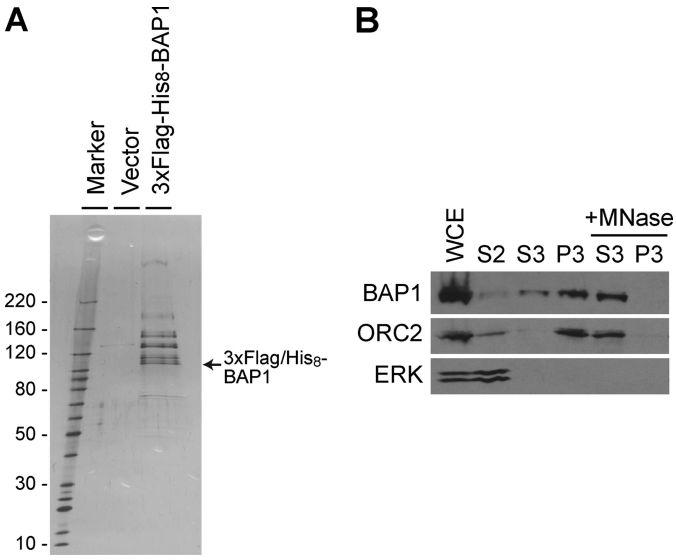

FIGURE 2. **Purification of BAP1-associated proteins.** *A*, shown is purification of BAP1 interactors. 293T cells were infected with retroviruses expressing 3× FLAG/His_8-tagged BAP1 and BAP1-associated proteins were purified by tandem affinity purification. Purified proteins were examined by silver staining. Mock purification from cells infected with empty viruses is shown as a control. *B*, shown is cell fractionation. Cellular proteins were fractionated into cytosolic- (S2), nucleoplasmic- (S3), and chromatin-enriched (P3) fractions as described under "Experimental Procedures." *WCE*, whole cell extracts. Where indicated, P3 fractions were treated with micrococcal nuclease (+*MNase*) to release chromatin-bound proteins into S3 fractions. ERK and ORC2 are shown as markers of cytosolic and chromatin-bound proteins, respectively.

TABLE 1
BAP1-associated proteins identified by mass spectrometry

Accession no.	Name	Description	Number of unique peptides
NP_005325.1	HCF-1	Host cell factor C1 (VP16-accessory protein)	25
NP_056153.2	ASXL1	Additional sex combs like 1	14
NP_060733.3	ASXL2	Additional sex combs like 2	13
NP_068814.2	HSPA2	Heat shock 70-kDa protein 2	8
NP_004505.2	FOXK2	Forkhead box K2 isoform 1	8
NP_071349.2	UBE2O	Ubiquitin-conjugating enzyme E2O	6
NP_001032242.1	FOXK1	Forkhead box K1	5
NP_004406.2	DSP	Desmoplakin isoform I	5
NP_694587.2	AOF1	Amine oxidase (flavin containing) domain 1	4
NP_003633.1	HAT1	Histone acetyltransferase 1 isoform a	3
NP_115593.3	ANKRD17	Ankyrin repeat domain protein 17 isoform a	3

