

BRCA1-Associated Protein-1 Is a Tumor Suppressor that Requires Deubiquitinating Activity and Nuclear Localization

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Abstract

BRCA1-associated protein-1 (BAP1), a deubiquitinating enzyme of unknown cellular function, is mutated in breast and lung cancers. In this study, we have shown for the first time that BAP1 has tumor suppressor activity *in vivo* by showing that BAP1 can suppress tumorigenicity of lung cancer cells in athymic nude mice. We show that BAP1 fulfills another criterion of a genuine tumor suppressor because cancer-associated *BAP1* mutants are deficient in deubiquitinating activity. We show for the first time that one of the two predicted nuclear targeting motifs is required for nuclear localization of BAP1 and that a truncation mutant found in a lung cancer cell line results in BAP1 that fails to localize to the nucleus. Furthermore, we show that deubiquitinating activity and nuclear localization are both required for BAP1-mediated tumor suppression in nude mice. We show that BAP1 exerts its tumor suppressor functions by affecting the cell cycle, speeding the progression through the G₁-S checkpoint, and inducing cell death via a process that has characteristics of both apoptosis and necrosis. Surprisingly, BAP1-mediated growth suppression is independent of wild-type BRCA1. Because deubiquitinating enzymes are components of the ubiquitin proteasome system, this pathway has emerged as an important target for anticancer drugs. The identification of the deubiquitinating enzyme BAP1 as a tumor suppressor may lead to further understanding of how the ubiquitin proteasome system contributes to cancer and aid in the identification of new targets for cancer therapy. [Cancer Res 2008;68(17):6953–62]

Introduction

Lung cancer is the leading worldwide source of cancer-related deaths (1), and breast cancer is the second leading cause of cancer-related deaths in women (2). BRCA1-associated protein-1 (BAP1) is a component of the ubiquitin proteasome system (UPS) that has been implicated in lung and breast cancers (3). Because mutations in UPS genes have been implicated in cancer, the UPS has emerged as a potential target for anticancer drugs (4, 5).

The UPS functions in the selective degradation of numerous short-lived proteins in eukaryotic cells (6). In this system, ubiquitin is covalently conjugated to target proteins (7). The attachment of ubiquitin to a substrate molecule can act as a targeting signal by delivering the modified protein to different locations in the cell or modifying its activity, macromolecular interactions, or half-life (8). One way to regulate ubiquitin-modified proteins is through deubiquitination, the removal of the ubiquitin modification, performed by deubiquitinating enzymes (DUB; ref. 9).

BAP1 is a DUB originally identified as a protein interacting with the RING finger domain of the breast cancer susceptibility gene product BRCA1 (3). BAP1 is a nuclear-localized DUB with an NH₂-terminal ubiquitin COOH-terminal hydrolase (UCH) domain and two predicted nuclear-localization signals (NLS). Because the full-length human BRCA1 is a ubiquitin ligase, it was hypothesized that autoubiquitinated BRCA1 might be a substrate for BAP1 deubiquitinating activity (3). It was later shown, however, that BAP1 does not seem to function in the deubiquitination of the BRCA1/BARD1 complex (10). There is no evidence that BRCA1 is a direct, biologically relevant substrate for BAP1, and therefore, the biological function of BAP1 remains to be identified.

Although its cellular function is still elusive, several lines of evidence support a role for BAP1 as a tumor suppressor: the *BAP1* gene locus undergoes frequent loss of heterozygosity in cancer; large rearrangements, deletions, and missense mutations in the *BAP1* gene locus have been found in lung and sporadic breast tumors and lung cancer cell lines (3, 11), and BAP1 suppresses growth of breast cancer cells *in vitro* (3). Despite the evidence to suggest that BAP1 is a tumor suppressor, it remains unknown whether BAP1 fulfills a critical criterion of tumor suppressors—the ability to suppress tumor growth *in vivo*. Furthermore, it is unknown whether cancer-associated BAP1 mutations affect protein function.

In this study, we sought to characterize the *in vivo* growth suppression phenotype of BAP1, to determine if it was a genuine tumor suppressor, to ask whether the mutations found in cancer cells inactivated protein function, and to ask whether BAP1 exerted its tumor suppressor activity through the BRCA1 pathway.

Materials and Methods

DNA constructs and antibodies. *BAP1* was amplified by PCR from pQE-30-BAP1 (3). For expression in *Escherichia coli*, full-length *BAP1* was amplified using primers that introduced an NH₂-terminal FLAG tag and COOH-terminal His₆ tag and subcloned into NdeI/BamHI-digested pRSET B vector (Invitrogen) to create pFLAG-BAP1-His₆. For localization studies, full length and truncated (1-393) *BAP1* were amplified using primers that introduced an NH₂-terminal HA tag and subcloned into BamHI/EcoRI-digested eGFP-N1 vector (Clontech) to create pHA-BAP1-GFP or pHA-BAP1 (1-393)-GFP. For growth and tumor suppression studies, *BAP1*

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doi:10.1158/0008-5472.CAN-08-0365

was amplified and subcloned into pLenti6/V5 vector using the pLenti6/V5 Directional TOPO Cloning kit (Invitrogen) to create pLenti-BAP1. All point mutations were introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and verified by DNA sequencing. Figure 1 shows all point and truncation mutants used in this study. Putative NLS1 and NLS2 (amino acids 656–661 and 717–722 of BAP1, respectively) were cloned upstream of two consecutive green fluorescent protein (GFP; called dGFP vector in this study; a gift from Dr. Anita H. Corbett, Emory University, Atlanta, GA) to form pNLS1-dGFP and pNLS2-dGFP. Anti-FLAG (M2; Sigma-Aldrich, Inc.) for detection of FLAG-BAP1-His₆, anti-BAP1 (C4; Santa Cruz Biotechnology, Inc.) anti-Myc (9B11) for detection of Myc-tagged BRCA1 in HCC1937-BRCA1 cells (Cell Signaling), and anti-Actin (Santa Cruz Biotechnology, Inc.) antibodies were used for immunoblotting.

Cell culture. Human cervical adenocarcinoma (HeLa) cells were maintained in MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Mediatech, Inc.). NCI-H226 [human non-small cell lung cancer (NSCLC)] cells were maintained in RPMI 1640 supplemented with 10% FBS. NCI-H226 cells from passage 1 to 10 were used for all experiments. NCI-H226 and HeLa cells were obtained from American Type Culture Collection. HCC1937 (human breast carcinoma) cells and HCC1937 cells stably transfected with Myc epitope-tagged wild-type (WT) *BRCA1* (HCC1937-BRCA1; gifts from Dr. Junjie Chen, Yale University, New Haven, CT) were maintained in RPMI 1640 supplemented with 10% FBS (12). Human embryonal kidney (293FT) cells were maintained in Complete Medium according to the manufacturer's protocol (Invitrogen). All cells were cultured in a humidified chamber at 37°C in 5% CO₂.

