MOLECULAR AND CELLULAR BIOLOGY, Apr. 2009, p. 2181–2192 Vol. 29, No. 8
0270-7306/09/$08.00+0 doi:10.1128/MCB.01517-08
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

Association of C-Terminal Ubiquitin Hydrolase BRCA1-Associated Protein 1 with Cell Cycle Regulator Host Cell Factor 1[▽]

Shahram Misaghi,[1] Søren Ottosen,[5] Anita Izrael-Tomasevic,[2] David Arnott,[2] Mohamed Lamkanfi,[1] James Lee,[3] Jinfeng Liu,[4] Karen O'Rourke,[1] Vishva M. Dixit,[1]* and Angus C. Wilson[5]*

Physiological Chemistry Department,[1] Protein Chemistry Department,[2] Molecular Biology Department,[3] and Bioinfomatics Department,[4] Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, and Department of Microbiology and NYU Cancer Institute, New York University School of Medicine, New York, New York 10016[5]

Received 29 September 2008/Returned for modification 27 October 2008/Accepted 26 January 2009

Protein ubiquitination provides an efficient and reversible mechanism to regulate cell cycle progression and checkpoint control. Numerous regulatory proteins direct the addition of ubiquitin to lysine residues on target proteins, and these are countered by an army of deubiquitinating enzymes (DUBs). BRCA1-associated protein-1 (Bap1) is a ubiquitin carboxy-terminal hydrolase and is frequently mutated in lung and sporadic breast tumors. Bap1 can suppress growth of lung cancer cells in athymic nude mice and this requires its DUB activity. We show here that Bap1 interacts with host cell factor 1 (HCF-1), a transcriptional cofactor found in a number of important regulatory complexes. Bap1 binds to the HCF-1 β-propeller using a variant of the HCF-binding motif found in herpes simplex virus VP16 and other HCF-interacting proteins. HCF-1 is K48 and K63 ubiquitinated, with a major site of linkage at lysines 1807 and 1808 in the HCF-1_C subunit. Expression of a catalytically inactive version of Bap1 results in the selective accumulation of K48 ubiquitinated polypeptides. Depletion of Bap1 using small interfering RNA results in a modest accumulation of HCF-1_C, suggesting that Bap1 helps to control cell proliferation by regulating HCF-1 protein levels and by associating with genes involved in the G_1-S transition.

The ubiquitin-mediated proteasomal degradation pathway plays an essential role in protein turnover in mammalian cells (10). The process begins with the conjugation of several ubiquitin moieties onto the substrate destined for degradation, and appropriately marked substrates are then recognized and degraded by the 26S proteasome. Ubiquitination is a reversible process, and a group of cysteine proteases called deubiquitinating enzymes (DUBs) catalyze the removal of ubiquitin chains from ubiquitinated proteins. This allows substrates to either be rescued from degradation by the proteasome or facilitates their degradation by removing the ubiquitin moiety as the substrate is being unfolded and fed through the proteasome core. Deubiquitination also allows ubiquitin to be recycled by hydrolyzing ubiquitin chains or peptide remnants that are attached to ubiquitin via isopeptide linkages (45).

Ubiquitin C-terminal hydrolases (UCHs) constitute a small class of DUBs that share a common and conserved UCH domain. In mammals, the four known members of this family are UCH-L1, UCH-L3, UCH37, and Bap1 (see Fig. 1A) (13, 23, 25). UCH-L1 and UCH-L3 are small proteins (~25 kDa) that share ~50% sequence identity to each other. Structurally, they are folded in a similar manner, but differences in positioning and geometry of catalytic residues within the active site suggest that their mode of function and/or substrate set may be different (6, 15, 31). Both UCH-L1 and UCH-L3 are believed to help sustain the pool of mono-ubiquitin species within the cell through ubiquitin recycling (24). UCH-L1 is implicated in neurodegenerative diseases and is itself mono-ubiquitinated, a reversible inhibitory modification that prevents UCH-L1 from binding to ubiquitinated targets (30). Through its unique C-terminal extension, UCH37 associates with the hRpn13 of the 26S proteasome (12, 25, 35). Although the exact function is unclear, UCH37 appears to regulate substrate degradation by disassembling ubiquitin chains from the distal end of polyubiquitin chain (19, 39).

The fourth member of the UCH family, BRCA1-associated protein 1 (Bap1), was discovered through its interaction with the RING finger domain of tumor suppressor protein BRCA1 (13). Involvement of Bap1 as a DUB in the BRCA1 pathway remains controversial and a firm link to breast cancer predisposition has not been established (4, 28, 33). However, it has been shown the BAP1 gene undergoes frequent loss of heterozygosity in human tumors, and missense mutations have been identified in lung, renal, and sporadic breast tumors (1, 3). Bap1 can suppress the growth of non-small-cell lung carcinoma NCI-H226 cells in culture and as solid tumors in athymic nude mice (41). In order for Bap1 to suppress growth, it must be localized to the nucleus and retain catalytic activity since lung cancer-derived Bap1 mutants fail to block proliferation or tumor growth. Although the mechanisms remains to be established, sustained expression of Bap1 induces profound alterations in the cell cycle and elevated rates of cell death.

Host cell factor 1 (HCF-1; HCFC1) is a conserved nuclear protein required for a variety of biological processes including

* Corresponding author. Mailing address for Vishva M. Dixit: Department of Physiological Chemistry, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080. Phone: (650) 225-1312. Fax: (650) 225-6127. E-mail: dixit.vishva@gene.com. Mailing address for Angus C. Wilson: Department of Microbiology, NYU School of Medicine, 550 First Ave., New York, NY 10016. Phone: (212) 263-0206. Fax: (212) 263-8276. E-mail: angus.wilson@med.nyu.edu.

▽ Published ahead of print on 2 February 2009.

cell cycle progression and maintenance of pluripotency (7, 11, 17). HCF-1 functions as a transcriptional cofactor and associates with a variety of histone-modifying activities, including the tri-thorax-related mixed-lineage leukemia (MLL) and Set1 histone H3 lysine 4 methyltransferases (H3K4 histone methyltransferases), the MOF histone acetyltransferase, and the mSin3 histone deacetylase complex (8, 37, 47, 50). HCF-1 is expressed as a large precursor protein that is proteolytically cleaved into two subunits, HCF-1_N and HCF-1_C, that remain stably associated in the mature protein (20, 44). HCF-1 does not bind DNA directly but instead associates with a variety of sequence-specific DNA-binding proteins through multiple interaction domains. The best studied is a six-bladed β-propeller domain in HCF-1_N that forms a docking surface for proteins carrying a tetrapeptide motif D/EHxY (where "x" denotes any residue), called the HCF-binding motif (HBM) (9, 26). The herpes simplex virus transactivator VP16 contains the prototype HBM and uses HCF-1 to assemble a potent activator complex on viral immediate-early genes (32, 46). Prominent cellular HBM-containing proteins include E2F1 and E2F4 (18, 27, 40), and HCF-1 plays a direct role in activation of E2F-regulated promoters through recruitment of the MLL family of H3K4 histone methyltransferases (40). Control of HCF-1 abundance and activity is poorly understood.

In the present study, we used affinity purification and mass spectrometry (MS) to search for potential substrate(s) for Bap1 UCH activity. We identified HCF-1 as a major interacting partner. Bap1 and HCF-1 are nuclear and interact through the β-propeller domain of HCF-1. Interaction is dependent on an evolutionarily conserved NHNY sequence resembling the aforementioned HBM. We show that HCF-1 is both lysine-48 (K48) and lysine-63 (K63) ubiquitinated and map a major ubiquitination site to lysine-1807 or lysine-1808 in HCF-1_C. Overexpression of a catalytically inactive Bap1 mutant results in the selective accumulation of K48 ubiquitinated HCF-1, implicating Bap1 in regulation of HCF-1 protein turnover. Depletion of Bap1 using small interfering RNAs (siRNAs) leads to an accumulation of both precursor and processed forms of HCF-1, suggesting that removal of ubiquitin chains is required for proteasomal degradation. Flow cytometry reveals a commensurate increase in the proportion of cells entering the S and G_2/M phases relative to the G_1 phase.

MATERIALS AND METHODS

Plasmids and siRNA. Hemagglutinin (HA)- and Flag-tagged human Bap1 expression plasmids were generated by PCR amplification from a Bap1 cDNA clone (Genentech, Inc.) using the following primers and subcloned into the XhoI and KpnI sites of pcDNA3.1(−). The amplification primers were as follows: Bap1-(XhoI-Kozak)-forward, 5′-CCGCTCGAGACCATGGCCAATAAGGGC TGGCTGGAGCTG-3′; Bap1-(KpnI-Stop-HA)-reverse, 5′-CGGGGTACCTT AGTCGAGTGCGTAGTCTGGTACGTCGTACGGATACTGGCGCTTGGC CTTGTAGG-3′; and Bap1-(KpnI-Stop-Flag)-reverse, 5′-CGGGGTACCTTAC TTGTCGTCATCGTCTTTGTAGTCCTGGCGCTTGGCCTTGTAGG-3′.

Active-site (C91A) and NHNY/AAAA mutations were introduced into Bap1-flag by using QuikChange site-directed mutagenesis (Stratagene) and the following primers: C91A-Bap1-forward, 5′-ACCAGCTGATACCCAACTCTGCT GCAACTCATGCCTTGCTGAGCGTGCTC-3′; C91A-Bap1-reverse, 5′-GAG CACGCTCAGCAAGGCATGAGTTGCAGCAGAGTTGGGTATCAGCTG GT-3′; NHNY/AAAA-Bap1-forward, 5′-CAGCGGCTGCCGGCCTTTCTAG ACGCTGCCGCTGCTGCCAAGTCCCCCATGCAGGAGGA-3′; and NHNY/AAAA-Bap1-reverse, 5′-TCCTCCTGCATGGGGGACTTGGCAGCAGCGG CAGCGTCTAGAAAGGCCGGCAGCCGCTG-3′.

Plasmids expressing HA-tagged HCF-1 truncations have been described previously (43). The HCF-1-V5 construct was kindly provided by Thomas M. Kristie (Laboratory of Viral Diseases, National Institutes of Health). The HCF-1-Myc expression construct was made by releasing the HCF-1 insert from the HCF-1-V5 construct using BamHI and XhoI and religating the fragment into pCMV-Tag 5A in-frame with a C-terminal Myc epitope tag. Expression plasmids encoding K48 HA-Ub and K63 HA-Ub were kindly provided by Nobuhiko Kayagaki (Genentech, Inc.). Pooled Bap1 siRNAs (Dharmacon J-058658) were introduced into HEK-293 cells by using Lipofectamine 2000 (Invitrogen) at a 100 nM final concentration and analyzed after 72 h.