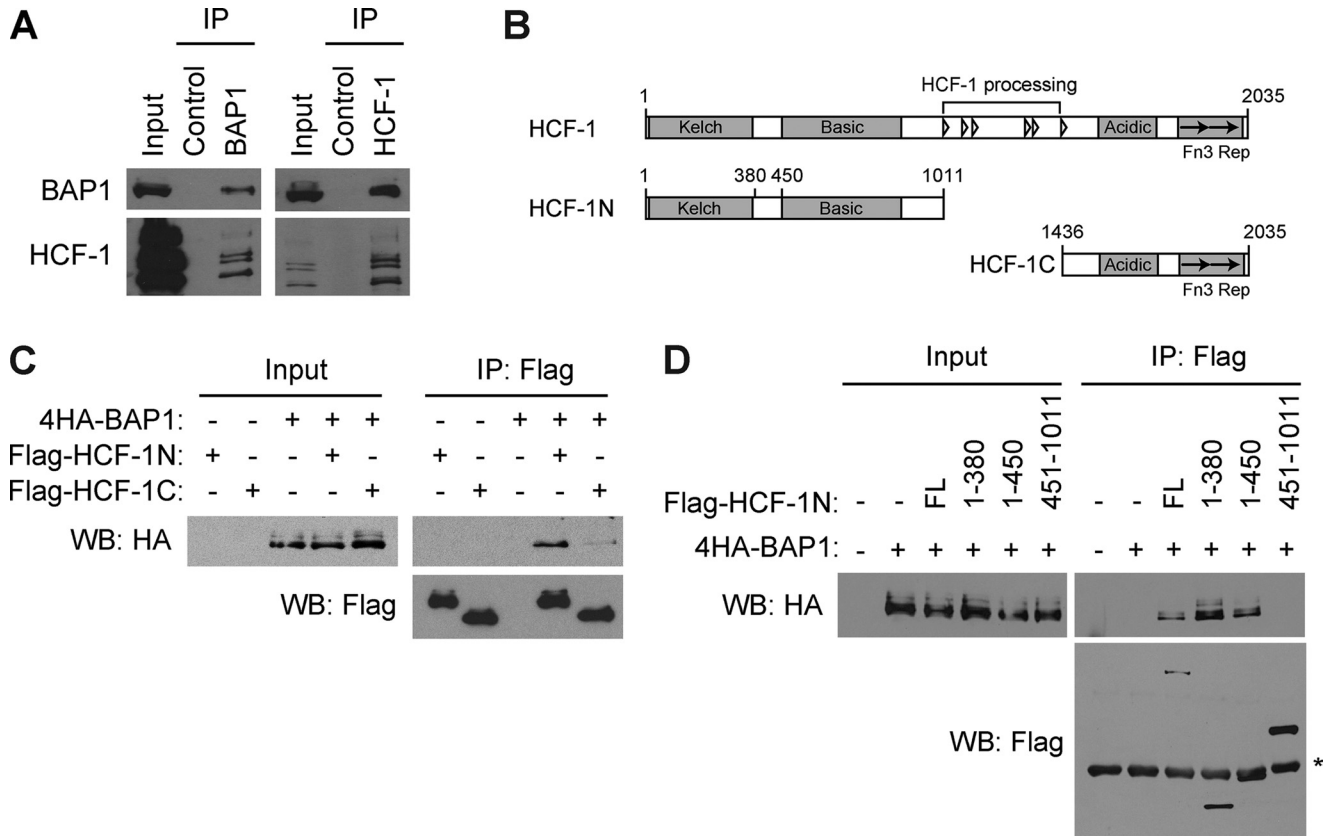

FIGURE 3. **Mapping the BAP1-interacting domain in HCF-1.** *A*, shown is the association of endogenous BAP1 and HCF-1. BAP1 and HCF-1 proteins were immunoprecipitated (*IP*) with specific antibodies, and the indicated proteins were examined by Western blotting. *B*, shown is a schematic representation of the HCF-1 domain structure. *C*, shown is *in vivo* association of BAP1 with HCF-1N. 293T cells were transfected with expression plasmids for the indicated proteins. HCF-1N and HCF-1C proteins were immunoprecipitated by anti-FLAG antibodies. Coprecipitation of BAP1 proteins was examined by anti-HA Western blotting (*WB*). A schematic representation of the HCF-1 domain structure is shown (*top*). *D*, shown is mapping the BAP1 binding domain of HCF-1N. Cells were transfected with plasmids expressing 4HA-BAP1 and various truncation mutants of HCF-1N tagged with FLAG-2NLS (nuclear localization signal). Association of BAP1 with each fragment was assessed by anti-FLAG immunoprecipitation followed by anti-HA Western blotting (*bottom*). An *asterisk* indicates Ig heavy chains. Note that FLAG-HCF 1–450 migrates just below the IgH chain. A schematic representation of the HCF-1N domain structure is shown (*top*). *FL*, full-length.

detected by anti-HA Western blotting. A smear of HA-ubiquitin signals was detected in the FLAG-HCF-1N pulldown assays, indicating that FLAG-HCF-1N is ubiquitinated *in vivo* (Fig. 5*A*). Ubiquitination of HCF-1N was also observed when it was produced by the expression of a full-length HCF-1, which was then processed to the HCF-1N fragment by proteolytic cleavage (supplemental Fig. S4). The Kelch domain appears to be the primary acceptor of ubiquitin in HCF-1N, as similar levels of ubiquitination were detected with HCF-1N and with the Kelch domain by itself (Fig. 5*A*). Additionally, the Kelch domain may also be sufficient to bind putative E3 enzyme(s) responsible for this ubiquitination.

We next determined the ubiquitin acceptor sites in the Kelch domain. FLAG-Kelch proteins transiently expressed in 293T cells were purified under denaturing conditions, separated by SDS-PAGE, and visualized by Coomassie staining (Fig. 5*B*). Besides the non-ubiquitinated form, several discrete bands (indicated as triangles in Fig. 5*B*) as well as a smear of proteins were detected specifically in cells expressing FLAG-Kelch. Mass spectrometry analysis of these discrete bands revealed ubiquitination at lysine 105, lysine 163, lysine 244, and lysine 363. Kelch domains have a six-bladed β-propeller structure with each blade composed of four β-strands (47). The ubiquitin acceptor sites identified by mass spectrometry are located in or close to the loops between β-strands (Fig. 5*C*).