Enzymatic activity assay. Enzyme activity assays for BAP1 were performed as previously described using ubiquitin COOH-terminal 7-amido-4-methylcoumarin substrate (Ub-AMC; ref. 13). Briefly, BL21(DE3) pLysS cells (Invitrogen) harboring the isopropyl-L-thio-β-D-galactopyranoside (IPTG)-inducible FLAG-BAP1-His₆ plasmid were grown to OD₆₀₀ of 0.8 and induced with 20 μmol/L IPTG at 15°C for 24 h. Bacteria were collected and the pellet resuspended to 1/20 volume (original culture) in lysis buffer [300 mmol/L NaCl, 50 mmol/L sodium phosphate, 20 mmol/L imidazole, 10 mmol/L β-mercaptoethanol, 1% Triton X-100 (pH 8.0)]. Lysates were

sonicated and centrifuged at 13,000 *g*. The amount of BAP1 protein in the crude extracts was estimated by immunoblot using anti-FLAG (M2). In some experiments, crude extracts were used for subsequent activity assays. In others, BAP1 was purified using Ni-NTA resin according to the QIAexpressionist protocol (Qiagen). Eluted BAP1 was extensively dialyzed [50 mmol/L TRIS-HCl (pH 7.5), 100 mmol/L NaCl, 0.1% TritonX-100, and 2 mmol/L β-mercaptoethanol] before use in activity assays. Ub-AMC was made according to previously published protocols (13). Ub-AMC (80 nmol/L) was incubated in reaction buffer [50 mmol/L Tris (pH 7.5), 1 mmol/L DTT, and 10 μg/mL ovalbumin] for 100 s. Purified WT FLAG-BAP1-His₆ (0.1 μg) or *E. coli* cell lysate (10 μg) expressing WT or mutant FLAG-BAP1-His₆ was added and the reaction monitored by measuring the increase in fluorescence at 440 nm ($\lambda_{\text{ex}} = 340 \text{ nm}$) using a fluorimeter (Aminco Bowman Series 2). Activity assays were carried out in triplicate at 37°C.

Direct fluorescent imaging. HeLa cells were seeded in 6-well plates (3×10^5 cells per well). Cells were transfected after 24 h with 2 μg purified WT or mutant HA-BAP1-GFP DNA or purified NLS-dGFP DNA using standard Fugene 6 transfection protocols (Roche). After 24 h, cellular DNA was labeled with 5 ng/μL bis-benzimide (Hoechst 33258) and incubated at 37°C for 15 min. Live cell imaging was performed with an Olympus fluorescent microscope IX-81 (Olympus America Inc.) and SlideBook 4.2 software.

Lentivirus production and transduction. Lentiviruses carrying vector alone (VC) or vector encoding WT or mutant BAP1 were produced with the ViraPower Lentiviral Expression System (Invitrogen). These vectors carry a Blasticidin-resistance gene for selection of stably transduced cells. Virus-containing medium was harvested 48 h posttransfection from 293FT cells according to the manufacturer's protocol. Each viral stock was titered separately by quantifying viral DNA in transduced cells (similar to Sastry and colleagues; ref. 14) and by quantifying the percentage of cells expressing BAP1 [via immunofluorescence with anti-BAP1 (C4) antibody]. NCI-H226 cells were infected with lentiviruses at a multiplicity of infection (MOI) of 1 to 3.

Growth suppression studies. NCI-H226 cells (1×10^5) were seeded in triplicate in 6-well plates. After 24 h, cells were infected with lentiviruses-carrying VC or vector encoding WT or mutant BAP1 so that ~10% of the

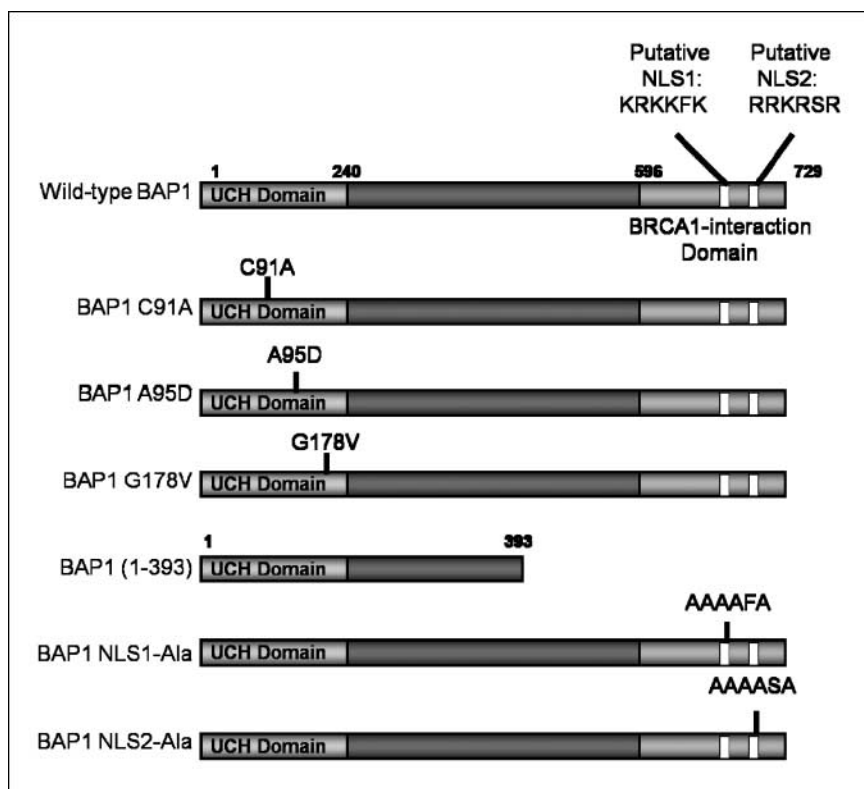
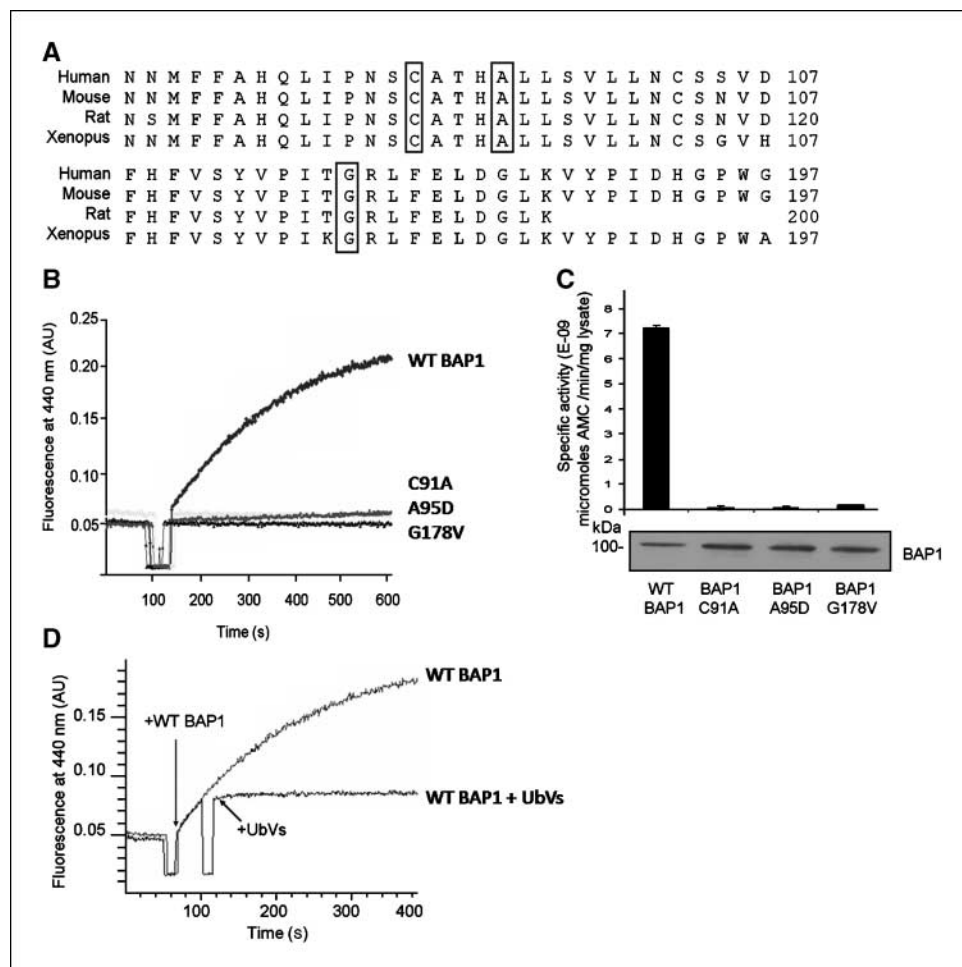


Figure 1. Depiction of BAP1 constructs used in this study. UCH (ubiquitin COOH-terminal hydrolase), NLS (nuclear localization signal).