Cell culture, immunoprecipitations, and antibodies. Human HEK-293 and HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 unit/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). For immunoprecipitation assays, cells were then lysed in either digitonin buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 2.5 mM $MgCl_2$, 1% digitonin) or NP-40 buffer (0.5% NP-40, 10 mM Tris [pH 8.0], 150 mM NaCl, 5 mM $MgCl_2$). Denatured lysates were prepared using NP-40 buffer supplemented with 1% sodium dodecyl sulfate (SDS) and boiled for 5 min. Prior to immunoprecipitation, SDS was diluted to 0.1% by addition of NP-40 buffer. Complete protease inhibitor tablets (Roche) were added to all lysis buffers. Proteasome inhibitor MG132 (Calbiochem) was used at a 20 μM final concentration as indicated. Agarose beads conjugated to anti-Flag (Sigma), anti-V5 (GeneTex), anti-HA (3F10; Roche), or anti-Myc (Clontech) antibodies were used for immunoprecipitations. Bound beads were washed three times in buffer prior to denaturation in sample loading buffer.

The following antibodies were used for immunoblotting: horseradish peroxidase (HRP)-conjugated anti-Flag antibody (M2, Sigma), HRP-conjugated anti-V5 antibody (GeneTex), HRP-conjugated anti-HA antibody (3F10; Roche), HRP-conjugated anti-Myc antibody (Invitrogen), HRP-conjugated anti-tubulin antibody (Abcam), and rabbit anti-Sp1 antibody (H-225; Santa Cruz Biotechnology). Mouse monoclonal antibody (M2) recognizes the C terminus of HCF-1 and has been described previously (44). Rabbit polyclonal anti-Bap1 antibody was raised against a synthetic polypeptide corresponding to Bap1 residues 322 to 436 (Yenzym Antibodies LLC). The antibodies used for immunofluorescence are described below.

Immunopurification and MS. For the analysis of Bap1-Flag-associated proteins, lysates derived from five 15-cm dishes were used for each pull-down analysis. For transient transfection, 15 μg of DNA was used per 15-cm dish. Cells were collected, washed in phosphate-buffered saline (PBS), and lysed in digitonin buffer (total, 30 ml) and incubated with anti-Flag antibody-conjugated beads (200 μl of 50% slurry per sample) for 2 h at 4°C. Beads were washed with 0.2% digitonin buffer and eluted (3 × 100 μl) with 0.2% digitonin buffer containing 0.5 mg of three-Flag peptide (Sigma)/ml. Eluted samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by Western blotting, silver staining, or MS.

For ubiquitylation site analysis, five 15-cm dishes of 293 cells stably expressing low levels of either wild-type or C91A mutant Bap1-Flag were used in each pull-down assay. Each plate was transiently transfected with 7.5 μg of HA-ubiquitin construct. After 48 h, cells were lysed and immunoprecipitated with anti-Flag antibody beads as described above. Associated proteins were released from the beads using 1% SDS in PBS (three times 300 μl) and boiled for 5 min. SDS was then diluted to 0.1% by addition of 10 ml of NP-40 buffer with 0.5 mM *N*-ethylmaleimide to the eluate. Lysates were incubated with 3F10 antibody conjugated to beads (100 μl of 50% slurry per sample) overnight at 4°C. Prior to analysis, the beads were washed and then eluted (three times 100 μl) with a buffer containing 25 mM Tris (pH 8.0), and 25 mM NaCl, and 1% SDS and analyzed by SDS-PAGE.

For analysis of HCF-1-V5 ubiquitination, five 15-cm dishes of 293 cells were transiently transfected with 4.0 μg of HA-ubiquitin and 16 μg of HCF-1-V5 or pcDNA3.1(−) constructs each. After 48 h, cells were treated with MG132 (20 μM) for 2 h and then washed with PBS and lysed in 4 ml of NP-40 buffer with 0.5 mM *N*-ethylmaleimide. Lysates were centrifuged at 13,000 × *g* for 10 min, and the supernatants were transferred to a new tube. SDS was added to a final concentration of 1%, and the lysates were boiled for 10 min. SDS was diluted to 0.1% with NP-40 buffer, and the lysates were incubated with anti-V5 antibody beads (100 μl) overnight at 4°C. The beads were washed and then eluted (four times 100 μl) with a buffer containing 5 mM Tris (pH 8.0), 30 mM NaCl, and 1% SDS prior to analysis.

For proteomic analysis, peptides were resolved by SDS-PAGE and stained with colloidal Coomassie blue, and bands were excised from the top to the bottom of each lane. Gel-isolated proteins were digested in situ with trypsin,

extracted, and analyzed by microcapillary reversed-phase liquid chromatography-electrospray tandem MS (LC-MS/MS) on a linear ion trap (LTQ; ThermoFisher, San Jose, CA) (29). Unbiased protein identification was performed with the mass spectrometer in "top-5" data-dependent mode; tandem mass spectra were searched against a database of mammalian proteins by using Mascot (Matrix Science, Ltd., London, United Kingdom), first without considering peptide modifications beyond S-alkylation and Met-sulfoxide, and subsequently allowing for S, T, and Y phosphorylation and glycyl-glycyl-lysine resulting from K ubiquitination. The results were viewed and validated with the Scaffold program (Proteome Software, Portland, OR). Targeted protein detection was accomplished by ion trap selected reaction monitoring of precursor ions and their fragments corresponding to known tryptic peptides from the target protein (2).

Subcellular fractionation. Cells were grown on a 15-cm dish, collected, and washed first in PBS and then briefly in hypotonic buffer (10 mM HEPES [pH 8.0], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT]). Cell pellets were resuspended in three packed cell volume equivalents of hypotonic buffer supplemented with a protease inhibitor cocktail (Roche). Cells were allowed to swell on ice for 15 min. One-fifth of the cells was then lysed in 1% digitonin buffer and saved as crude lysate. The remaining cells were lysed by Dounce homogenization until 90% lysis, as judged by trypan blue exclusion. Nuclei were separated from the cytoplasm fraction by centrifugation for 15 min at 5,000 × *g*. Purified nuclei were then lysed by addition of 1% digitonin. Bap1-flag was immunoprecipitated from each of these fractions as explained above.

Ubiquitin-AMC assay. Wild-type and mutant Bap1-flag proteins were immunopurified with anti-Flag beads (30 μl) and eluted in four washes (50 μl each) of NP-40 buffer containing 2 mM DTT, 5% glycerol, and 0.5 mg of three-Flag peptide/ml. To assess the UCH activity, the fluorogenic substrate 7-amido-4-methylcoumarin derivatized ubiquitin (ubiquitin-AMC; Boston Biochem) was diluted to a final concentration of 340 nM in 190 μl of the assay buffer (50 mM HEPES [pH 7.5], 0.5 mM EDTA, and 1 mM DTT). Samples were incubated for 2 h at room temperature, and the levels of hydrolyzed AMC were measured by excitation at 380 nm and emission at 460 nm. UCH-L3 (400 μg; Boston Biochem) was used as a positive control, and 10 μl of Bap1-Flag eluate was used in each assay. All samples were tested in triplicate.

Indirect immunofluorescence microscopy. HeLa cells were plated on glass coverslips and 24 h later transfected using PolyFect (Qiagen). For immunofluorescence, cells were fixed on the coverslip using 3.7% paraformaldehyde in PBS. The cells were permeabilized with 0.1% Triton X-100 and preblocked in blocking buffer (10% fetal bovine serum and 0.25% saponin in PBS). Both primary and secondary antibodies were incubated 1 h at 37°C in blocking buffer at the following dilutions: mouse monoclonal anti-HA (1:500), mouse monoclonal anti-Flag (1:500), rabbit polyclonal anti-HCF-1 N18 (1:200), goat anti-mouse Alexa 555 (1:500; Invitrogen), and goat anti-rabbit Alexa 633 (1:500; Invitrogen). Before addition of the secondary antibody, cells were washed three times in 7% gelatin–0.025% saponin in PBS. After the secondary antibody, the slides were washed in PBS and mounted in DakoCytomation fluorescent mounting medium. Fluorescence and differential interference contrast micrographs were collected on a Zeiss 510 Meta laser-scanning confocal microscope, using either a 63× or a 100× objective lens and Zeiss AIM software.

Cell cycle analysis. HeLa cells were plated at 2.5×10^5 per 6-cm plate and cotransfected with control or Bap1 siRNA together with a green fluorescent protein (GFP) expression plasmid. After 48 h, samples were harvested by trypsinization and fixed using 1% paraformaldehyde, followed by permeabilization with 70% ethanol. The DNA was stained with 25 μg of propidium iodide/ml in the presence of 60 μg of RNase A/ml. Flow cytometry was performed by using a FACSCalibur flow cytometer (Becton Dickinson), and transfected cells were selected for analysis by gating the GFP fluorescence. The DNA content was correlated to cell cycle distribution using FlowJo software (Tree Star, Inc.) using the Watson pragmatic model.

RESULTS

Identification of HCF-1 as a Bap1 interacting protein. Bap1 belongs to the UCH family of DUBs, which are characterized by a UCH catalytic domain at the N terminus (Fig. 1A). Within the family, the UCH domain of UCH37 is the most similar to that of Bap1 (42.5% identity), and the two proteins share an additional region of similarity (here termed the UCH37-like domain [ULD]) near the C terminus (Fig. 1B). UCH37 is one of three DUBs associated with the proteasome and does so through the interaction of a putative coiled-coil domain with Rpn13 (Adrm1, GP110), a component of the nine-subunit proteasome base (12, 49). By analogy, the C-terminal region of Bap1 including the ULD might therefore also function as a protein-protein interaction domain and residues 598 to 729 mediate the association with BRCA1 (13). Lastly, a block of basis residues (717 to 722) serves as a nuclear localization signal (41).

To identify interacting partners and potential substrates of Bap1, we expressed a Flag-epitope tagged version of full-length human Bap1 (Bap1-Flag) in 293 cells and used anti-Flag antibody coupled beads to immunoprecipitate Bap1-containing complexes from cell lysates that had been prepared using 1% digitonin as a mild detergent (Fig. 1C). Bap1-Flag and coassociated proteins were eluted from the anti-Flag beads with free Flag peptide, separated by SDS-PAGE, and visualized by silver staining. A parallel coimmunoprecipitation was performed using a lysate from cells that do not express Bap1-Flag (control). Protein bands detected in the Bap1-Flag sample or the corresponding area of the control, were excised from the gel, digested with trypsin, and identified by MS/MS. Multiple peptides corresponding to HCF-1 were identified in regions of the gel above 100-kDa marker but not from the control. The analysis was repeated three times using either transiently or stably expressed Bap1-Flag and, in all cases, HCF-1 was identified as the principal Bap1-interacting protein (Fig. 2A and data not shown). Collectively, 27 unique peptides corresponding to 25% of the 2,035-amino-acid HCF-1 precursor sequence were identified and derive from both the $HCF\text{-}1_N$ and the $HCF\text{-}1_C$ subunits. Peptides from several additional proteins were also obtained but yielded fewer peptides than HCF-1 and were not analyzed further. Under these immunoprecipitation conditions, BRCA1, a previously described partner for Bap1, was not detected.