Depending on which lysine residue of ubiquitin is utilized for chain formation, different types of polyubiquitin chains direct proteins to distinct fates. For example, Lys-48-linked ubiquitin chains mainly trigger degradation by proteasomes, whereas Lys-63-linked chains are involved in totally distinct processes such as kinase signaling (48). HCF-1C was recently shown to be conjugated with Lys-48- and -63-linked polyubiquitin chains (12). We, therefore, examined the linkage of the polyubiquitin chains on the HCF-1 Kelch domain by using a variety of lysine mutants of ubiquitin in the *in vivo* ubiquitination assays. Expression of lysine to arginine mutants of ubiquitin inhibits polyubiquitin chain elongation if the mutated lysine residues are used in the normal elongation process. Because ubiquitin is an abundant protein *in vivo*, expression of ubiquitin mutants is not expected to inhibit elongation completely but should reduce polyubiquitin chain length due to capping.

First, we examined the effect of the K48R mutant in which lysine-48 is mutated to arginine. Utilizing this mutant caused an increase of ubiquitinated Kelch proteins in the low molecular weight range (Fig. 5*D*), suggesting that lysine 48 is involved in polyubiquitin chain formation on the Kelch domain. The

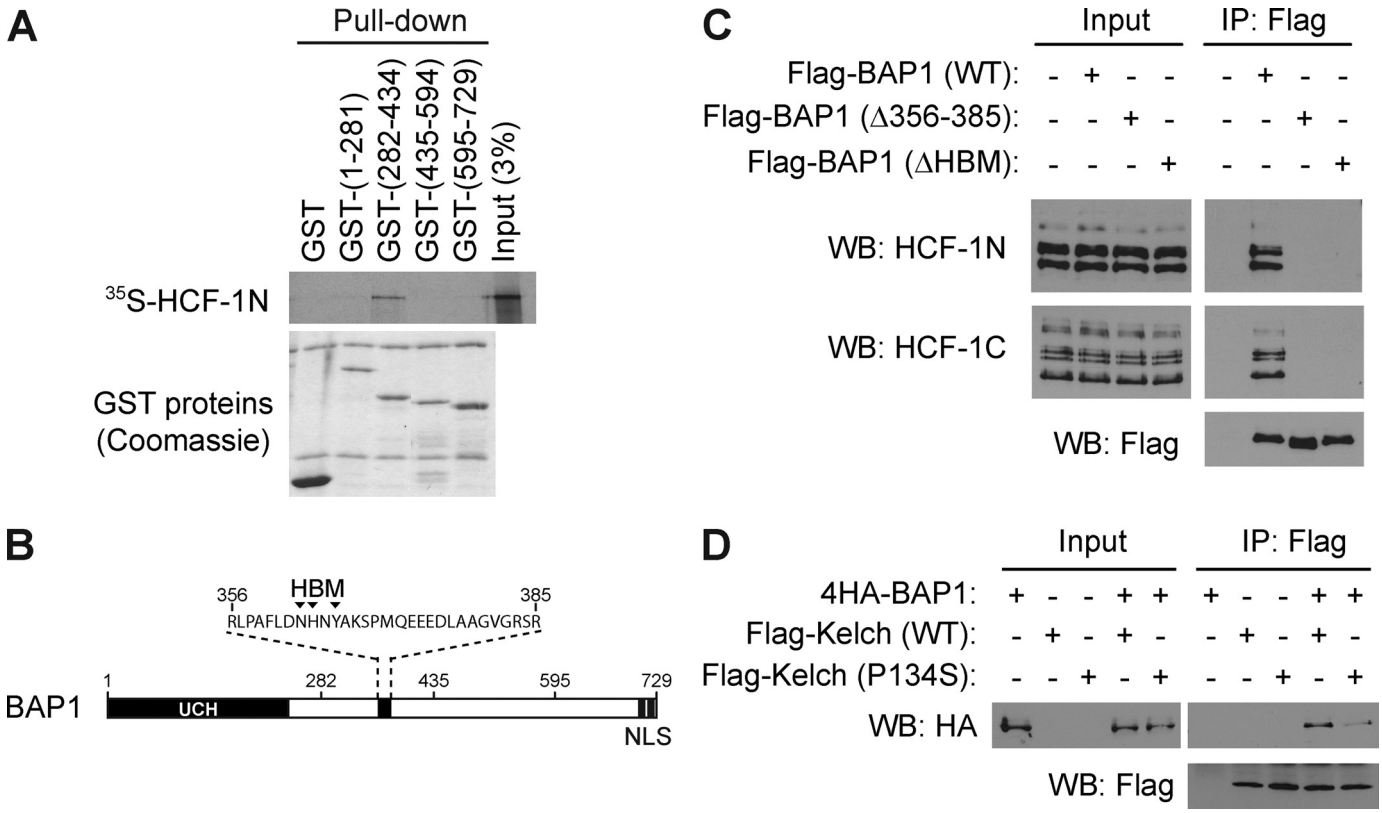

FIGURE 4. **Mapping the HCF-1-interacting domain in BAP1.** *A*, shown is *in vitro* association of BAP1 fragments with HCF-1N. GST pulldown assays were performed using GST fusion of the indicated BAP1 fragments and HCF-1N protein produced by *in vitro* translation. HCF-1N proteins in the pulldown fractions were examined by autoradiography. Precipitated GST fusion proteins are shown by Coomassie staining. *B*, shown is a schematic representation of BAP1 protein indicating the highly conserved sequence surrounding the HBM. *NLS*, nuclear localization signal. *C*, shown is *in vivo* interaction of HCF-1 with various mutants of BAP1. 293T cells were transfected with expression plasmids for indicated proteins. FLAG-BAP1 proteins were pulled down from cell lysates using anti-FLAG antibodies and probed with indicated antibodies. *IP*, immunoprecipitate; *WB*, Western blot; *WT*, wild type. *D*, shown is *in vivo* interaction of BAP1 and the HCF-1 Kelch domain. Wild type or P134S mutant of HCF-1 Kelch domain was coexpressed with BAP1, immunoprecipitated with anti-FLAG antibodies, and associated BAP1 proteins examined by anti-HA Western blotting.