Figure 2. Cancer-associated mutations in BAP1 result in protein lacking deubiquitinating activity. **A**, alignment of BAP1 NH₂-terminal region from different species showing that the catalytic cysteine 91 and residues mutated in cancer cell lines alanine 95 and glycine 178 are conserved. The alignment was performed using ClustalW alignment and MegAlign 7.1.0 software. National Center for Biotechnology Information (NCBI) accession numbers are as follows: Human BAP1, AAH01596; Mouse BAP1, NP_081364; Rat BAP1, NP_001100762; *Xenopus* BAP1, NP_001008206. **B**, deubiquitinating activity assay. WT and mutant BAP1 proteins were expressed in *E. coli* by IPTG induction. Cells were lysed and deubiquitinating activity of crude extracts was measured as follows: ubiquitin Ub-AMC (80 nmol/L) was added to crude extracts (10 µg) and hydrolysis of Ub-AMC was monitored by measuring the increase in fluorescence at 440 nm (λ_{ex} = 340 nm). C91A is the active site point mutant, whereas A95D and G178V are two mutants associated with lung cancers. **C**, specific activities were calculated from the slope and expressed as micromoles AMC/min/mg lysate. Columns, mean ($n = 3$); bars, SD. Immunoblot of *E. coli* cell lysates shows that similar levels of each BAP1 variant were expressed (5 µg of cell lysate per lane). **D**, DUB activity of purified BAP1 is inhibited by the irreversible inhibitor Ub-Vs. BAP1 was purified using Ni-NTA chromatography. Enzyme activity was measured by adding 0.1 µg purified BAP1 to 80 nmol/L Ub-AMC. Hydrolysis of Ub-AMC was measured as described in **B**. Ub-Vs was able to label WT BAP1 and inhibit the hydrolysis of Ub-AMC.



cells in each well were infected. An additional control well was not infected with virus. Viral medium was removed after 24 h and replaced with RPMI 1640/FBS. After 24 h, this was replaced with selective medium (RPMI-1640/FBS supplemented with 1 µg/mL Blasticidin). Selective medium was replaced every 3 to 4 d until all noninfected cells were dead. Cells were washed with PBS and stained with crystal violet according to the ViraPower protocol. The number of surviving colonies in each well was counted with a colony counter (Bel-Art Products).

Tumorigenicity assay. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Emory University School of Medicine. NCI-H226 cells (1×10^6) infected with lentiviruses-carrying VC, WT BAP1, or mutant BAP1 were injected s.c. on the rear flank of 5- to 6-wk-old athymic *nu/nu* female mice ($n = 9$), and tumorigenesis was monitored for 50 d. NCI-H226 cells were infected with an MOI of 3 with almost 100% cells expressing BAP1 protein. Cells injected into mice were not subjected to antibiotic selection and, therefore, represent pooled clones. Each mouse was identified by tattooing as previously described (15). Mice were weighed and tumors measured every 10 d starting 2 wk after injection. The mean tumor volume was calculated assuming an ellipsoid morphology ($\text{length} \times \text{width}^2 \times 1/2$). Each data set was tested for outliers with the Grubb's test (extreme studentized deviate), and each contained one outlier (in every case the mouse bearing the largest tumor) with a P value of <0.05 , single tailed. After removal of the outliers, statistical analysis was performed using ANOVA with repeated measurements: $P = 0.0009$. Differences in measurements with a P value of <0.05 were considered significant. Animals were sacrificed in a CO₂ chamber and tumors were removed aseptically in a laminar flow hood.

Cell cycle analysis. NCI-H226 cells expressing VC or WT BAP1 were harvested by trypsinization 24 h after Blasticidin selection and washed twice with ice-cold PBS. Cells were fixed in 70% ethanol at 4°C overnight, washed twice in PBS, resuspended (1×10^5 cells/mL) in staining buffer [1 mg/mL RNase A, 0.05 mg/mL propidium iodide (PI), and 0.3% Triton X-100 in PBS], incubated at room temperature for 45 min in the dark, and analyzed by flow cytometry (BD FACSCalibur System; Becton Dickinson) using Cell Quest software.

AnnexinV binding assay. NCI-H226 cells (1×10^6) were infected with lentiviruses-carrying VC or vector-encoding WT, or mutant BAP1. Cells were analyzed for externalization of phosphatidylserine 48 and 120 h after Blasticidin selection by staining with 5 mg AnnexinV-FITC (Calbiochem) and 1 µg/mL PI in 100 µL HEPES buffer [10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂ (pH 7.4)] at 4°C for 15 min. After incubation, an additional 300 µL of HEPES buffer were added. Cells were analyzed by flow cytometry.

Cell growth and cell viability assays. NCI-H226 cells (0.5×10^5 cells per well) were infected with lentiviruses-carrying empty vector or WT BAP1 constructs at an MOI of 3 so that almost 100% cells were infected. Total cell numbers were quantified via hemacytometer after 6, 12, 14, and 21 d. At each time point, cells were stained with 0.8 mmol/L trypan blue (Sigma-Aldrich, Inc.) to distinguish live and dead cells.

Results

Cancer-associated BAP1 mutants have diminished deubiquitinating activity. Recombinant BAP1 has previously been

shown to display ubiquitin hydrolase activity toward a model DUB substrate (3). Mutations in *BAP1* that result in single amino acid substitutions (A95D and G178V) within the UCH domain have been identified in lung cancers (16). These residues are close to the

active site cysteine (C91; Fig. 1) and are highly conserved in *BAP1* orthologues (Fig. 2A).

To test how these cancer-associated mutations affect deubiquitinating activity of *BAP1*, we measured activity using the *in vitro*