HCF-1 and Bap1 can interact and colocalize together. To confirm the interaction of HCF-1 with Bap1, we transiently expressed Bap1-Flag and V5-tagged HCF-1 (HCF-1-V5) in 293 cells. Expression was confirmed by immunoblotting with anti-Flag or anti-V5 antibodies (Fig. 2B, left panel). HCF-1-V5 was recovered using anti-Flag beads only when coexpressed with Bap1-Flag (Fig. 2B, right panel), confirming that the two proteins interact within the cell. Next, we examined the subcellular localization of Bap1 and HCF-1 by immunofluorescence microscopy (Fig. 2C). Bap1 has been found in both the nuclear and the cytoplasmic compartments (13), whereas HCF-1 is predominantly nuclear protein (21, 22, 42). HA-tagged Bap1 (Bap1-HA) was transiently expressed in HeLa cells and detected using a mouse monoclonal anti-HA primary antibody and a Alexa 555 secondary antibody (depicted in green), and endogenous HCF-1 was detected by using a rabbit polyclonal against the $HCF\text{-}1_N$ subunit (anti-N18) (11) detected using an Alexa 633-conjugated secondary (red). These images clearly shown that HCF-1 (red) and Bap1 (green) are similarly distributed throughout the nucleoplasm (yellow in the merge image), with additional HA signal in the cytoplasm. Both proteins were excluded from the nucleoli. Nuclear localization was confirmed by subcellular fractionation of 293 cells that stably express Bap1-Flag at low levels (Fig. 2D). Nuclear transcription factor Sp1 was used as a positive control, and the majority was retained in the nuclear fraction (top panel). As

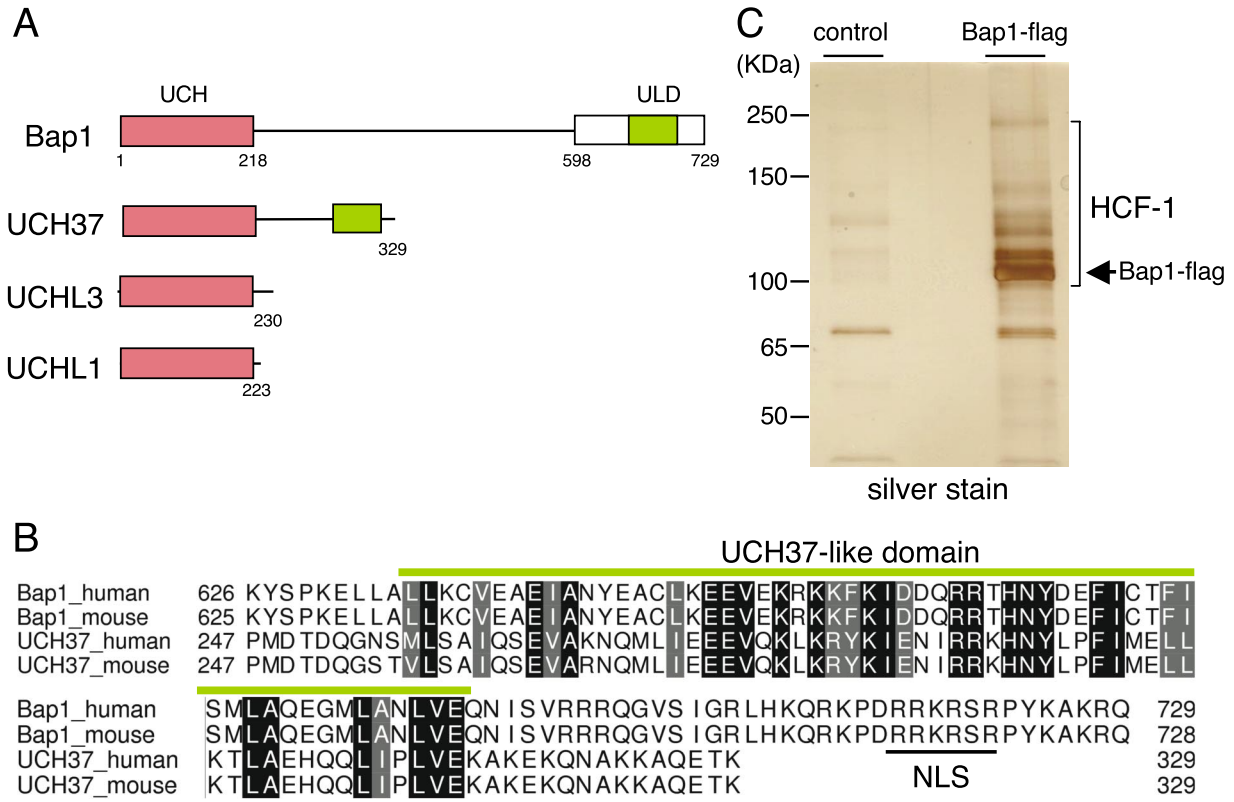

FIG. 1. Identification of HCF-1 as a Bap1 interacting protein. (A) Schematic comparing structures of human UCH family members: Bap1 (RefSeq accession no. NP_004647), UCH37 (NP_057068), UCHL1 (NP_004172), and UCHL3 (CAG33136). The Bap1 UCH domain is most similar to that of UCH37 (42.5% sequence identity) and includes the critical cysteine, histidine, and aspartate residues of the active site. Bap1 and UCH37 share an additional region of homology at the C terminus (ULD, green box). In UCH37, this region folds as a coiled coil that interacts with the Rpn13 subunit of the proteasome. (B) Sequences of the C-terminal homology region (ULD) from human and mouse Bap1 (accession numbers NP_004647 and NP_081364) and UCH37 (accession numbers NP_057068 and AAD31534). Residues in UCH37 that are identical (filled) or similar (shaded) to Bap1 are indicated. (C) Purification of Flag-tagged Bap1 (Bap1-Flag). Silver-stained SDS-PAGE gel (4 to 12% gradient) showing eluted proteins after anti-Flag coimmunoprecipitation from a 1% digitonin lysate of Bap1-Flag-expressing 293 cells. Bands from a Bap1-Flag sample and corresponding regions of the control sample were excised, digested with trypsin, and subjected to peptide analysis by MS/MS. In three independent experiments, multiple bands in the 100-kDa or larger size range were identified as HCF-1 from the Bap-1 elution but not the control.

expected, HCF-1 was also found predominantly in the nuclear fraction, whereas Bap1 was both nuclear and cytoplasmic. These results confirm that HCF-1 and Bap1 are both found in the nucleus with a similar distribution through the nucleoplasm and is consistent with the physical interaction detected by co-immunoprecipitation.

HCF-1 interacts with Bap1 through its β-propeller domain. To identify the Bap1 interaction domain(s) within HCF-1, we performed coimmunoprecipitations with Bap-Flag and a series of HA-tagged HCF-1 fragments (Fig. 3A). Lysates (1% digitonin) were prepared from Bap1-Flag-expressing 293 cells transiently transfected with plasmids encoding each HCF-1 truncation. Expression of the recombinant proteins was confirmed by immunoblotting with anti-HA antibody (Fig. 3B, top panel). Bap1 was then immunoprecipitated using anti-Flag beads, and the precipitates were probed by immunoblotting with α-HA antibody (middle panel). All of the HCF-1 truncations were recovered except for construct 4 (HA-HCF-$1_{N450-1011}$). The only domain common to all three truncations recovered with Bap1-Flag is the β-propeller (residues 1 to 380), and this is likely to be the principal interaction domain.

We also sought to determine whether Bap1-Flag could associate with endogenous HCF-1. In addition to wild-type Bap1, we also tested a Bap1 mutant in which the essential cysteine-91 of the active site had been changed to alanine (C91A), thereby abolishing UCH enzymatic activity (Fig. 3C). Lysates were prepared from 293 cells stably expressing wild-type or C91A Bap1-Flag, immunoprecipitated using anti-Flag beads, and probed by immunoblotting with a mouse monoclonal antibody (anti-HCF-1, antibody M2) (44) that recognizes an epitope in the C terminus of HCF-1. Blotting for HCF-1 (lower panel) revealed the characteristic ladder of HCF-1_C fragments in the wild-type and C91A immunoprecipitates but not in the mock (untransfected) 293 cell lysate. This result indicates that Bap1-flag interacts with endogenous HCF-1 and that the catalytic activity of the UCH domain is not required.

NHNY consensus sequence in Bap1 is responsible for its interaction with HCF-1. The HCF-1 β-propeller domain is critical for HCF-1 function, mediating a variety of protein-protein interactions, including the association with VP16 (36, 43). Many of the interacting proteins contain a tetrapeptide HBM consisting of an acidic residue (Asp or Glu), followed by