polyubiquitin chains, however, do not appear to be exclusively Lys-48-linked because the effect of the K48R mutant was not as strong as the K0 mutant. Next, we examined the effect of the ubiquitin K48 mutant, in which all lysine residues except Lys-48 have been substituted with arginines. As such, only Lys-48 and the N terminus are available for ubiquitin conjugation. In this case, the K48 mutant did not alter the pattern of Kelch polyubiquitination (Fig. 5*D*). In contrast, expression of the K63 mutant resulted in increase in the hypo-ubiquitinated forms, and the effect was nearly similar to that of the K0 mutant. Based on these results, we concluded that Lys-48 is a major but not exclusive site for polyubiquitin chain linkage on the Kelch domain.

BAP1 Deubiquitinates HCF-1N in Vivo—Having established assays to determine the ubiquitination levels of HCF-1N *in vivo*, we next tested whether coexpression of BAP1 affects the ubiquitination level of HCF-1N. Wild-type BAP1 induced a dramatic decrease in the ubiquitination levels of both FLAG-HCF-1N and FLAG-Kelch (Fig. 6*A* and supplemental Fig. S5, *middle panel*). In contrast, the C91S mutant, a catalytically inactive form of BAP1, did not reduce ubiquitination. Rather, BAP1 C91S increased the amount of ubiquitinated Kelch protein in the high molecular weight range. This is likely due to the dominant negative effect of the mutant, caused by interference of the overexpressed variant protein with endogenous BAP1 action. This experiment, therefore, suggests that BAP1 decreases the ubiquitination levels of the HCF-1 Kelch domain.

Next we tested whether direct interaction between BAP1 and HCF-1 is required for the reduction of HCF-1 polyubiquitination by BAP1. First, using *in vivo* deubiquitination assays, we examined the importance of the HBM for BAP1-mediated reduction of FLAG-Kelch domain ubiquitination. Mutations in the HBM abolished BAP1-mediated reduction in the ubiquitination level (Fig. 6*B*, *middle panel*). Similarly, introduction of the ΔHBM mutation diminished the dominant negative effect of BAP1 C91S on the ubiquitination level of HCF-1 Kelch (Fig. 6*C*). These results suggest that the direct interaction between BAP1 and Kelch is required for the BAP1-mediated reduction of HCF-1N ubiquitination. Consistent with this, the Kelch domain harboring the P134S mutation exhibited greater ubiquitination in the *in vivo* ubiquitination assays (Fig. 6*D*) likely due to the weaker association with endogenous BAP1 (Fig. 4*D*). In summary, the HBM of BAP1 and Pro-134 of HCF-1 Kelch domain are required for the reduction of HCF-1N ubiquitination by BAP1. Because the UCH activity of BAP1 is also required (Fig. 6*A*), we conclude that BAP1 associates with and deubiquitinates HCF-1 *in vivo*.