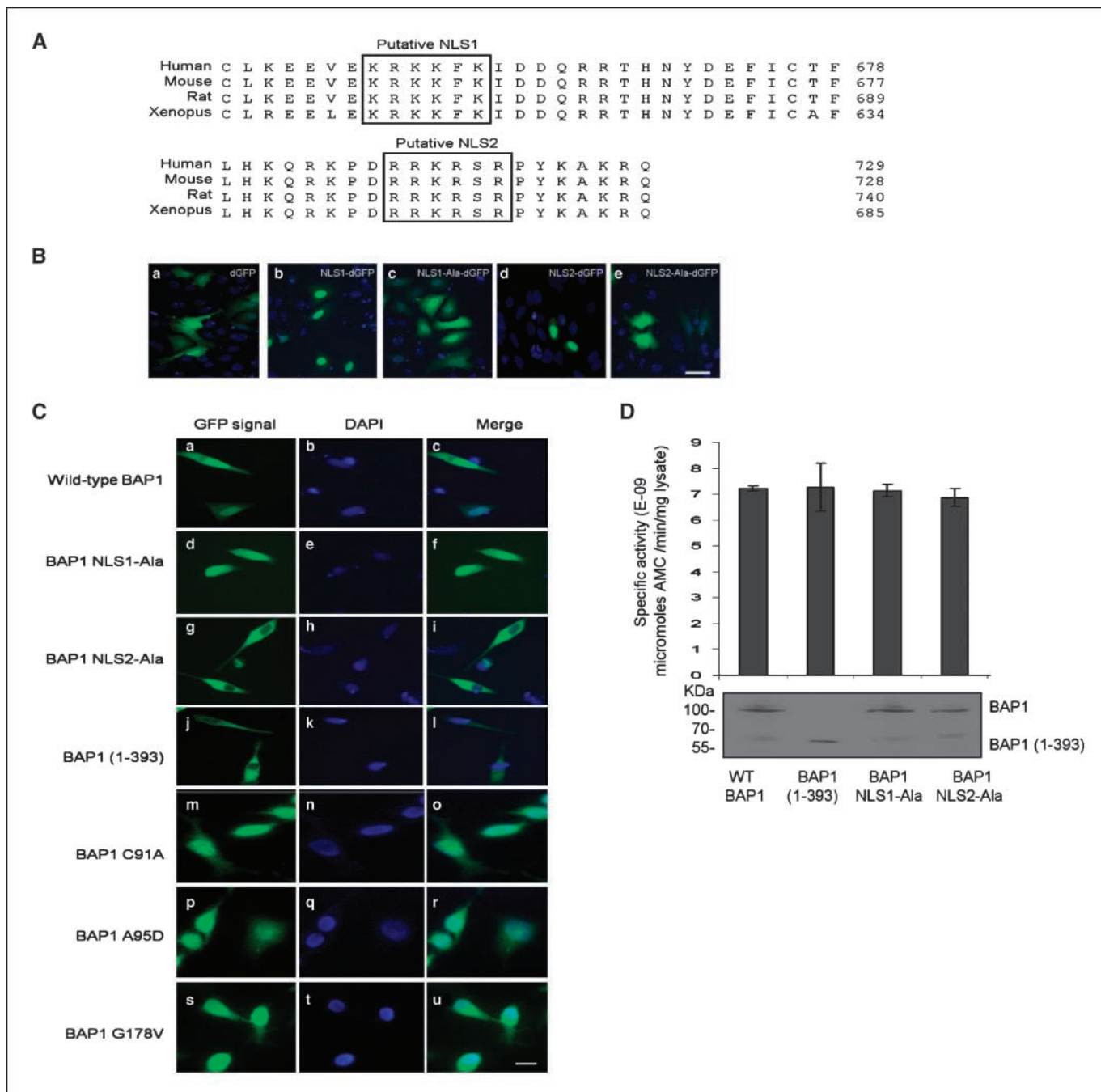


Figure 3. NLS2 is required for nuclear localization of *BAP1*. **A**, alignment of COOH-terminal region of *BAP1* orthologues showing conservation of predicted nuclear targeting signals. The alignment was performed using ClustalW alignment and MegAlign 7.1.0 software. NCBI accession numbers are as follows: Human *BAP1*, AAH01596; Mouse *BAP1*, NP_081364; Rat *BAP1*, NP_001100762; *Xenopus* *BAP1*, NP_001008206. **B**, both putative NLS of *BAP1* can localize GFP to the nucleus. WT NLS sequences or mutant NLS sequences (where all basic residues of the NLS were mutated to alanine) were cloned into the dGFP vector. Each construct (2 μ g) was transfected into HeLa cells (3×10^5 cells per well of 6-well plate). After 24 h, DNA was stained with 5 ng/ μ L bis-benzimide (Hoechst 33258) and the localization of GFP was assessed by direct fluorescent microscopy. *Scale bar*, 20 μ m. **C**, full-length WT or mutant GFP-tagged *BAP1* was expressed in HeLa cells, and protein localization was analyzed by direct fluorescence microscopy, as described in (B). *BAP1* mutants are as follows: NLS1-Ala, mutant in which all basic residues in predicted NLS1 have been converted to alanine; NLS2-Ala, mutant in which all basic residues in predicted NLS2 have been converted to alanine; (1–393), truncation at amino acid 393, discovered in NSCLC NCI-H1466; C91A, active-site point mutant; A95D and G178V, point mutants found in cancer cell lines. *Scale bar*, 10 μ m. **D**, *BAP1* mutants that affect nuclear localization retain deubiquitinating activity. *E. coli* cell lysates (10 μ g/ μ L) expressing WT *BAP1*, *BAP1* (1–393), *BAP1* NLS1-Ala, or *BAP1* NLS2-Ala were used in the Ub-AMC hydrolysis assay. Specific activities of WT and mutant *BAP1* are represented as micromoles AMC/min/mg lysate. Immunoblot analysis of *E. coli* cell lysates expressing WT or mutant *BAP1* shows similar levels of expression (5 μ g of cell lysate per lane); columns, mean ($n = 3$); bars, SD.

DUB substrate Ub-AMC (13). Wild-type BAP1 was able to hydrolyze Ub-AMC, whereas the active site point mutant (C91A) had no activity and the cancer-associated BAP1 mutants (A95D and G178V) were much less active (Fig. 2B). Immunoblot analysis of *E. coli* cell lysates showed equivalent amounts of WT or mutant BAP1 protein expressed (Fig. 2C). The specific activity of BAP1-expressing *E. coli* lysates was determined (Fig. 2C). BAP1 G178V showed low activity, whereas C91A and A95D were essentially inactive. Thus, these cancer-associated mutations reduce catalytic activity by over two orders of magnitude.

To test whether BAP1 DUB activity could be inhibited by the DUB-specific irreversible inhibitor ubiquitin vinyl sulfone (Ub-VS), BAP1 protein was purified by Ni-NTA chromatography and tested for the ability to hydrolyze Ub-AMC in the presence or absence of Ub-VS. As shown in Fig. 2D, purified BAP1 was able to hydrolyze Ub-AMC and this was inhibited by the addition of saturating amounts of Ub-VS. Whereas WT BAP1 was irreversibly labeled by Ub-VS, the active site point mutant (C91A) and the two cancer-associated mutants that had significantly reduced DUB activity (A95D and G178V) were not labeled (data not shown), implying that the structure of the BAP1 active site is altered in these mutants. Together, our data show that cancer-associated BAP1 mutants have diminished deubiquitinating activity, suggesting that deubiquitinating activity of BAP1 may be important in cancer pathogenesis.

BAP1 has a functional nuclear localization signal. BAP1 is primarily localized to the nucleus at steady-state in Rh30 human rhabdomyosarcoma cells (3). To understand the importance of localization for BAP1 function, we first had to define a nuclear targeting signal. Previous studies defined two putative NLS motifs: NLS1 (KRKKFK) and NLS2 (RRKRSR; ref. 3), which are conserved in BAP1 orthologues (Fig. 3A).

To determine whether the NLS sequences of BAP1 are functional, each NLS was cloned into dGFP to produce NLS1-dGFP and NLS2-dGFP. Localization of the expressed proteins in HeLa cells was assessed by direct fluorescence microscopy. Each NLS sequence was sufficient to localize GFP to the nucleus (Fig. 3B, *b* and *d*). Mutant forms of each fusion protein were created in which all five basic residues were mutated to alanine. These mutant NLS-dGFP fusion proteins (Fig. 3B, *c* and *e*) generated a signal similar to that of the dGFP VC.

To determine whether these predicted NLS motifs are required for nuclear localization of full-length BAP1, HA-BAP1-GFP fusion proteins were prepared in which each predicted NLS was mutated separately (BAP1 NLS1-Ala and BAP1 NLS2-Ala; see Fig. 1) and the effect on localization of BAP1 in HeLa cells was assessed. Wild-type BAP1 localized primarily in the nucleus with some protein in the cytoplasm (Fig. 3C, *a-c*). Mutant BAP1 in which all basic residues in the first predicted NLS had been converted to alanine (BAP1 NLS1-Ala) localized to the nucleus (Fig. 3C, *d-f*). Mutant BAP1 in which all basic residues in the second predicted NLS had been converted to alanine (BAP1 NLS2-Ala) showed a significant decrease in nuclear accumulation of BAP1 (Fig. 3C, *g-i*), suggesting that only NLS2 is required for nuclear localization of BAP1.