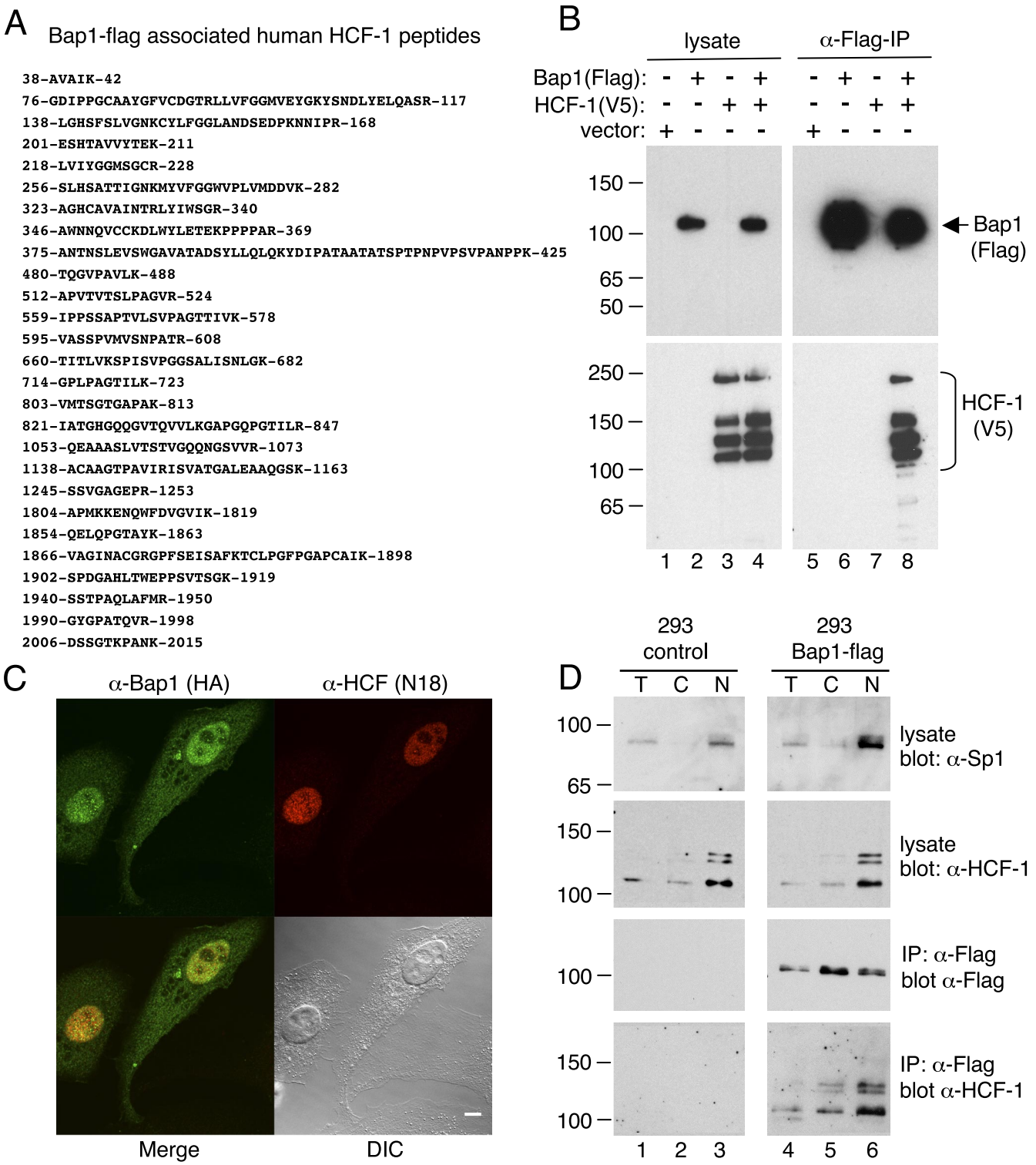

FIG. 2. HCF-1 associates with Bap1 in transfected cells. (A) Sequences of 27 HCF-1-derived peptides identified by MS/MS from proteins coimmunoprecipitated with Bap1-Flag. The amino acid positions in the prototype human HCF-1 protein sequence (RefSeq accession no. NP_005325) are indicated. (B) Coassociation of Bap1-Flag and HCF-1-V5 in transfected cells. Bap1-Flag and HCF-1-V5 were expressed individually or together in human 293 cells, and their expression was confirmed in immunoblotting of the 1% digitonin lysates (left panels). Bap1-Flag was immunoprecipitated using Flag antibody and precipitated proteins detected by immunoblotting with V5 or Flag (right panels). (C) Bap1 and HCF-1 colocalize within the cell. HeLa cells were transiently transfected with a Bap1-HA expression plasmid, fixed and subjected to indirect immunofluorescence using an α-HA monoclonal antibody to detect Bap1 and anti-HCF-1 (N18) polyclonal antibody to detect endogenous HCF-1. Scale bar, 5 μm. (D) Bap1-Flag associates with endogenous HCF-1 both in cytoplasm and nucleus. Whole-cell lysates (T) or cytoplasmic (C) and nuclear (N) fractions were prepared from control 293 cells or cells stably expressing low levels of Bap1-Flag and probed by immunoblotting either directly or after immunoprecipitation with anti-Flag beads. Sp1 serves as a nuclear protein control.

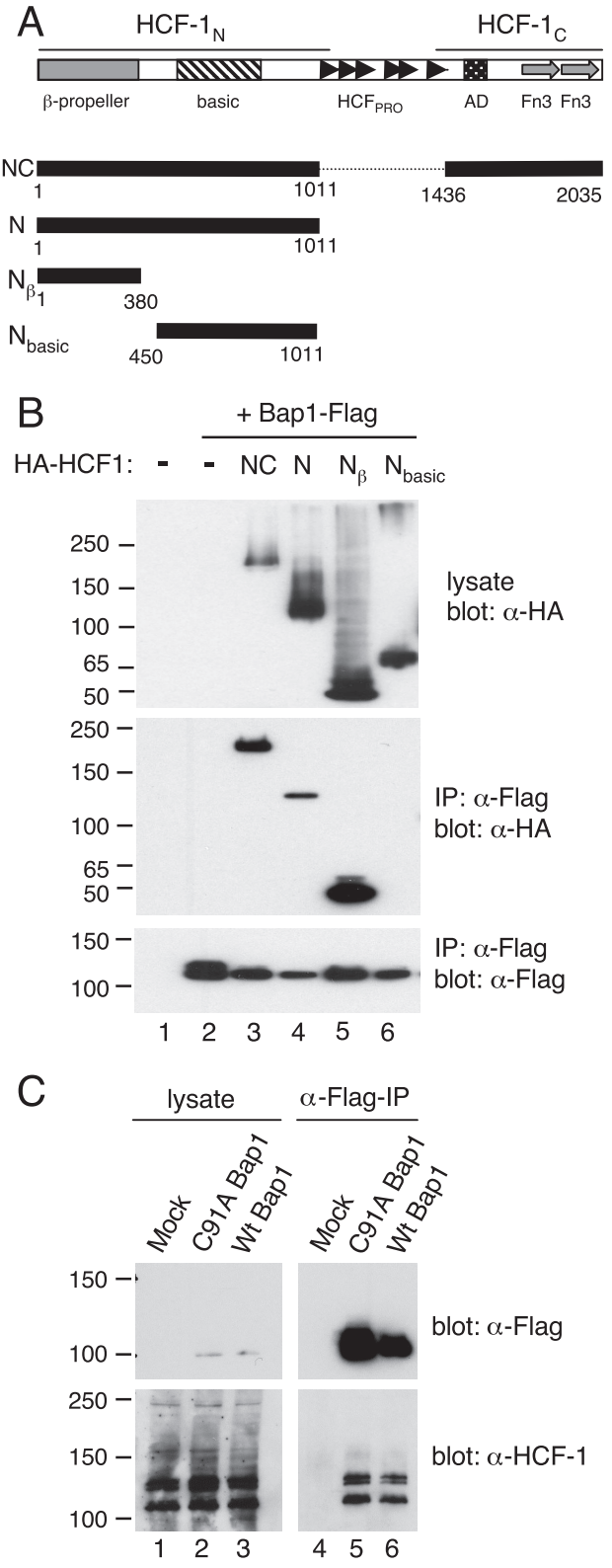

FIG. 3. The β-propeller domain of HCF-1 is sufficient for interaction with Bap1. (A) Schematic representation of HCF-1 to indicate the positions of the β-propeller, basic region, HCF_{PRO} repeats (arrowheads), transactivation domain (AD), and tandem Fn3 repeats. Four HCF-1 truncations used in panel B have been described previously (43) and correspond to HCF-1Δrep (NC), $HCF\text{-}1_{N1011}$ (N), $HCF\text{-}1_{N380}$ (N_β), and $HCF\text{-}1_{N450\text{-}1011}$ (N_{basic}). An HA-epitope tag is included at the N terminus of each HCF-1 polypeptide. (B) The HCF-1 β-propeller domain is sufficient for interaction with Bap1. Plasmids encoding Bap1-Flag and selected HA-tagged HCF-1 truncations were transiently transfected into 293 cells, and their association was examined by coimmunoprecipitation. Cells were lysed in 1% digitonin and Bap1-Flag immunoprecipitated from the clarified lysates using anti-Flag beads. Recovered proteins were detected by immunoblotting anti-HA and anti-Flag antibodies. (C) Catalytic activity of Bap1 is not required for association with endogenous HCF-1. Control 293 cells (mock) or cells stably expressing low levels of wild-type (Wt) or active site mutant (C91A) Bap1-Flag were lysed in 1% digitonin and immunoprecipitated using anti-Flag beads. Endogenous HCF-1 was detected by immunoblotting with anti-HCF-1 antibody.

an invariant histone separated from an invariant tyrosine by one variable position (consensus D/EHXY, where "X" is any residue) (9, 26, 27). Although Bap1 lacks an exact match, we noted that it does contain the sequence Asn-His-Asn-Tyr (NHNY, amino acids 363 to 366) in the unique region separating the UCH domain from the C-terminal domains (Fig. 4A). This NHNY sequence and surrounding residues are perfectly conserved in other vertebrate Bap1 proteins, suggesting conservation of function. To determine whether the HBM-like sequence contributes to the interaction of Bap1 with HCF1, we substituted all four residues with alanine (NHNY/AAAA) in the context of Bap1-Flag, and the resulting mutant was coexpressed with Myc epitope-tagged HCF-1 (HCF-1-Myc) in 293 cells. Wild-type Bap1-Flag and the UCH-inactive mutant C91A were included as controls. Each Bap1-Flag protein was immunoprecipitated from a mild detergent cell lysate and probed by immunoblotting with either anti-Flag or anti-Myc antibodies. HCF-1-Myc was recovered efficiently using wild-type and C91A versions of Bap1 but not with the NHNY/AAAA mutant (Fig. 4B, middle panels). As an additional control, we measured the intrinsic ubiquitin cleavage activity of each immunopurified Bap1-Flag protein using the fluorescent substrate ubiquitin-AMC (5). As shown in Fig. 4C, wild-type Bap1 and the NHNY/AAAA mutant displayed robust activity, comparable to that of the control protein UCH-L3. As expected, the C91A mutant was inactive in this assay. Thus, mutation of the NHNY sequence abolishes the association with HCF-1 but does not alter the intrinsic enzymatic activity of the UCH domain.