The BAP1 HBM Is Required for BAP1-mediated Growth Regulation—Biochemical evidence shown above suggests that BAP1 binds to the HCF-1 Kelch domain via HBM and deubiq-

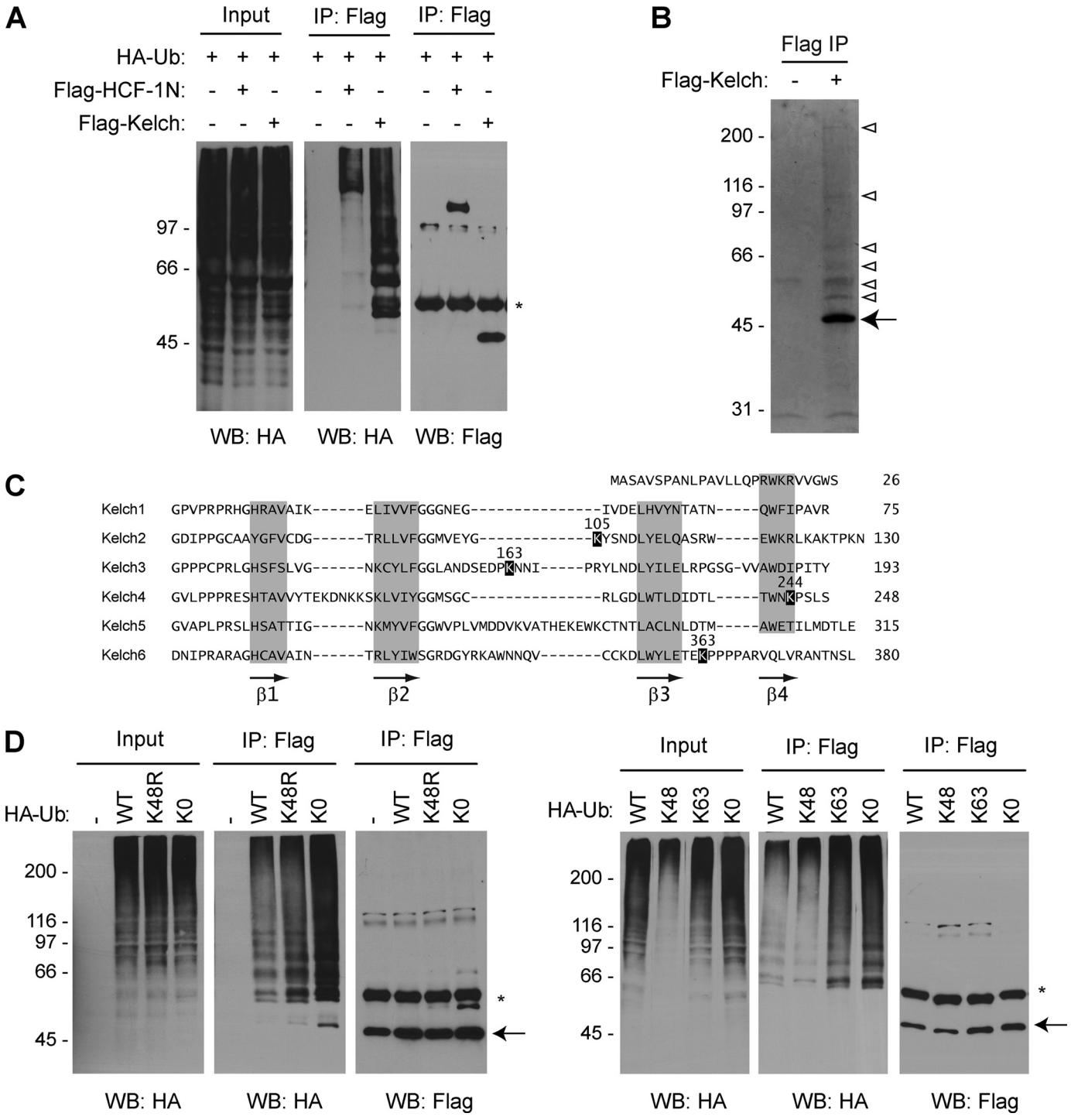

FIGURE 5. **Ubiquitination of HCF-1N *in vivo*.** *A*, shown is mapping the ubiquitination domain of HCF-1N. Plasmids expressing the indicated proteins were cotransfected to 293T, and ubiquitination (*Ub*) of HCF-1 proteins was examined by Western blotting (*WB*) after anti-FLAG immunoprecipitation (*IP*). *B*, shown is purification of ubiquitinated FLAG-Kelch from cells. 293T cells were transfected with plasmids expressing HA-ubiquitin and FLAG-Kelch. FLAG-Kelch proteins were purified with anti-FLAG antibodies, separated by SDS-PAGE, and visualized by Coomassie staining. Unmodified Kelch is indicated by an *arrow*. *Triangles* indicate bands analyzed by mass spectrometry. *C*, shown is the amino acid sequence of the HCF-1 Kelch domain. The alignment was adapted from Wilson *et al.* (52). The ubiquitin acceptors identified in this study are highlighted in *black*. β-Strands are indicated by *gray boxes*. *D*, examination of ubiquitin linkages on the HCF-1 Kelch domain is shown. FLAG-Kelch proteins were expressed in combination with various ubiquitin mutants. Ubiquitination of FLAG-Kelch was analyzed as in *A*. The unmodified Kelch domain is indicated by *arrows*. *Asterisks* indicate Ig heavy chains. *WT*, wild type.