A homozygous 8-bp deletion was detected in BAP1 cDNA from the non-small cell lung carcinoma cell line NCI-H1466 (3) that would result in a truncation at residue 393 and a protein lacking the nuclear targeting signal (NLS2). To test whether or not amino acids 1 to 393 of BAP1 could efficiently be targeted to the nucleus, an HA-BAP1-GFP construct was created that expressed residues 1 to 393 [BAP1 (1-393); Fig. 1] and its localization in HeLa cells was

assessed. As predicted, BAP1 (1-393) was restricted to the cytoplasm (Fig. 3C, *j-l*). This confirms that the COOH terminus of BAP1 contains a signal required for nuclear localization and suggests that nuclear localization of BAP1 may be important in cancer pathogenesis. In addition to affecting nuclear localization of BAP1, a truncation at amino acid 393 may also affect other protein-protein interactions (such as the interaction with BRCA1), and therefore, the effect of BAP1 nuclear localization on cancer pathogenesis cannot be inferred from this observation alone.

To determine whether mutants that affect DUB activity of BAP1 also have an effect on localization of the protein, the following HA-BAP1-GFP point mutants were made: C91A, the active site point mutant, and A95D and G178V, two cancer-associated mutants with greatly reduced DUB activity. These mutants were expressed in HeLa cells and localization was analyzed by direct fluorescence microscopy. All three BAP1 mutants defective in deubiquitinating activity still retained nuclear localization (Fig. 3C, *m-u*), implying that the effect of BAP1 A95D and BAP1 G178V in cancer is primarily due to the lack of deubiquitinating activity.

We next analyzed whether mutations that abrogate nuclear localization also have an effect on deubiquitinating activity *in vitro* (Fig. 3D). BAP1 (1-393), BAP1 NLS1-Ala, and BAP1 NLS2-Ala were expressed in *E. coli*, and crude cell extracts were tested for the ability to hydrolyze Ub-AMC. All three mutants were able to hydrolyze Ub-AMC and their specific activities were similar to that of WT BAP1. Immunoblot analysis of *E. coli* cell lysates showed equivalent levels of expression of WT and mutant BAP1 (Fig. 3D). Together, our data show for the first time that BAP1 has a functional classic NLS, and that a cancer-associated truncation mutant lacks nuclear localization but not deubiquitinating activity, whereas other cancer-associated point mutants lack deubiquitinating activity but not nuclear localization. These results suggest that nuclear localization of BAP1 is important in cancer pathogenesis.

Deubiquitinating activity and nuclear localization are required for BAP1-mediated growth suppression of NCI-H226 cells *in vitro*. NCI-H226 is a NSCLC cell line that harbors a deletion at the *BAP1* locus (3). We confirmed that this cell line does not express BAP1 protein (Fig. 4A, *lane 1*). We used a lentivirus expression system to reintroduce *BAP1* into this null background. To validate our expression system, NCI-H226 cells were infected with lentiviruses-carrying VC or vector encoding WT BAP1, and after 48 hours, the levels of BAP1 protein were analyzed by immunoblotting. Using the lentivirus expression system, BAP1 was expressed at levels comparable with endogenous WT BAP1 protein found in HeLa and 293 FT cells (Fig. 4A). We estimate that expression of BAP1 is on the same order of magnitude as in other cells and tissues, suggesting that the effects observed are not due to massive overexpression.

Using colony formation assays, BAP1 was previously shown to suppress growth of MCF7 breast cancer cells *in vitro* (3). To ask if BAP1 could suppress growth of NCI-H226 cells *in vitro*, NCI-H226 cells were infected with lentiviruses-carrying VC or vector encoding WT or mutant BAP1. Immunoblot analysis showed equivalent amounts of mutant or WT BAP1 48 hours after infection (Fig. 4B). After growth in selective medium, the number of surviving colonies was quantified using crystal violet staining, and the data represented graphically (Fig. 4C). The mean vector control colony number was set at 100% and the number of surviving colonies in all other groups was represented as a percentage of the control mean. Expression of WT BAP1 resulted in strong growth suppression

(almost 100%), whereas the expression of active site or nuclear localization mutants had very slight or no effect (0–30%; Fig. 4C). We observed some correlation between the level of enzyme activity and ability to suppress growth. Recombinant BAP1 C91A and BAP1 A95D had almost undetectable deubiquitinating activity, but the specific activity of BAP1 G178V was ~10-fold higher (Fig. 2B and C). Correspondingly, the BAP1 G178V mutant showed some growth suppression of NCI-H226 cells (~30%), compared with the C91A (0%) and A95D (5%) mutants (Fig. 4C), thus supporting a requirement for BAP1 deubiquitinating activity in growth suppression. BAP1 NLS2-Ala, a mutant that is unable to localize to the nucleus showed ~10% growth suppression, suggesting that DUB activity and nuclear localization of BAP1 are both required for growth suppression of NCI-H226 cells *in vitro*. Finally, to confirm that BAP1-mediated growth suppression was independent of cell-type, the growth suppression assay was repeated using HeLa cells. We found that overexpression of WT BAP1 resulted in ~60% growth suppression in HeLa cells (data not shown). Together, our data not only confirm that BAP1 has a growth suppressive affect

in vitro (3) but also show that mutants with decreased catalytic activity or mutants defective in nuclear localization also have decreased growth suppressor activity.

BAP1-mediated growth suppression is independent of WT BRCA1 expression. BAP1 has been hypothesized to function in BRCA1-mediated processes (3). To test whether BAP1-mediated growth suppression was dependent on BRCA1 function, the growth suppression assay was repeated using the human breast carcinoma cell line, HCC1937 that lacks WT BRCA1 (17) as well as BRCA1-reconstituted HCC1937 cells (HCC1937-BRCA1). It is prudent to note that although HCC1937 cells lack WT BRCA1, they express a truncated form of the protein that lacks the functional BRCT motifs. Several lines of evidence show that reexpressed full-length tagged BRCA1 in HCC1937-BRCA1 is functionally active: one study showed that this cell line (but not in HCC1937) is able to ubiquitinate CtIP [a binding partner that interacts with the BRCT motifs (18)]; another study showed that this cell line (but not in HCC1937) could efficiently repair double strand breaks after ionizing radiation (19). The mean colony number of HCC1937