HCF-1 is K48 and K63 ubiquitinated. Our finding that the HCF-1 stably associates with Bap1 raises the intriguing question of whether HCF-1 is itself modified by ubiquitin and might be a natural substrate for the deubiquitination activity of Bap1. To assess the ubiquitination status of HCF-1, we transfected 293 cells with plasmids encoding HCF-1-V5 and one of two versions of HA-tagged ubiquitin, in which six of the seven lysine residues had been changed to arginine, leaving only lysine-48 (K48 HA-Ub) or lysine-63 (K63 HA-Ub) available for covalent linkage. At 48 h after transfection, the cells were treated with proteasome inhibitor MG132 for 2 h in order to stabilize ubiquitinated proteins and then lysed in the presence of 1% SDS and boiled to fully dissociate any noncovalent associations. HCF-1-V5 was recovered by immunoprecipitation with anti-V5 beads, separated by gel electrophoresis, and probed by immunoblotting with anti-HA antibody to detect

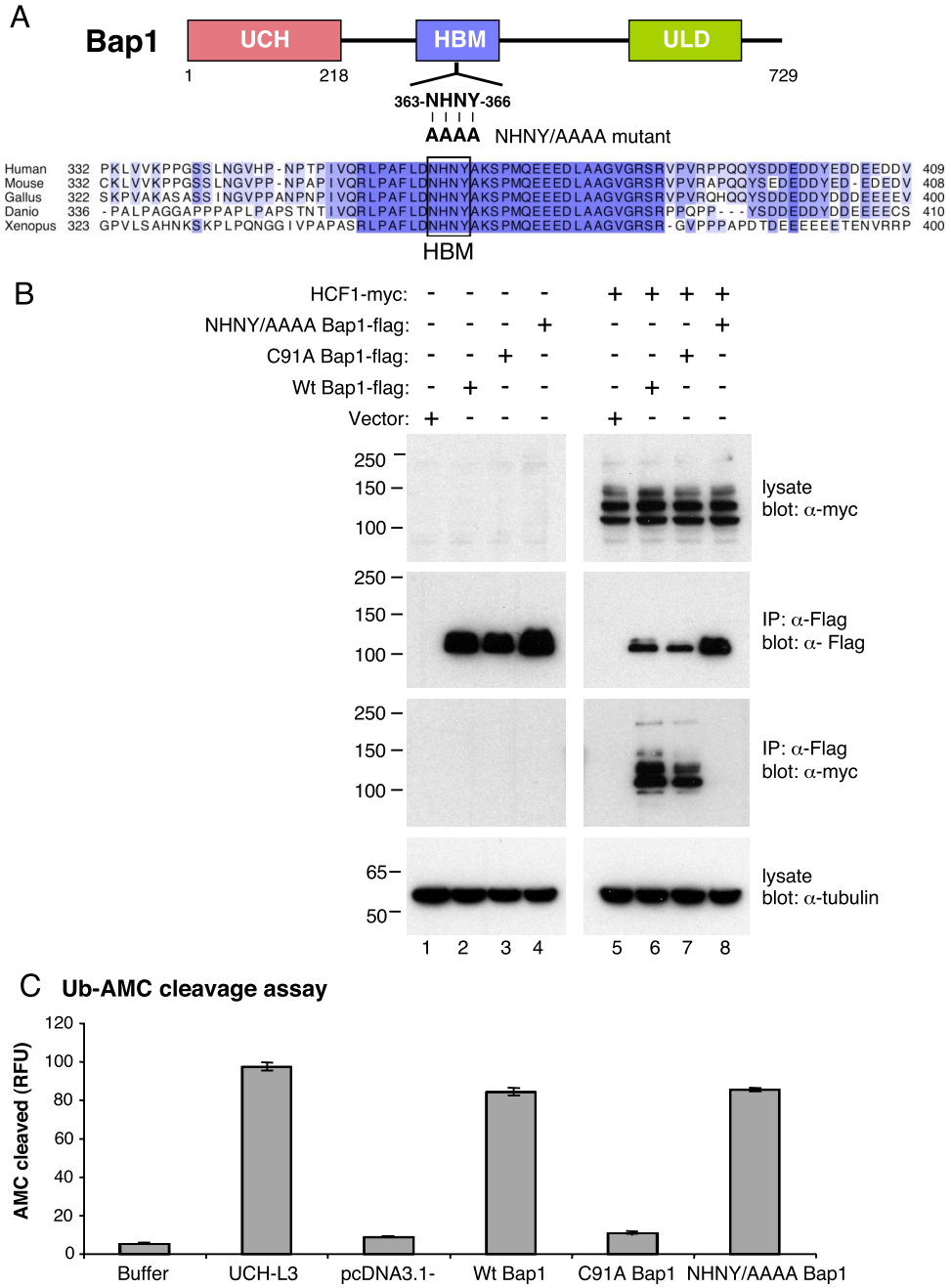

FIG. 4. Association with HCF-1 requires a conserved tetrapeptide HBM in the central region of Bap1. (A) An HBM-like sequence is located near the center of Bap1, within a region that is highly conserved in vertebrate Bap1 sequences. Accession numbers are as follows: human (*Homo sapiens*), NP_004647; mouse (*Mus musculus*), NP_081364; chicken (*Gallus gallus*), NP_001025761; zebrafish (*Danio rerio*), XP_687254; and African clawed toad (*Xenopus laevis*), NP_001089388. (B) Mutation of the NHNY sequence abrogates the interaction with HCF-1. Empty Flag vector (lanes 1 and 5) or vectors encoding wild-type Bap1-Flag (lanes 2 and 6), C91A Bap1-Flag (lanes 2 and 6), or NHNY/AAAA Bap1-Flag (lanes 4 and 8) were transiently cotransfected into 293 cells, together with either empty Myc tag vector (lanes 1 to 4) or HCF1-Myc vector (lanes 5 to 8). Cells were lysed in buffer containing 1% digitonin, immunoprecipitated with anti-Flag antibody beads, and coprecipitated HCF-1-Myc detected by immunoblotting with anti-Myc antibody. (C) The NHNY/AAAA Bap1 mutant retains enzymatic activity. Immunopurified Flag-tagged proteins were washed extensively, eluted from the beads with free three-Flag peptide, and assayed for the ability to cleave a ubiquitin-AMC fluorogenic substrate. Purified UCH-L3 and cells transfected with empty vector served as positive and negative controls. Values correspond to the average of three independent assays, and the standard deviations are shown.

covalently linked HA-Ub moieties and anti-V5 antibody to detect the ectopic HCF-1. As shown in Fig. 5A, we detected abundant K48 and K63 polyubiquitinated species in the 150 kDa or greater size range, with some additional K63-linked products at ~110 kDa.

Selective accumulation of K48-ubiquitinated HCF-1 in the presence of catalytically inactive Bap1. To determine whether Bap1 might influence the levels of ubiquitinated HCF-1, we repeated the experiment, cotransfecting wild-type or C91A mutant Bap1 with HCF-1-V5 and K48 HA-Ub (Fig. 5B). After

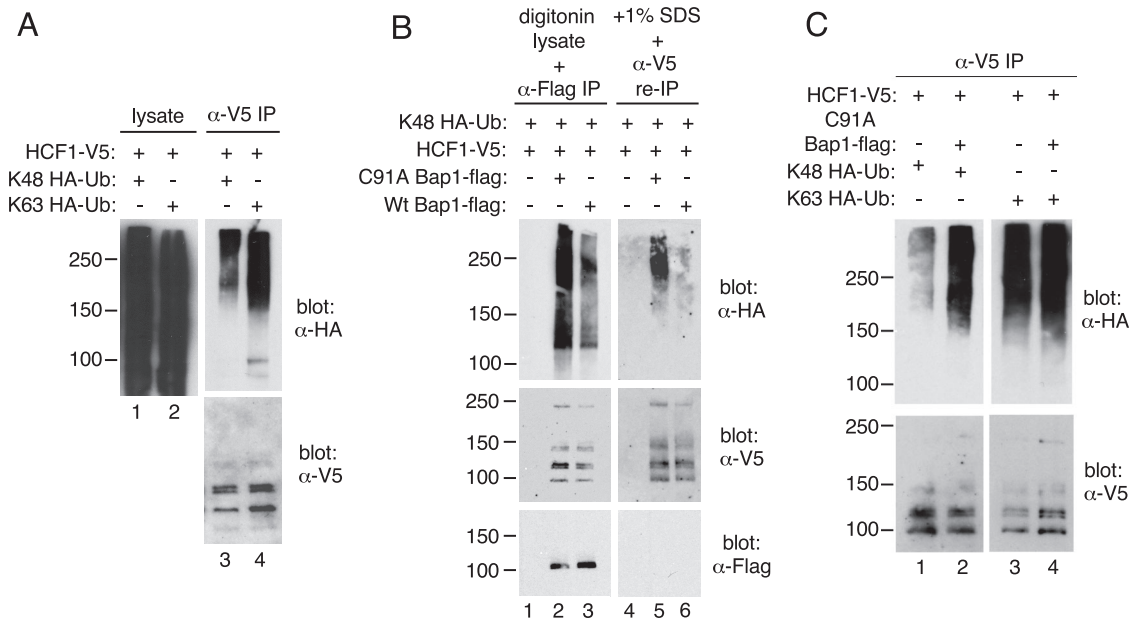

FIG. 5. Catalytically inactive Bap1 promotes the selective accumulation of K48 ubiquitinated HCF-1. (A) HCF-1 is ubiquitinated through both lysine-48 (K48) and lysine-63 (K63) linkages. 293 cells were transiently transfected with plasmid encoding HCF-1-V5 and HA-tagged K48 or K63-ubiquitin. Cells were treated for 2 h with 20 μM MG132 prior to lysis in NP-40-containing buffer supplemented with 1% SDS. After removal of the insoluble material, the lysates were diluted in 10 volumes of buffer lacking SDS, and V5-tagged HCF-1 was recovered by anti-V5 immunoprecipitation. Linkage of ubiquitin moieties was detected by immunoblotting with anti-HA antibody. (B) Ectopic expression of Bap1 alters the level of HCF-1 K48 ubiquitination. Plasmids encoding HCF-1-V5, K48 HA-Ub, and wild-type or C91A mutant Bap1-Flag were introduced into 293 cells and processed as described for panel A. (C) Bap1 expression only affects levels of K48 ubiquitinated HCF-1. HCF-1-V5, C91A Bap1-Flag, K48 HA-Ub, or K63 HA-Ub were expressed as indicated in 293 cells. Prior to lysis, cells were treated for 2 h with 20 μM MG132. HCF-1-V5 was immunoprecipitated (NP-40 + 1% SDS lysis, followed by 10-fold dilution in buffer without SDS) with anti-V5 antibody, and equal amounts of HCF-1-V5 were loaded on gels, followed by Western blotting with anti-HA antibody. The results show that levels of K48 ubiquitinated HCF-1 are considerably increased when C91A Bap1 is expressed; however, no noticeable effects on the levels of K63 ubiquitinated HCF-1 were observed.

MG132 treatment, Bap1-Flag was recovered by immunoprecipitation under mild conditions, and the precipitates were analyzed by immunoblotting with anti-HA. A ladder of K48-Ub-linked species were recovered from cells expressing either version of Bap1-Flag (lanes 2 and 3) but not in the control lysate (lane 1). Although wild-type and C91A Bap1-Flag were expressed at similar levels (see the anti-Flag immunoblot), ubiquitinated species were significantly more abundant in the presence of the C91A catalytic mutant. To examine ubiquitinated HCF-1 directly, the same lysates were fully denatured by boiling in 1% SDS and then diluted to 0.1% SDS and reimmunoprecipitated with anti-V5 antibody to purify HCF-1_C polypeptides (lanes 4 to 6). Again, a significantly higher level of K48-ubiquitinated material was recovered in the presence of C91A Bap1-Flag (lane 5) compared to the wild type (lane 6). We interpret this result as evidence that the active form of Bap1 is able to continuously remove K48 linkages from HCF-1. We hypothesize that the catalytically inactive mutant can still associate with the HCF-1 β-propeller but cannot remove ubiquitin chains.