uitinates HCF-1N *in vivo*. We next determined whether the interaction between BAP1 and HCF-1 is important for the role of BAP1 in cell proliferation. In Fig. 1*B* we have shown that overexpression of the BAP1 C91S mutant in MCF10A suppresses cell growth. This is a dominant negative effect of the mutant, caused by sequestration of critical substrates from endogenous BAP1. If interaction with HCF-1 is important for BAP1 functions, the HBM would be required for the BAP1 C91S mutant to have a dominant negative effect on cell proliferation. We, therefore, tested whether deletion of the HBM

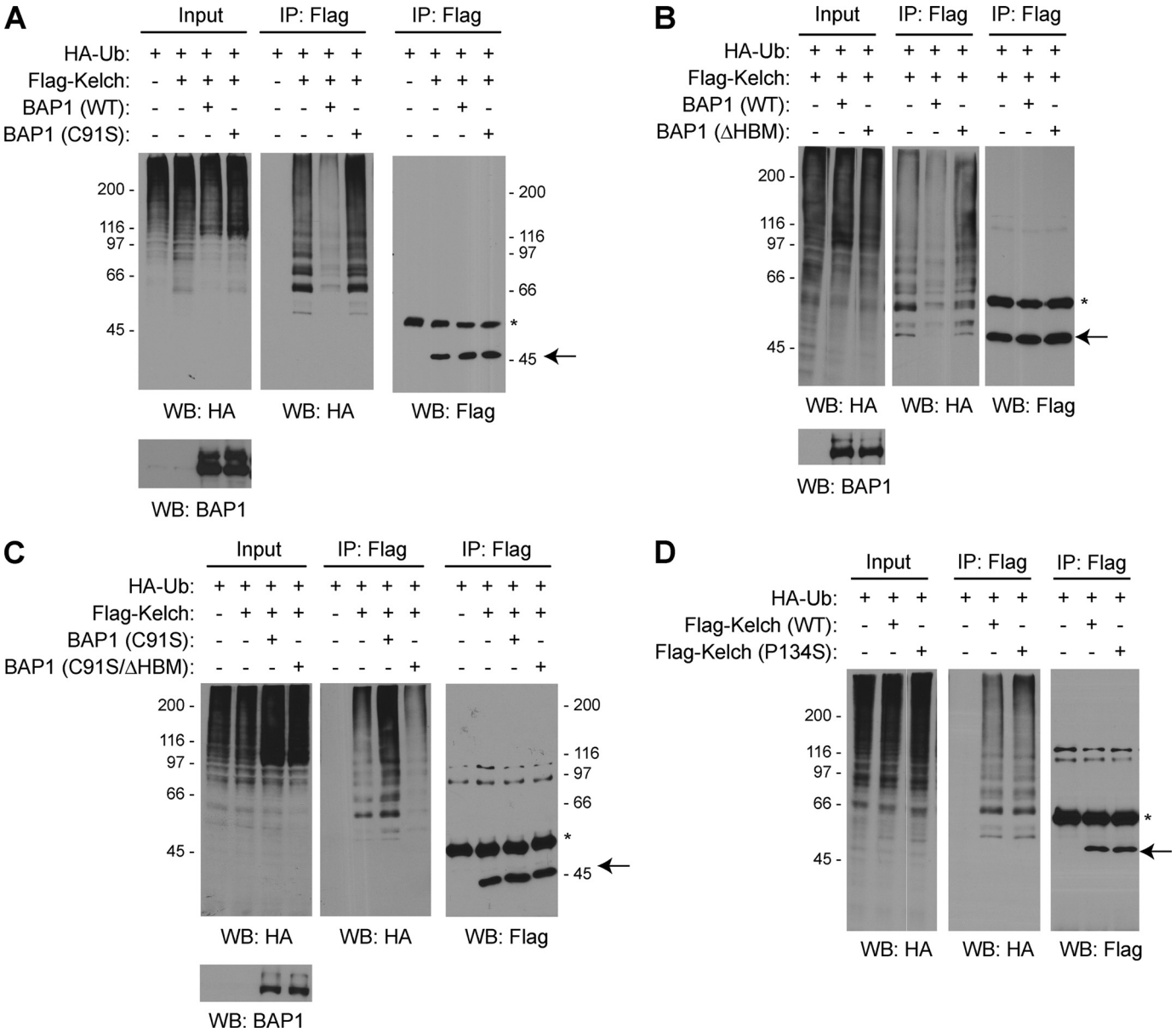

FIGURE 6. **Deubiquitination of HCF-1N by BAP1.** *A*, shown is *in vivo* ubiquitination assays. 293T cells were cotransfected with indicated plasmids. FLAG-Kelch was immunoprecipitated (*IP*) by anti-FLAG antibodies and examined by Western blotting (*WB*). *WT*, wild type. *Ub*, ubiquitin. *B*, shown is the effect of the ΔHBM mutation on the BAP1-mediated reduction of Kelch ubiquitination. *In vivo* ubiquitination assays were performed in cells expressing the indicated proteins. *C*, shown is the effect of the ΔHBM mutation on the dominant negative activity of BAP1 C91S. *In vivo* ubiquitination assays were performed as in *A*. *D*, shown is the effect of the P134S mutation on the Kelch ubiquitination. Cells were transfected with plasmids for the indicated proteins. Ubiquitination of the Kelch domains was examined as in *A*. Unmodified FLAG-Kelch is indicated by an *arrow*. *Asterisks* indicate Ig heavy chains.