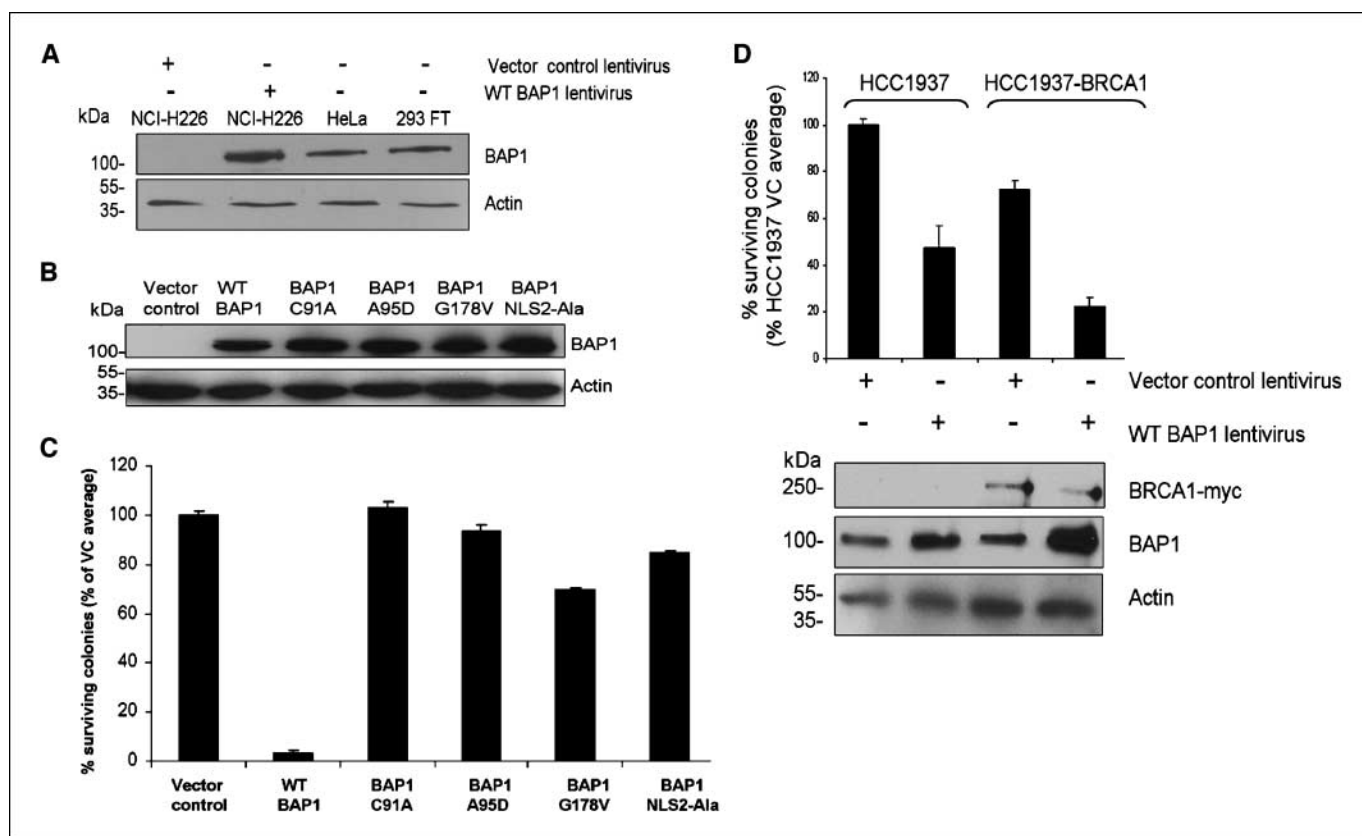


Figure 4. Deubiquitinating activity and nuclear localization are required for BAP1-mediated growth suppression of NCI-H226 cells *in vitro*. **A**, immunoblot analysis of lentivirus-expressed BAP1 in NCI-H226 cells compared with endogenous WT BAP1 in cultured cell lines: NCI-H226 cells (BAP1-null) expressing VC (lane 1) or WT BAP1 (lane 2), HeLa cells (lane 3), and 293FT cells (lane 4; 5 μ g total cell lysate per lane). **B**, NCI-H226 cells (1×10^5) were seeded in triplicate in 6-well plates. After 24 h, cells were infected with lentiviruses-carrying VC, or vector-encoding WT BAP1 (WT), or one of the following mutants: C91A, active-site point mutant; A95D, cancer-associated, catalytically inactive mutant; G178V, another cancer-associated, catalytically inactive mutant or BAP1 NLS2-Ala, mutant BAP1 that does not localize to the nucleus. An additional control well was not infected with any virus. Immunoblot analysis shows similar expression levels of WT, and mutant BAP1 protein is attained 48 h after infection (10 μ g cell lysate per lane). **C**, the number of surviving colonies was quantified after incubation in selective medium (until all noninfected cells were dead), using crystal violet according to the ViraPower kit protocol. Results are represented graphically as a percentage of the VC mean; columns, mean ($n = 3$); bars, SD. **D**, BAP1-mediated growth suppression is independent of BRCA1. Human breast carcinoma cells HCC1937 and HCC1937-BRCA1 cells (HCC1937 cells expressing Myc epitope-tagged WT BRCA1) were seeded in triplicate in 6-well plates (1×10^5 per well). After 24 h, cells were infected with lentiviruses-carrying VC or vector encoding WT BAP1. An additional control well was not infected with any virus. Immunoblot analysis was performed 48 h after infection (10 μ g cell lysate per lane). After incubation in selective medium (until all noninfected cells were dead), the number of surviving colonies was quantified using crystal violet according to the ViraPower kit protocol. Results are represented graphically as a percentage of the HCC1937-VC mean. Columns, mean ($n = 3$); bars, SD.

cells infected with VC-carrying viruses (HCC1937-VC) was set at 100% (interpreted as 0% growth suppression), and the number of surviving colonies in the other groups was represented as a percentage of this (Fig. 4D). Overexpression of BAP1 had a growth suppressive effect (~60%) on HCC1937 as well as on HCC1937-BRCA1 cells (~80%), Fig. 4D (lanes 2 and 4), demonstrating that BAP1-mediated growth suppression does not require WT BRCA1.

Deubiquitinating activity and nuclear localization are required for BAP1-mediated tumor suppression *in vivo*. One important property of a tumor suppressor is the ability to suppress tumorigenesis *in vivo*. NCI-H226 cells are tumorigenic in nude mice (20) so we were able to test the hypothesis that deubiquitinating activity and nuclear localization of BAP1 are required for tumor suppression. NCI-H226 cells expressing VC, WT, or mutant BAP1 were injected s.c. into *nu/nu* mice. A subset of each cell population was analyzed with trypan blue vital stain to confirm that viable cells were being injected into the mice. Tumors were analyzed by two variables: tumor growth rate (mm^3/day ; Fig. 5A) and final tumor volume (mm^3 ; Fig. 5B). We found that expression of WT BAP1 significantly abolished tumorigenicity of NCI-H226 cells, whereas expression of BAP1 that lacks deubiquitinating activity (C91A) or nuclear localization (NLS2-Ala) did not suppress tumorigenicity. After 50 days of growth, WT BAP1 tumors reached a final volume of only 37 mm^3 . This was significantly smaller than control (VC), BAP1 C91A, or BAP1 NLS2-Ala tumors (Fig. 5C), which reached final volumes of 567, 426, and 338 mm^3 , respectively (P values are 0.0006, 0.0001, and 0.016, respectively, when compared with WT BAP1). The other P values showed that there was not a significant difference between the other tumors: VC versus C91A, 0.1143; VC versus NLS2-Ala, 0.1206; C91A versus NLS2-Ala, 0.6844. We conclude that BAP1 is a tumor suppressor that requires its deubiquitinating activity and nuclear localization for tumor suppression in NCI-H226 cells.

BAP1 suppresses cell viability and growth. To define the molecular mechanisms behind BAP1-mediated growth suppression, (a) flow cytometry analysis was used to investigate the effect of BAP1 on alterations in DNA content, cell cycle distribution, and apoptosis, and (b) a growth curve of BAP1-expressing cells versus control cells was done.

Expression of WT BAP1 resulted in a cell cycle defect compared with cells expressing VC or mutant BAP1 (C91A or NLS2-Ala). There were ~20% fewer cells in G_1 compared with the other cell lines, a slight accumulation of cells in S phase and ~10% increase in the sub- G_1 population (indicative of apoptosis; Fig. 6A).

Cells were analyzed for binding to AnnexinV at 48 and 120 hours after Blasticidin selection. AnnexinV-positive/PI-negative cells are indicative of early apoptosis, whereas AnnexinV-positive/PI-positive cells are indicative of late apoptosis and/or necrosis. At 48 hours, WT BAP1-expressing cells showed a significant increase (43%) in early apoptosis compared with VC (9%), C91A (18%), and NLS2-Ala (26%) cells. By 120 hours, most WT BAP1-expressing cells were in late apoptosis/necrosis (55%). Control and mutant cell lines showed similar profiles at 48 hours (data not shown) and 120 hours (Fig. 6B). To further confirm the effect of BAP1 on apoptosis, cleavage of caspase-3 was assessed; however, no evidence of significant cleavage was observed compared with control cells (data not shown). Our data imply that BAP1-mediated cell death displays properties of both apoptosis and necrosis.