To determine the specificity of the Bap1 UCH activity, we repeated the experiment but included K63 HA-Ub (Fig. 5C). V5-tagged HCF-1 was immunoprecipitated from the denatured lysates and probed for covalently linked HA-tagged ubiquitin. As in Fig. 5B, we observed a marked increase in the level of K48 ubiquitinated HCF-1 in the presence of C91A Bap1 (compare lanes 1 and 2) but found no significant change in the levels of K63 ubiquitinated HCF-1 (lanes 3 and 4). Thus, although HCF-1 is subject to both K48 and K63 ubiquitination, Bap1 appears to selectively remove only K48-linked ubiquitin.

Endogenous HCF-1_C is ubiquitinated at lysine-1807 and/or lysine-1808. To map the major sites of HCF-1 ubiquitination, endogenous HCF-1 was affinity purified from 293 cell lysates by way of association with Bap1. Control 293 cells or 293 cells that stably express low levels of either wild-type or C91A Bap1-Flag were transiently transfected with HA-Ub vector. Flag-tagged complexes were then immunoprecipitated from mild detergent lysates and recovery of endogenous HCF-1 (Fig. 3C), and HA-Ub was confirmed by immunoblotting (data not shown). Next, the immunoprecipitated complexes were fully denatured using 1% SDS and reimmunoprecipitated with anti-HA antibody to enrich for the ubiquitinated forms of HCF-1. Recovery of HCF-1 in the doubly immunoprecipitated wild-type Bap1 sample was also verified by targeted MS/MS (Fig. 6B). Immunoprecipitated proteins were resolved by SDS-PAGE, and bands greater than 100 kDa were isolated, digested with trypsin, and subjected to LC-MS/MS. Ions corresponding to the predicted masses of two tryptic peptides (1875-GPFSE ISAFK-1884 and 1990-GYGPATQVR-1998) derived from the C-terminal subunit of HCF-1 were isolated repeatedly. For each peptide, three fragments were observed at the same retention times, and these were absent in the control sample. This result confirms the previous immunoblotting results showing that HA-Ub can be covalently conjugated to endogenous HCF-1_C.

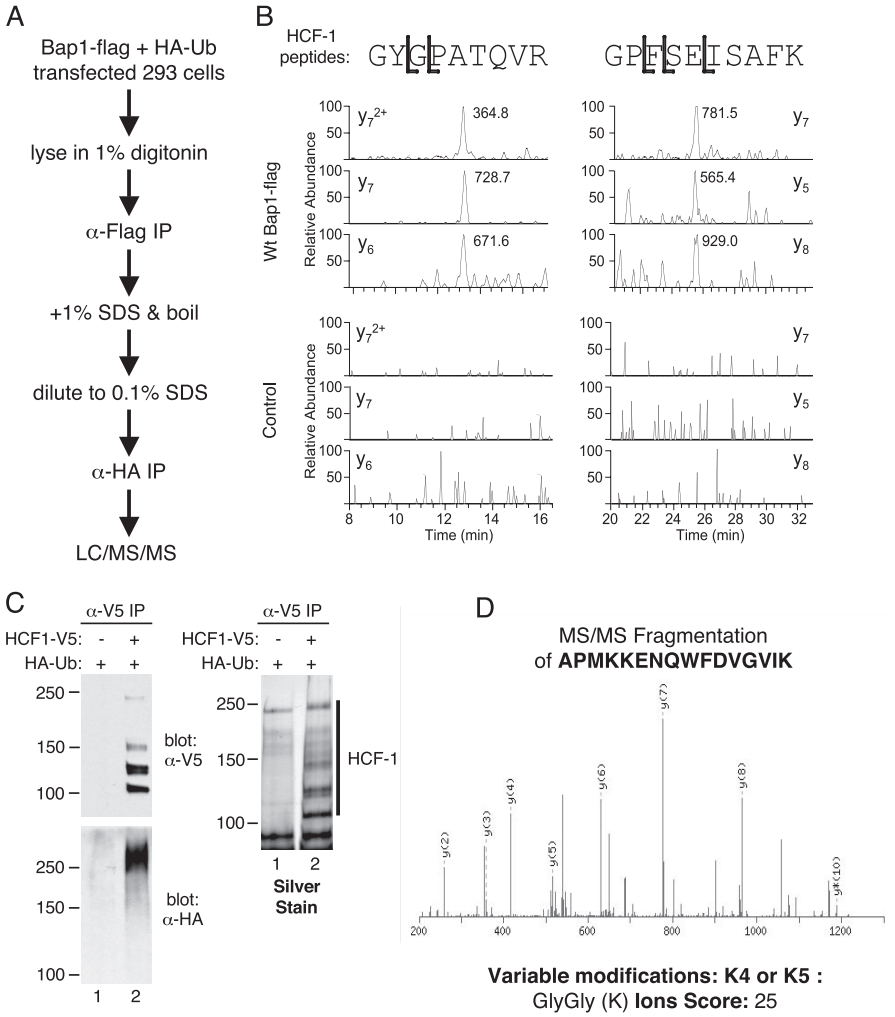

FIG. 6. Endogenous HCF-1 is ubiquitinated, and the major site of HCF-1 ubiquitination is mapped to K1807 or K1808 residues. (A) Scheme for partial purification of ubiquitinated HCF-1 through association with Bap1-Flag. (B) Samples from Bap1-Flag-expressing or control 293 cells were subjected to sequential anti-Flag and anti-HA immunoprecipitation as summarized for panel A and analyzed by MS/MS. Peptide masses and fragment ions characteristic of two tryptic peptides derived from HCF-1_C were monitored throughout an LC-MS/MS experiment. Three fragment ions for each peptide were detected in the samples from Bap1-Flag-expressing cells but not the controls. (C) Purification of HCF-1-V5 for ubiquitination site mapping. HA-Ub was expressed in 293 cells with or without HCF-1-V5. HCF-1-V5 polypeptides were recovered from denatured lysates by immunoprecipitation with anti-V5 beads. After extensive washing, bound proteins were eluted and detected by immunoblotting with anti-V5 and anti-HA antibodies (left panel) or directly visualized by silver staining (right panel). (D) Identification of lysine-1807 and/or lysine-1808 of HCF-1_C as major sites of ubiquitination. Eluted HCF-1-V5 and control samples (from C) were analyzed by MS/MS, and a single HCF-1 peptide was identified to be ubiquitinated. Highlighted fragment ions are derived from HCF-1_C peptide 1804-APMKKENQWFDVGVIK-1809 and display a mass shift indicative of a ubiquitin linkage at lysine-1807 and/or lysine-1808.

To more precisely map site(s) of ubiquitin linkage, we cotransfected HCF-1-V5 with HA-Ub and recovered the V5-tagged HCF-1_C species by immunoprecipitation under denaturing conditions. Recovery of ubiquitinated species was confirmed by immunoblotting with anti-HA (Fig. 6C, left panel) and by silver staining (right panel). These samples were then subjected to LC-MS/MS, and the data were searched by using Mascot for evidence of ubiquitination in the form of lysines that have been modified by the C-terminal diglycyl residues that remain after trypsin digestion of ubiquitinated proteins. A peptide corresponding to HCF-1 residues 1804 to 1819 was detected with the appropriate mass for ubiquitination. The sequence contains two internal lysines that are ordinarily cleaved by trypsin but are expected to resist proteolysis when hindered by ubiquitin linkage. A series of sequence-specific fragment ions confirmed the identity of the peptide and localized the site of ubiquitination to lysine-1807 or its neighbor lysine-1808 (Fig. 6D). No fragment ions were detected that could unambiguously assign the ubiquitination site to one lysine or the other. We conclude that the lysine residues at positions 1807 and/or 1808 in HCF-1_C serve as prominent sites of ubiquitin chain attachment. Reanalysis of the original MS data obtained from the samples in Fig. 1 revealed a mixture of ubiquitinated and nonubiquitinated versions of these same peptides, indicating that these sites are similarly modified in endogenous HCF-1 (data not shown).

Stabilization of HCF-1 through depletion of Bap1. Because K48 ubiquitin linkage typically leads to protein degradation by