from the BAP1 C91S mutant abolishes the effect on cell growth. We expressed BAP1 with various combinations of mutations: wild-type, C91S, ΔHBM, C91S/ΔHBM (Fig. 7*A*). The C91S mutant affected cell growth as expected, whereas the BAP1 construct with both C91S and ΔHBM mutations failed to inhibit cell growth (Fig. 7*B*). This result indicates that interaction with HCF-1 is essential for BAP1 function in cell growth regulation.

DISCUSSION

BAP1 plays important roles in cell growth and tumor suppression. In this study we investigated the molecular mechanisms of BAP1-mediated growth regulation. We reported here that BAP1 is associated with factors involved in chromatin regulation and transcription. While this manuscript was under review, Sowa *et al.* (49) published a global proteomic analysis of human deubiquitinating enzyme complexes and reported similar BAP1-associated proteins. We further showed that interaction with HCF-1 is important for BAP1-mediated control of cell growth. HCF-1N is ubiquitinated *in vivo*, and the ubiquitination level is decreased by BAP1. We propose that HCF-1 is a critical target of BAP1 in growth regulation.

Using a targeted small interfering RNA screen, we found that BAP1 is important for cell proliferation. Previous genome-wide RNAi screening performed by another group also identified BAP1 as a gene that is important for cell proliferation in other

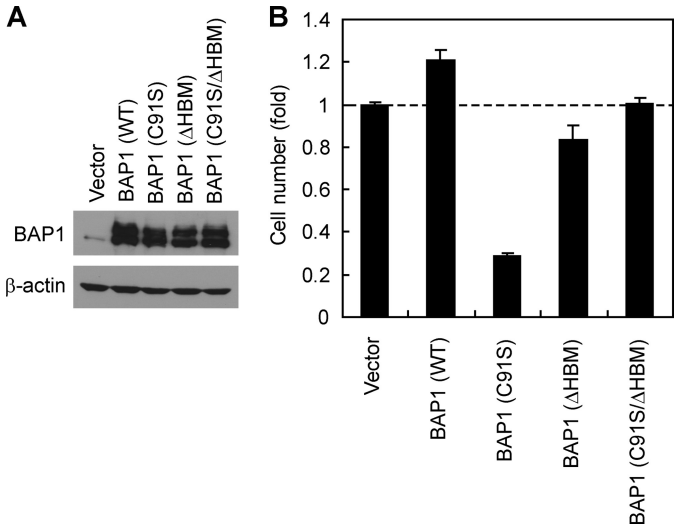

FIGURE 7. **Overexpression of the BAP1 C91S mutant suppresses cell growth in an HBM-dependent manner.** *A*, shown is overexpression of various BAP1 mutant proteins. BAP1 mutants were expressed in MCF10A by retrovirus vectors and examined by Western blotting (*WB*). β-Actin is shown as a loading control. *IP*, immunoprecipitate. *Ub*, ubiquitin. *B*, shown is effect of BAP1 proteins harboring various mutations on cell proliferation. Proliferation of MCF10A cells expressing the indicated proteins were measured by counting cells after a 11-day culture. Values are the means ± S. D. ($n = 3$).

cell lines (50, 51). However, it was not clear whether the role of BAP1 in cell proliferation is dependent on its deubiquitinating activity. In this study we showed, using a dominant negative form of BAP1, that the intact UCH activity of BAP1 is necessary for proper cell proliferation (Fig. 1*B*). Several studies have implicated BAP1 in cell-cycle regulation; one study reported that BAP1 depletion slows down S phase, whereas another showed that BAP1 expression accelerates S phase entry (11, 13). These facts, therefore, point to the notion that BAP1 is involved in the transition from G_1 to S phase. Similarly, HCF-1N has been implicated in G_1 phase progression (15). Thus, BAP1 may control the G_1/S transition by regulating the function of HCF-1N. Interestingly, BAP1 is not absolutely essential for cell proliferation. A number of cancer cell lines are viable without BAP1 (8), and the lack of BAP1 is even advantageous to those cells for proliferation and tumor formation (11). It is likely that those cancer cells have additional genetic or epigenetic alterations in other genes that overcome the requirement of BAP1 for cell proliferation. BAP1 may have dual roles in growth control; it is involved in the regulation of the normal cell cycle, whereas it prevents uncontrolled cell growth, thereby ensuring proper cell proliferation.