To confirm that the effects of BAP1 on cell viability were not simply due to stress induced by antibiotic selection, we assessed

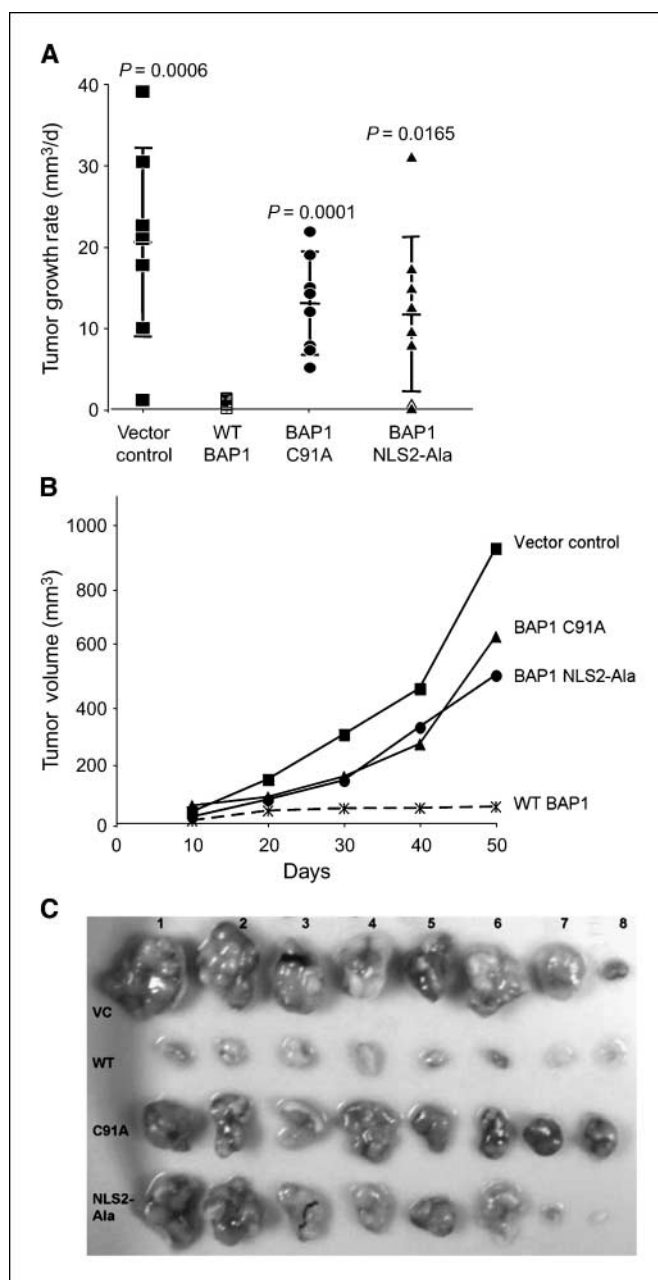


Figure 5. BAP1-mediated suppression of NCI-H226 cell tumorigenicity *in vivo* requires deubiquitinating activity and nuclear localization. Athymic *nu/nu* female mice (5- to 6-wk-old) were injected s.c. with 1×10^6 NCI-H226 cells carrying VC, or vector encoding WT BAP1, active-site point mutant BAP1 (C91A), or mutant BAP1 that does not localize to the nucleus (NLS2-Ala). A, tumor growth rates represented as $\text{mm}^3/\text{d} \pm \text{SD}$. B, the mean tumor volume was plotted as a function of time using the formula ($\text{length} \times \text{width}^2 \times 1/2$). P values shown are compared with WT BAP1 tumors. Other P values are as follows: VC versus C91A, 0.1143; VC versus NLS2-Ala, 0.1206; C91A versus NLS2-Ala, 0.6844; $n = 8$. C, representative tumors excised from mice. Numbers at top represent mouse number.

whether BAP1 affects the growth of NCI-H226 cells in the absence of antibiotic selection. NCI-H226 cells were infected with control and WT BAP1-carrying lentiviruses so that almost 100% of the cells were infected. Total cell numbers were counted over time. We found that control cells doubled approximately every 72 hours, whereas WT-BAP1-expressing cells hardly grew (Fig. 6C). The number of dead cells in the WT BAP1 sample increased in a linear fashion,

implying that growth was counterbalanced by cell death and that the rate of cell death was constant throughout the experiment.

Discussion

Previous evidence has implicated *BAP1* as a tumor suppressor gene: the *BAP1* gene is located on human chromosome 3 (3p21.3); deletions in 3p are detected in almost 100% of small-cell lung cancers, renal cell carcinomas, over 90% of NSCLC cell lines, and >80% of breast carcinomas (21). Our study shows, for the first time, that *BAP1* exhibits another key characteristic of tumor suppressors, the ability to inhibit tumor growth in an animal model. This is not a trivial extension of the growth suppression studies. First, growth in soft agar is an incomplete model for tumorigenesis. Any number

of factors provided in the milieu of the whole animal can affect tumor growth, and the results we have presented make it clear that the growth properties observed in cell culture are mirrored in the whole animal. Second, because the cell population injected into the animals was not subject to selection by antibiotic, the strength of the effects seen is even more impressive. At the MOI used, we anticipate virtually complete transfection of the cells with *BAP1* expression vectors, but certainly a few cells will remain untransfected or express lower levels of *BAP1* than the bulk pool of cells. The nearly complete suppression of tumor growth by WT *BAP1* reexpression makes it clear that this is a powerful tumor suppressor. The partial effects of expressing catalytically dead *BAP1*, or the NLS mutants, may be due to statistical variation (*P* values indicate the differences are not statistically significant) or