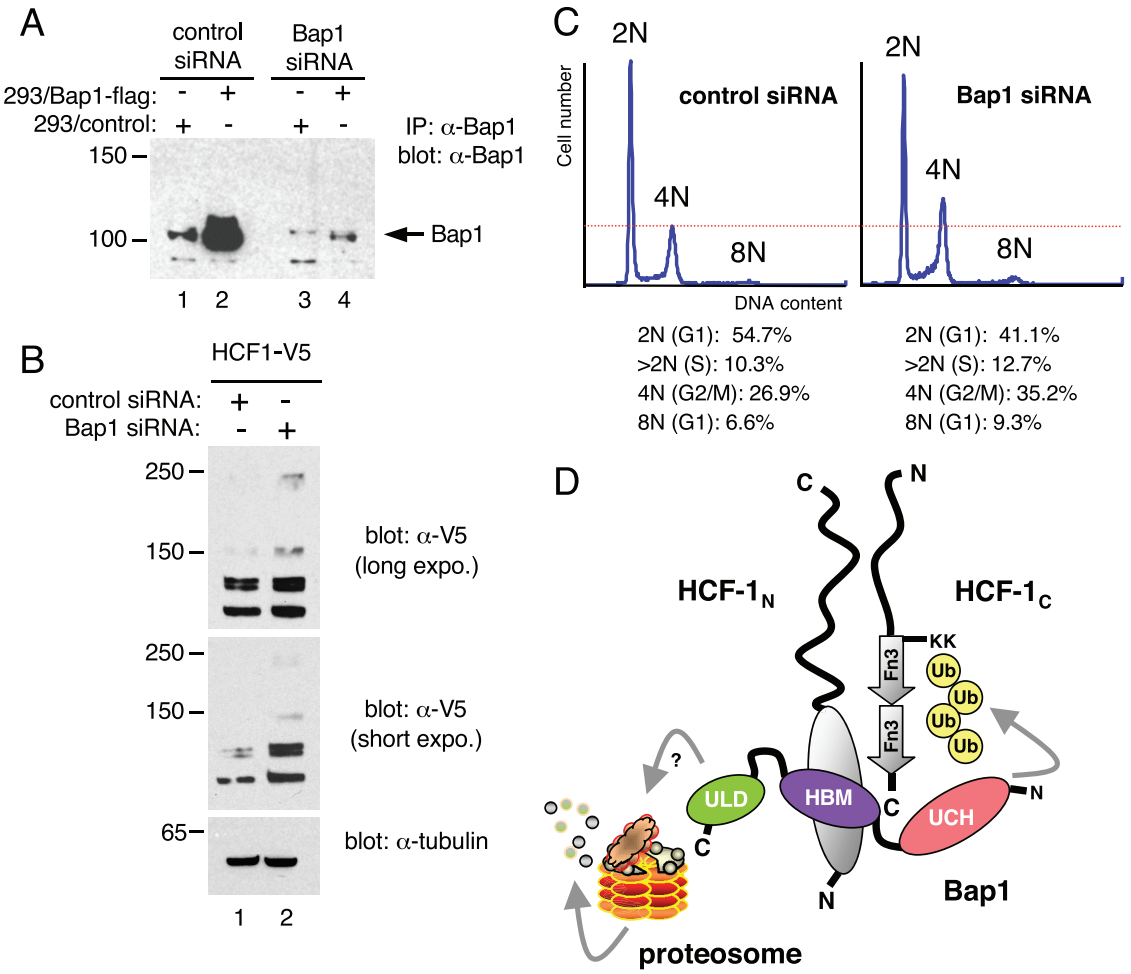

FIG. 7. Bap1 contributes to HCF-1 stability. (A) Depletion of Bap1 using siRNA. Control 293 cells or cells stably expressing Bap1-Flag were treated with control or Bap1 siRNA for 3 days. Endogenous and ectopic Bap1 were immunoprecipitated and blotted with a rabbit polyclonal antibody 419 against Bap1 (α-Bap1). (B) Depletion of Bap1 leads to a modest stabilization of HCF-1. 293 cells were treated with control siRNA or Bap1 siRNA for 2 days and then transfected with HCF-1-V5. After 48 h, cells were lysed, and the clarified lysates were probed by immunoblotting with anti-V5 antibody. Blotting for tubulin served as a loading control. (C) Depletion of Bap1 alters cell cycle progression. Subconfluent HeLa cells were cotransfected with a GFP expression plasmid and either a Bap1 siRNA or a control siRNA. After 48 h, cells were fixed and stained with propidium iodide, and GFP-positive cells were analyzed for DNA content by flow cytometry. For easier comparison of the two FACScalibur profiles, a dotted line marks the 4N peak in the mock siRNA sample. (D) Schematic showing the proposed association of Bap1 with the two subunits of HCF-1. Bap1 contains a nonconsensus HBM that is recognized by the β-propeller domain of the HCF-1_N subunit and required for association of Bap1 with HCF-1. By analogy to UCH37, the C-terminal coiled-coiled (C-C) region of Bap1 may facilitate recruitment to the proteasome, whereas the UCH domain catalyzes the removal of K48-linked ubiquitin moieties. A candidate substrate might be the HCF-1_C subunit, which is shown to be ubiquitinated at conserved lysine-1808 and/or lysine-1809 in the first Fn3 repeat.

way of the proteasome, our findings suggest that Bap1 might help to determine HCF-1 levels in the cell. To examine this further, we depleted Bap1 in 293 cells using siRNA. The analysis was hampered by very low levels of endogenous Bap1 protein in these cells as revealed by immunoblotting with a rabbit polyclonal anti-Bap1 antibody. The effectiveness of the siRNA (~80% reduction) was more clearly demonstrated in cells stably expressing Bap1-Flag (Fig. 7A). As shown in Fig. 7B, treatment with Bap1 siRNA resulted in a modest stabilization of transiently transfected HCF-1-V5 (compare lanes 1 and 2).

The consequence of Bap1 depletion on cell cycle progression was also tested by cotransfecting HeLa cells with the Bap1 or control siRNAs and a GFP expression plasmid to distinguish transfected from untransfected cells. After 48 h, the cells were fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry (Fig. 7C). Cells transfected with control siRNA showed the typical profile of an asynchronously proliferating culture. Treatment with the Bap1 siRNA increased the proportion of cells in S phase and G_2/M phase, with a commensurate decrease in the G_1 population. This results provides further support for the idea that Bap1 has a role in proliferation control. Knockdown of Bap1 results in a modest increase in the steady-state levels of HCF-1, and this presumably helps to promote the transition from G_1 to S phase.

DISCUSSION

Ordered progression through the eukaryotic cell cycle involves an intricate and tightly orchestrated program of protein modifications that alter the stability, localization, and/or activ-

ity of regulatory factors. DUBs have emerged as key players in this process, extending protein half-lives and terminating ubiquitin-dependent signals (38). The coactivator HCF-1 is required for cell proliferation, acting first at the G_1-S transition, where it associates with E2F1, E2F3, and E2F4 and Miz-1 proteins and helps to recruit both activator and repressor complexes to gene promoters, and then again at the M phase by directing the expression of histone methyltransferases involved in chromosome condensation (16, 18, 34, 40). Steady-state levels of HCF-1 appear to correlate with proliferative capacity, and the highest levels are found in rapidly dividing cells such as transformed cell lines (48).

As shown schematically in Fig. 7C, we demonstrate here that the β-propeller domain of the HCF-1_N subunit forms a stable complex with the C-terminal ubiquitin hydrolase Bap1, and this is dependent on a divergent HBM positioned near the center of the Bap1 polypeptide within a block of evolutionarily conserved sequence. Our MS analysis detected 17 peptides from the HCF-1_N subunit and 10 from HCF-1_C, indicating that Bap1 is most likely associated with the heteromeric HCF-1 complex rather than just the β-propeller containing HCF-1_N subunit. Furthermore, using the association with Bap1 as a means to affinity purify HCF-1, we show that HCF-1_C subunit is conjugated to both K48- and K63-linked polyubiquitin chains and have mapped a major site of ubiquitin linkage to lysine-1807 and/or lysine-1808 within the first of the two fibronectin type III (Fn3) repeats. These juxtaposed lysine residues are perfectly conserved in all known vertebrate HCF-1 sequences, hinting that covalent modification of these residues might be important for function and/or regulation. Depletion of Bap1 results in a modest increase in the steady-state level of the proteolytically processed and unprocessed forms of HCF-1, suggesting that Bap1 might help to fine tune HCF-1 levels. The changes, however, are not dramatic, which is consistent with the relatively long half-life of HCF-1, its overall abundance, and fact that only a subset HCF-1_C molecules are ubiquitinated at this position as evident from our MS data. Even though the effects on HCF-1 steady-state levels are modest, they appear to be enough to alter cell cycle dynamics. Depletion of Bap1 leads to small but reproducible shift in the proportion of cells in the S and G_2/M phases relative to the G_1 phase. This is consistent with the proposed role of HCF-1 in promoting the G_1-to-S transition by acting as a coactivators for E2F1 (40).

The unique central region of Bap1 is important for the interaction with HCF-1 and includes a functional variant of the HCF-binding tetrapeptide motif (D/EHxY) in which the acidic residue at the first position is replaced by an asparagine (363-NHNY-366 [the variant position is underlined]). Mutation of these four residues to alanine ablates the Bap1-HCF-1 interaction, confirming that this is a bona fide HBM. Thus, despite the limited length of the motif and apparent lack of sequence requirement at the third position, there appears to be additional flexibility at the first position. Transcription factors E2F3a and E2F3b have also been shown to interact with the HCF-1 β-propeller and contain a putative HBM (GHQY) that substitutes glycine for the acidic residue (40). Flexibility at two of the four positions of the HBM suggests that overall sequence context is critical and distinguishes a genuine HBM from irrelevant sequences. Only the histidine and tyrosine residues appear to be invariant, although this has not been tested rigorously. Identification of multiple sequences capable of functioning as an HBM raises the intriguing possibility that there might be more than one docking site on the HCF-1 β-propeller domain, perhaps related to the domain's sixfold symmetry. Sequential coimmunoprecipitation studies have shown that two HBM-containing proteins, VP16 and hSet1, can interact with HCF-1 in a single complex (47). Along the same lines, the association of Bap1 with the β-propeller could bring the UCH domain into close proximity with other HBM-containing proteins that would serve as substrates for the deubiquitination activity.

There is mounting genetic and functional evidence that Bap1 functions as a tumor suppressor protein (13, 41). The chromosomal region 3p21.3 containing the *BAP1* gene locus is frequently deleted or rearranged in lung, renal, and sporadic breast cancers, and inactivating missense mutations in the UCH domain have been identified in lung carcinomas (1, 3, 14). More recently, Wilkinson and coworkers showed that growth of human NCI-H226 squamous lung carcinoma cells, either in tissue culture or as tumors in mice, is blocked by the expression of Bap1 (41). NCI-H226 cells harbor a deletion in the *BAP1* locus, and no protein is expressed. Restoring Bap1 expression using a lentivirus leads to a gross disturbance in the cell cycle with a concordant loss of cell viability. Growth suppression in this context is independent of BRCA1 but does require Bap1 to be nuclear and retain its DUB activity. Cell cycle alterations have also been observed by Nishikawa et al., who showed that Bap1 depletion leads to increased sensitivity of HeLa cells to ionizing radiation and to delayed progression through the S phase after release from a double thymidine block (33). The findings presented here offer a mechanistic framework for these proliferation control functions by proposing that Bap1 is recruited to the regulatory regions of genes involved in cell cycle progression and/or check point control through a physical association with the transcriptional cofactor HCF-1. Further elucidation of the physiological targets for the Bap1 DUB activity in complex with HCF-1 will help to define its role in preventing cancer development.

ACKNOWLEDGMENTS

We thank Sebastian Guelman for useful discussions, Tom Kristie, and Nobuhiko Kayagaki for their gifts of plasmids.

This study was supported by Genentech and grants from the National Institutes of Health to A.C.W. (GM61139, S10 RR017970).