Recently, the interaction between BAP1 and HCF-1 was also reported by Misaghi *et al.* (12). Consistent with their study, our data indicates that the HCF-1 Kelch domain recognized the noncanonical HBM sequence of BAP1 (Fig. 4). This conclusion was further strengthened by another experiment in which we showed that the interaction between BAP1 and the Kelch domain was affected by substitution of Proline-134 with serine (P134S), a mutation in the HCF-1 Kelch domain known to disrupt Kelch-HBM interactions (20, 21) (Fig. 4*D*). The function of the BAP1 HBM is unique among the other known HBMs commonly found in DNA binding proteins (25) because it is used for an enzyme-substrate interaction rather than for tethering HCF-1 to a promoter. Importantly, we further showed that the interaction with HCF-1 is important for regulation of cell growth by BAP1, as the dominant negative effect of BAP1 C91S on cell growth was entirely dependent on the BAP1 HBM (Fig. 7*B*). This result links BAP1 enzyme activity with HCF-1 and is in line with the notion that BAP1 is a deubiquitinating enzyme for HCF-1.

We present several lines of evidence to support the idea that HCF-1 is a substrate of BAP1 *in vivo*. First, BAP1 binds to HCF-1N *in vivo* (Figs. 3 and 4). Second, coexpression of BAP1 reduced ubiquitination levels of the HCF-1N in a UCH- and HBM-dependent manner (Fig. 6, *A* and *B*). Third, ubiquitination of the Kelch domain was increased when coexpressed with a dominant negative form of BAP1 (Fig. 6*A*), and this effect is dependent on the BAP1-HCF-1 interaction (Fig. 6*C*). Fourth, the Kelch domain harboring the P134S mutation, which reduces association between BAP1 and the HCF-1 Kelch domain, exhibited a higher degree of ubiquitination than wild type (Fig. 6*D*). Based on these results, we concluded that BAP1 directly deubiquitinates HCF-1N *in vivo*, although we cannot exclude the possibility that the effect of BAP1 on the HCF-1 ubiquitination may also be mediated by indirect effects.

Misaghi *et al.* (12) recently reported that HCF-1C is also ubiquitinated; thus, HCF-1 appears to be ubiquitinated on both subunits. Our data indicated that the Kelch domain is sufficient for ubiquitination (Fig. 5*A*), suggesting that the putative E3 ligase binds to the Kelch domain. Our analysis using ubiquitin mutants revealed that the polyubiquitin chains on the Kelch domain are mainly Lys-48-mediated but could potentially include other types of linkages as well (Fig. 5*D*). Indeed, Lys-48- and -63-linked ubiquitin peptides were detected in the mass spectrometry analyses of the ubiquitinated Kelch domain shown in Fig. 4 (data not shown). In the previous study the polyubiquitin chains on HCF-1C were reported to be Lys-48- and -63-linked (12). Because Lys-48-linked polyubiquitin chains are known as a signal for proteasome-dependent degradation, BAP1 may be a stabilization factor for HCF-1. However, we did not observe a reduction in the total levels of HCF-1 subunits after either BAP1 depletion or overexpression of dominant negative BAP1.[3] Likewise, BAP1 RNAi did not reduce (but instead increased) the HCF-1C levels in the recent report by Misaghi *et al.* (12). Although we cannot rule out the possibility that BAP1 regulates HCF-1 levels during only a small part of the cell-cycle, on the whole BAP1 appears to control cell growth via deubiquitination of HCF-1 without affecting its overall level. Alternatively, a small fraction of HCF-1 that is bound to a specific promoter may be regulated by BAP1. It is also possible that deubiquitination of HCF-1N may modulate completely different properties of the protein, such as localization of HCF-1 or interactions with Kelch-binding proteins. In any case, the BAP1/HCF-1 interaction serves to underscore the increasingly complex and dynamic nature of ubiquitination and deubiquitination in the cellular environment.

In summary, we have shown that the BAP1-HCF-1 interaction plays a pivotal role in regulation of cell growth by BAP1. We also showed that HCF-1 is modified with polyubiquitin chains, which are reduced by BAP1. It is worth noting that, although the dominant negative effect of BAP1 C91S on cell

growth was totally dependent on the BAP1-HCF-1 interaction, this study does not undermine the potential importance of other BAP1 interactors. BAP1 C91S may have different levels of dominant negative effects on each BAP1 interactor depending on the strength of their respective interactions. Also, BAP1 may have a function independent of its deubiquitinating enzyme activity. Such a function is not expected to be affected by BAP1 C91S overexpression. Further characterization of other BAP1 interactors will be needed for a more complete understanding of BAP function in cell growth and tumor suppression.

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