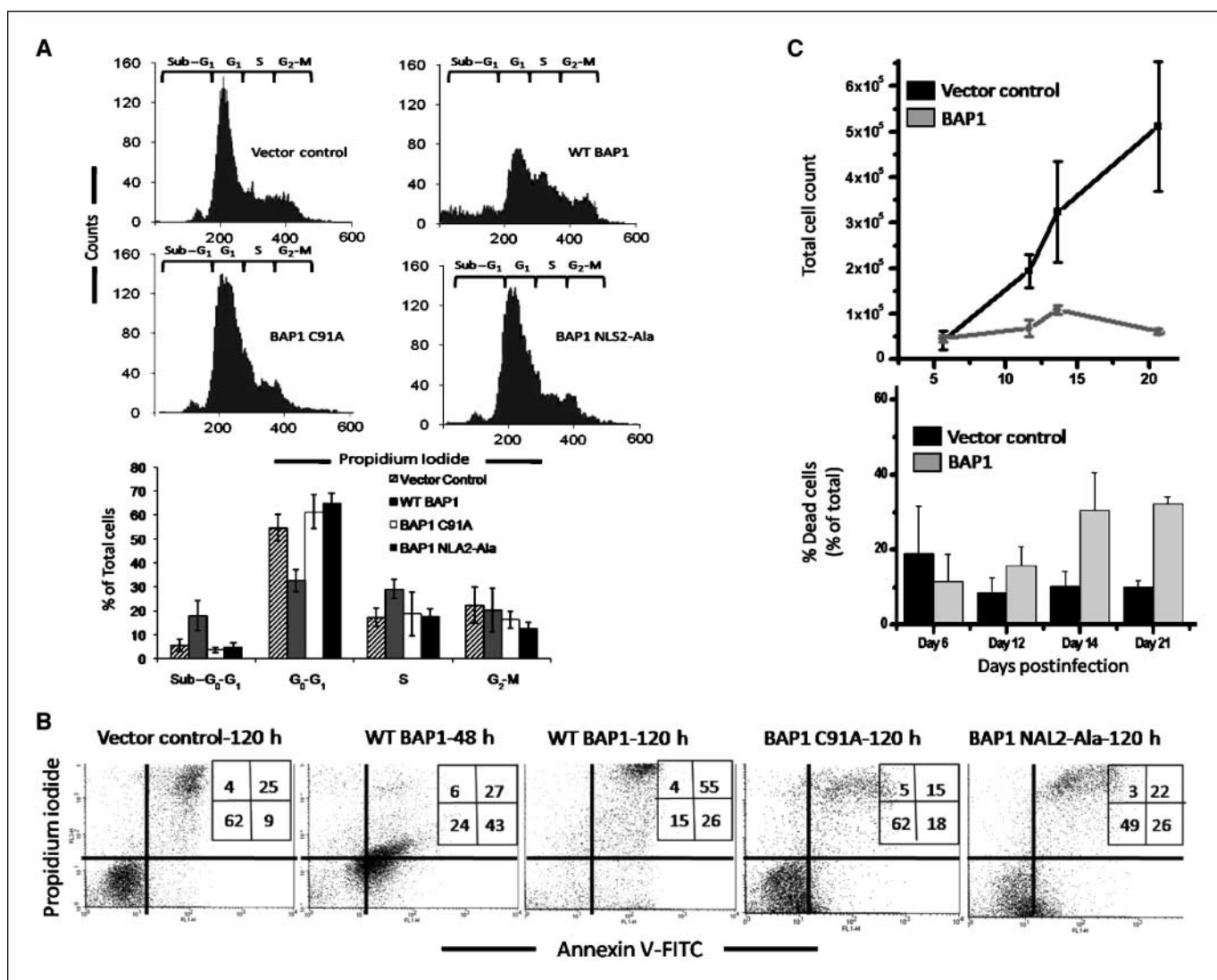


Figure 6. *BAP1* alters cell cycle distribution and induces cell death in NCI-H226 cells. NCI-H226 (*BAP1*-null) cells (1×10^6) were infected with lentiviruses-carrying VC or vector encoding WT, C91A, or NLS2-Ala *BAP1*. An additional control well was not infected with any lentivirus. Cells were grown in selective medium (until noninfected cells were dead) then analyzed by three variables: *A*, cells were fixed and treated with PI to study alterations in DNA content and cell cycle distribution. Gates are as follows: M1, sub-G₁; M2, G₁; M3, S phase; M4, G₂-M. Graph represents relative % of total cell counts in respective gates. *B*, cells were treated with AnnexinV-FITC and PI and analyzed by flow cytometry at the indicated time points. Quadrants are as follows: *bottom left*, viable cells; *bottom right*, early apoptotic; *top right*, late apoptotic/necrotic. Numbers in quadrants represent percent of total cells. *C*, cells were infected with viruses carrying empty vector (VC) or WT *BAP1* at an MOI of 3 so that almost 100% cells were infected. Cells were not subjected to antibiotic selection. Growth and cell viability were assessed by trypan blue exclusion.

a small dominant negative effect due to expression of the inactive or mislocalized protein.

Like BAP1, other deubiquitinating enzymes have recently been shown to be *bona fide* tumor suppressors, including the tumor-suppressor gene, *CYLD* (22) and the herpes virus-associated ubiquitin-specific protease (ubiquitin-specific protease 7; ref. 23). The UPS is an integral part of normal cellular functions including cell cycle progression, signal transduction, response to extracellular stress, and DNA repair, and mutations that affect UPS components have been found to either enhance the effect of oncoproteins or reduce the function or amount of tumor suppressors (4).

Although BAP1 has been hypothesized to function in BRCA1-mediated processes (3), our results, and others', support the possibility of BRCA1-independent functions of BAP1. First, Mallery and colleagues (10), have shown that BAP1 does not seem to function in the deubiquitination of the BRCA1/BARD1 complex. Second, we have shown that BAP1-mediated growth suppression is independent of WT BRCA1 (Fig. 4D). Because there is no evidence that BRCA1 is a biologically relevant substrate for BAP1, we suspect that there are other *in vivo* targets of BAP1-deubiquitinating activity.

Deubiquitinating activity of BAP1 seems to be important in cancer pathogenesis. Missense mutations that abolish the deubiquitinating activity of BAP1 (A95D and G178V) have been identified in cancer cell lines (16). We have shown that these cancer-associated BAP1 mutants are not only defective in deubiquitinating activity (Fig. 2B and C) but are also defective in tumor suppressor activity (Fig. 5), implying that DUB activity of BAP1 is important in cancer pathogenesis and further supporting BAP1 as a genuine tumor suppressor.

BAP1 is a nuclear-localized DUB. Although two nuclear localization signals had previously been predicted (3), we have shown, for the first time, that BAP1 contains a functional classic NLS (Fig. 3C) and that localization to the nucleus is required for BAP1-mediated tumor suppression (Fig. 5). The NLS2 motif of BAP1 is a hydrophobic PY-NLSS, which uses karyopherin β 2 as an import carrier (24). The identification of a functional classic NLS in BAP1 is consistent with BAP1 having a nuclear substrate and supports a mechanism, whereby BAP1 must enter the nucleus and its deubiquitinating activity is involved in functions that lead to tumor suppression. These functions may include DNA damage repair, regulation of apoptosis, and/or senescence or cell cycle regulation.

We hypothesize that expression of BAP1 in NCI-H226 cells induces early exit out of G₁, thereby bypassing the G₁-S checkpoint and causing an accumulation of unrepaired DNA damage and eventual induction of cell death. Several gene expression array studies have implicated BAP1 as a cell cycle regulator (25–27), and our preliminary studies support a role for BAP1 in cell cycle regulation (Fig. 6A). Our data also imply that BAP1-mediated tumor suppression results from an increase in cell death by

apoptosis (Fig. 6B). However, other characteristics of apoptosis such as cleavage of caspase-3, a molecular marker of apoptosis, were not observed. This may be related to the relatively small portion of the cell population that is undergoing cell death at any time. Most studies on apoptosis inflict catastrophic insult on the cells, and all cells undergo a synchronized apoptosis where molecular markers are evident by 24 to 72 hours. In the experiments reported here, the presence of BAP1 is speeding the transition to S phase and some of those cells have accumulated some degree of DNA damage. Therefore, only a portion of these cells will undergo apoptosis at each division, leading to a low level of apoptotic markers detectable at any time. It is also prudent to note that expression of BAP1 increased the population of cells that stain positive for both AnnexinV and PI, an indication of late apoptosis and/or necrosis. More in-depth analysis of BAP1-mediated cell death is warranted to determine the specific mechanism of BAP1-mediated growth inhibition. For example, other studies have described cases of alternative (nonapoptotic) programs of cell death, which also exhibit features characteristic of necrosis (28).

In summary, we have provided strong evidence that BAP1 fulfills three major criteria for a genuine tumor suppressor: (a) it is mutated in cancer, (b) these cancer-associated mutations inactivate a key growth suppressive activity of the protein, and (c) restoration of the protein expression in mutant tumor cells antagonizes tumor growth. We have shown that BAP1 has a functional classic NLS and that deubiquitinating activity and nuclear localization are both required for its tumor suppressor activity. BAP1 acts as a cell cycle regulator and this may be one of the mechanisms through which it carries out its tumor suppressor function. BAP1-mediated tumor suppression seems to be a complex sequence of events that may activate multiple pathways, thereby regulating the cell cycle in a manner that results in apoptosis, necrosis, or both. The identification of the deubiquitinating enzyme BAP1 as a tumor suppressor may further our understanding of how the UPS functions in cancer development and/or progression as well as help identify potential new targets for anticancer drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 1/30/2008; revised 6/20/2008; accepted 6/21/2008.

Grant support: NIH GM30308 (K.D. Wilkinson), CA86335 and CA116804 (E.G. Van Meir), and 5K12GM000680 (K.L. Friedrich).

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We thank Sean R. Stowell Ph.D. for his assistance and useful discussions.

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Cancer Res 2008;68:6953-6962.

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