REFERENCES

1. **Angeloni, D.** 2007. Molecular analysis of deletions in human chromosome 3p21 and the role of resident cancer genes in disease. Brief Funct. Genomic Proteomic **6:**19–39.
2. **Arnott, D., A. Kishiyama, E. A. Luis, S. G. Ludlum, J. C. Marsters, Jr., and J. T. Stults.** 2002. Selective detection of membrane proteins without antibodies: a mass spectrometric version of the Western blot. Mol. Cell. Proteomics **1:**148–156.
3. **Buchhagen, D. L., L. Qiu, and P. Etkind.** 1994. Homozygous deletion, rearrangement and hypermethylation implicate chromosome region 3p14.3-3p21.3 in sporadic breast-cancer development. Int. J. Cancer **57:**473–479.
4. **Coupier, I., P. Y. Cousin, D. Hughes, P. Legoix-Ne, A. Trehin, O. M. Sinilnikova, and D. Stoppa-Lyonnet.** 2005. BAP1 and breast cancer risk. Fam. Cancer **4:**273–277.
5. **Dang, L. C., F. D. Melandri, and R. L. Stein.** 1998. Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes. Biochemistry **37:**1868–1879.
6. **Das, C., Q. Q. Hoang, C. A. Kreinbring, S. J. Luchansky, R. K. Meray, S. S.**

Ray, P. T. Lansbury, D. Ringe, and G. A. Petsko. 2006. Structural basis for conformational plasticity of the Parkinson's disease-associated ubiquitin hydrolase UCH-L1. Proc. Natl. Acad. Sci. USA **103:**4675–4680.
7. **Dejosez, M., J. S. Krumenacker, L. J. Zitur, M. Passeri, L. F. Chu, Z. Songyang, J. A. Thomson, and T. P. Zwaka.** 2008. Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. Cell **133:**1162–1174.
8. **Dou, Y., T. A. Milne, A. J. Tackett, E. R. Smith, A. Fukuda, J. Wysocka, C. D. Allis, B. T. Chait, J. L. Hess, and R. G. Roeder.** 2005. Physical association and coordinate function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase MOF. Cell **121:**873–885.
9. **Freiman, R. N., and W. Herr.** 1997. Viral mimicry: common mode of association with HCF by VP16 and the cellular protein LZIP. Genes Dev. **11:**3122–3127.
10. **Glickman, M. H., and A. Ciechanover.** 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. **82:**373–428.
11. **Goto, H., S. Motomura, A. C. Wilson, R. N. Freiman, Y. Nakabeppu, K. Fukushima, M. Fujishima, W. Herr, and T. Nishimoto.** 1997. A single-point mutation in HCF causes temperature-sensitive cell-cycle arrest and disrupts VP16 function. Genes Dev. **11:**726–737.
12. **Hamazaki, J., S. Iemura, T. Natsume, H. Yashiroda, K. Tanaka, and S. Murata.** 2006. A novel proteasome interacting protein recruits the deubiquitinating enzyme UCH37 to 26S proteasomes. EMBO J. **25:**4524–4536.
13. **Jensen, D. E., M. Proctor, S. T. Marquis, H. P. Gardner, S. I. Ha, L. A. Chodosh, A. M. Ishov, N. Tommerup, H. Vissing, Y. Sekido, J. Minna, A. Borodovsky, D. C. Schultz, K. D. Wilkinson, G. G. Maul, N. Barlev, S. L. Berger, G. C. Prendergast, and F. J. Rauscher III.** 1998. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. Oncogene **16:**1097–1112.
14. **Jensen, D. E., and F. J. Rauscher III.** 1999. Defining biochemical functions for the BRCA1 tumor suppressor protein: analysis of the BRCA1-binding protein BAP1. Cancer Lett. **143**(Suppl. 1)**:**S13–S17.
15. **Johnston, S. C., C. N. Larsen, W. J. Cook, K. D. Wilkinson, and C. P. Hill.** 1997. Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 Å resolution. EMBO J. **16:**3787–3796.
16. **Julien, E., and W. Herr.** 2004. A switch in mitotic histone H4 lysine 20 methylation status is linked to M phase defects upon loss of HCF-1. Mol. Cell **14:**713–725.
17. **Julien, E., and W. Herr.** 2003. Proteolytic processing is necessary to separate and ensure proper cell growth and cytokinesis functions of HCF-1. EMBO J. **22:**2360–2369.
18. **Knez, J., D. Piluso, P. Bilan, and J. P. Capone.** 2006. Host cell factor-1 and E2F4 interact via multiple determinants in each protein. Mol. Cell. Biochem. **288:**79–90.
19. **Koulich, E., X. Li, and G. N. Demartino.** 2008. Relative structural and functional roles of multiple deubiquitylating proteins associated with mammalian 26S proteasome. Mol. Biol. Cell **19:**1072–1082.
20. **Kristie, T. M., J. L. Pomerantz, T. C. Twomey, S. A. Parent, and P. A. Sharp.** 1995. The cellular C1 factor of the herpes simplex virus enhancer complex is a family of polypeptides. J. Biol. Chem. **270:**4387–4394.
21. **Kristie, T. M., J. L. Vogel, and A. E. Sears.** 1999. Nuclear localization of the C1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency. Proc. Natl. Acad. Sci. USA **96:**1229–1233.
22. **La Boissiere, S., T. Hughes, and P. O'Hare.** 1999. HCF-dependent nuclear import of VP16. EMBO J. **18:**480–489.
23. **Larsen, C. N., B. A. Krantz, and K. D. Wilkinson.** 1998. Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. Biochemistry **37:**3358–3368.
24. **Larsen, C. N., J. S. Price, and K. D. Wilkinson.** 1996. Substrate binding and catalysis by ubiquitin C-terminal hydrolases: identification of two active site residues. Biochemistry **35:**6735–6744.
25. **Li, T., N. I. Naqvi, H. Yang, and T. S. Teo.** 2000. Identification of a 26S proteasome-associated UCH in fission yeast. Biochem. Biophys. Res. Commun. **272:**270–275.
26. **Lu, R., P. Yang, S. Padmakumar, and V. Misra.** 1998. The herpesvirus transactivator VP16 mimics a human basic domain leucine zipper protein, luman, in its interaction with HCF. J. Virol. **72:**6291–6297.
27. **Luciano, R. L., and A. C. Wilson.** 2003. HCF-1 functions as a coactivator for the zinc finger protein Krox20. J. Biol. Chem. **278:**51116–51124.
28. **Mallery, D. L., C. J. Vandenberg, and K. Hiom.** 2002. Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. EMBO J. **21:**6755–6762.
29. **Maun, H. R., C. Eigenbrot, H. Raab, D. Arnott, L. Phu, S. Bullens, and R. A. Lazarus.** 2005. Disulfide locked variants of factor VIIa with a restricted beta-strand conformation have enhanced enzymatic activity. Protein Sci. **14:**1171–1180.
30. **Meray, R. K., and P. T. Lansbury, Jr.** 2007. Reversible monoubiquitination regulates the Parkinson disease-associated ubiquitin hydrolase UCH-L1. J. Biol. Chem. **282:**10567–10575.
31. **Misaghi, S., P. J. Galardy, W. J. Meester, H. Ovaa, H. L. Ploegh, and R. Gaudet.** 2005. Structure of the ubiquitin hydrolase UCH-L3 complexed with a suicide substrate. J. Biol. Chem. **280:**1512–1520.
32. **Narayanan, A., W. T. Ruyechan, and T. M. Kristie.** 2007. The coactivator host cell factor-1 mediates Set1 and MLL1 H3K4 trimethylation at herpesvirus immediate-early promoters for initiation of infection. Proc. Natl. Acad. Sci. USA **104:**10835–10840.
33. **Nishikawa, H., W. Wu, A. Koike, R. Kojima, H. Gomi, M. Fukuda, and T. Ohta.** 2009. BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity. Cancer Res. **69:**111–119.
34. **Piluso, D., P. Bilan, and J. P. Capone.** 2002. Host cell factor-1 interacts with and antagonizes transactivation by the cell cycle regulatory factor Miz-1. J. Biol. Chem. **277:**46799–46808.
35. **Qiu, X. B., S. Y. Ouyang, C. J. Li, S. Miao, L. Wang, and A. L. Goldberg.** 2006. hRpn13/ADRM1/GP110 is a novel proteasome subunit that binds the deubiquitinating enzyme, UCH37. EMBO J. **25:**5742–5753.
36. **Simmen, K. A., A. Newell, M. Robinson, J. S. Mills, G. Canning, R. Handa, K. Parkes, N. Borkakoti, and R. Jupp.** 1997. Protein interactions in the herpes simplex virus type 1 VP16-induced complex: VP16 peptide inhibition and mutational analysis of host cell factor requirements. J. Virol. **71:**3886–3894.
37. **Smith, E. R., C. Cayrou, R. Huang, W. S. Lane, J. Cote, and J. C. Lucchesi.** 2005. A human protein complex homologous to the *Drosophila* MSL complex is responsible for the majority of histone H4 acetylation at lysine 16. Mol. Cell. Biol. **25:**9175–9188.
38. **Song, L., and M. Rape.** 2008. Reverse the curse–the role of deubiquitination in cell cycle control. Curr. Opin. Cell Biol. **20:**156–163.
39. **Stone, M., R. Hartmann-Petersen, M. Seeger, D. Bech-Otschir, M. Wallace, and C. Gordon.** 2004. Uch2/Uch37 is the major deubiquitinating enzyme associated with the 26S proteasome in fission yeast. J. Mol. Biol. **344:**697–706.
40. **Tyagi, S., A. L. Chabes, J. Wysocka, and W. Herr.** 2007. E2F activation of S phase promoters via association with HCF-1 and the MLL family of histone H3K4 methyltransferases. Mol. Cell **27:**107–119.
41. **Ventii, K. H., N. S. Devi, K. L. Friedrich, T. A. Chernova, M. Tighiouart, E. G. Van Meir, and K. D. Wilkinson.** 2008. BRCA1-associated protein-1 is a tumor suppressor that requires deubiquitinating activity and nuclear localization. Cancer Res. **68:**6953–6962.
42. **Wilson, A. C., M. Boutros, K. M. Johnson, and W. Herr.** 2000. HCF-1 amino- and carboxy-terminal subunit association through two separate sets of interaction modules: involvement of fibronectin type 3 repeats. Mol. Cell. Biol. **20:**6721–6730.
43. **Wilson, A. C., R. N. Freiman, H. Goto, T. Nishimoto, and W. Herr.** 1997. VP16 targets an amino-terminal domain of HCF involved in cell cycle progression. Mol. Cell. Biol. **17:**6139–6146.
44. **Wilson, A. C., M. G. Peterson, and W. Herr.** 1995. The HCF repeat is an unusual proteolytic cleavage signal. Genes Dev. **9:**2445–2458.
45. **Wing, S. S.** 2003. Deubiquitinating enzymes: the importance of driving in reverse along the ubiquitin-proteasome pathway. Int. J. Biochem. Cell Biol. **35:**590–605.
46. **Wysocka, J., and W. Herr.** 2003. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. Trends Biochem. Sci. **28:**294–304.
47. **Wysocka, J., M. P. Myers, C. D. Laherty, R. N. Eisenman, and W. Herr.** 2003. Human Sin3 deacetylase and trithorax-related Set1/Ash2 histone H3-K4 methyltransferase are tethered together selectively by the cell-proliferation factor HCF-1. Genes Dev. **17:**896–911.
48. **Wysocka, J., P. T. Reilly, and W. Herr.** 2001. Loss of HCF-1-chromatin association precedes temperature-induced growth arrest of tsBN67 cells. Mol. Cell. Biol. **21:**3820–3829.
49. **Yao, T., L. Song, W. Xu, G. N. DeMartino, L. Florens, S. K. Swanson, M. P. Washburn, R. C. Conaway, J. W. Conaway, and R. E. Cohen.** 2006. Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1. Nat. Cell Biol. **8:**994–1002.
50. **Yokoyama, A., Z. Wang, J. Wysocka, M. Sanyal, D. J. Aufiero, I. Kitabayashi, W. Herr, and M. L. Cleary.** 2004. Leukemia proto-oncoprotein MLL forms a SET1-like histone methyltransferase complex with menin to regulate Hox gene expression. Mol. Cell. Biol. **24:**5639–5649.