BAP1 is a tumor suppressor that requires deubiquitinating activity and nuclear localization

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Abstract

BAP1 (BRCA1-associated protein-1), a deubiquitinating enzyme of unknown cellular function, is mutated in breast and lung cancers. In this study, we have demonstrated 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 mutations in *BAP1* result in a protein 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 demonstrate 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. Since deubiquitinating enzymes are components of the ubiquitin proteasome system, this pathway has emerged as an important target for anti-cancer 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.

Keywords

BAP1; BRCA1; ubiquitin; cancer

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). BAP1 (BRCA1-associated protein-1) 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 anti-cancer 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 (DUBs) (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 N-terminal ubiquitin C-terminal hydrolase (UCH) domain and two predicted nuclear-localization signals (NLSs). 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 appear 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.

While 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 (LOH) 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 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 *E. coli*, full-length *BAP1* was amplified using primers that introduced an N-terminal FLAG tag and C-terminal His6 tag and subcloned into NdeI/BamHI digested pRSET B vector (Invitrogen, Carlsbad, CA) to create pFLAG-BAP1-His6. For localization studies, full length and truncated (1–393) *BAP1* were amplified using primers that introduced an N-terminal HA tag and subcloned into BamHI/EcoRI digested eGFP-N1 vector (Clontech, Mountain View, CA) to create pHA-BAP1-GFP or pHA-BAP1 (1–393)-GFP. For growth and tumor suppression studies, *BAP1* 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, La Jolla, CA) 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 GFPs (called dGFP vector in this study; a gift from Dr. Anita H. Corbett, Emory University) to form pNLS1-dGFP and pNLS2-dGFP. Anti-FLAG (M2) (Sigma-Aldrich, Inc., St. Louis, MO), for detection of FLAG-BAP1-His6, anti-BAP1 (C4) (Santa Cruz

Biotechnology, Inc, Santa Cruz, CA), anti-Myc (9B11), for detection of Myc-tagged BRCA1 in HCC1937-BRCA1 cells (Cell Signaling, Danvers, MA), and anti-Actin (Santa Cruz Biotechnology, Inc) antibodies were used for immunoblotting.

Cell culture

HeLa (human cervical adenocarcinoma) cells were maintained in Minimum Essential Medium supplemented with 10% heat inactivated fetal bovine serum (FBS) (Mediatech Inc., Manassas, VA). NCI-H226 (human non-small cell lung cancer) cells were maintained in RPMI-1640 supplemented with 10% FBS. NCI-H226 cells from passage one to ten were used for all experiments. NCI-H226 and HeLa cells were obtained from American Type Culture Collection (Manassas, VA). HCC1937 (human breast carcinoma) cells and HCC1937 cells stably transfected with Myc epitope–tagged wild-type *BRCA1* (HCC1937-BRCA1) (gifts from Dr. Junjie Chen, Yale University) were maintained in RPMI-1640 supplemented with 10% FBS (12). 293FT (human embryonal kidney) 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 Ub-AMC substrate (ubiquitin C-terminal 7-amido-4-methylcoumarin) (13). Briefly, BL21(DE3) pLysS cells (Invitrogen) harboring the IPTG-inducible FLAG-BAP1-His6 plasmid were grown to OD600 of 0.8 and induced with 20 µM IPTG at 15°C for 24 h. Bacteria were collected and the pellet resuspended to 1/20 volume (original culture) in lysis buffer (300 mM NaCl, 50 mM sodium phosphate, 20 mM imidazole, 10 mM beta-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, Valencia, CA). Eluted BAP1 was extensively dialyzed (50 mM TRIS-HCl pH 7.5, 100 mM NaCl, 0.1% TritonX-100, 2 mM beta-mercaptoethanol) before use in activity assays. Ub-AMC was made according to previously published protocols (13). Ub-AMC (80 nM) was incubated in reaction buffer (50 mM Tris pH 7.5, 1 mM DTT, 10 µg/ml ovalbumin) for 100 sec. Purified wild-type FLAG-BAP1-His6 (0.1 µg) or *E. coli* cell lysate (10 µg) expressing wild-type or mutant FLAG-BAP1-His6 was added and the reaction monitored by measuring the increase in fluorescence at 440 nm (λex= 340 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 six-well plates (3×10^5 cells per well). Cells were transfected after 24 h with 2 µg purified wild-type or mutant HA-BAP1-GFP DNA or purified NLS-dGFP DNA using standard Fugene 6 transfection protocols (Roche, Indianapolis, IN). 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 or vector encoding wild-type or mutant BAP1 were produced with the ViraPower[TM] Lentiviral Expression System (Invitrogen). These vectors carry a Blasticidin-resistance gene for selection of stably transduced cells. Virus-containing medium was harvested 48 h post transfection 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 *et al.* (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 1–3.

Growth suppression studies

NCI-H226 cells (1×10^5) were seeded in triplicate in six-well plates. After 24 h cells were infected with lentiviruses carrying vector alone (VC) or vector encoding wild-type or mutant BAP1 so that approximately 10% of the 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 (RMPI-1640/FBS supplemented with 1 µg/ml Blasticidin). Selective medium was replaced every three-four days until all non-infected cells were dead. Cells were washed with PBS and stained with crystal violet according to the ViraPower^{TM} 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 vector alone, WT BAP1 or mutant BAP1 were injected subcutaneously (s.c.) on the rear flank of 5–6-week-old athymic *nu/nu* female mice (n=9) and tumorigenesis was monitored for 50 days. 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 ten days starting two weeks after injection. The mean tumor volume was calculated assuming an ellipsoid morphology (length x width^2 x ½). 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 p-value < 0.05, single tailed. After removal of the outliers, statistical analysis was performed using ANOVA with repeated measurements: p-value = 0.0009. Differences in measurements with p-value < 0.05 were considered significant. Animals were sacrificed in a CO2 chamber and tumors removed aseptically in a laminar flow hood.

Cell cycle analysis

NCI-H226 cells expressing vector alone or wild-type BAP1 were harvested by trypsinization 24h after Blasticidin selection and washed twice with ice-cold PBS. Cells were fixed in 70% ethanol at 4°C overnight, washed twice in PBS, re-suspended (1×10^5 cells/ml) in staining buffer (1 mg/ml RNase A, 0.05 mg/ml propidium iodide, 0.3% Triton X-100 in PBS), incubated at room temperature for 45 min in the dark and analyzed by flow cytometry (BD FACSCalibur^{TM} System, Becton Dickinson, San Jose, CA) using Cell Quest software.

AnnexinV binding assay

NCI-H226 cells (1×10^6) were infected with lentiviruses carrying vector alone or vector encoding WT, or mutant BAP1. Cells were analyzed for externalization of phosphatidylserine 48 h and 120 h after Blasticidin selection by staining with 5 mg AnnexinV-FITC (Calbiochem, San Diego, CA) and 1 µg/ml propidium iodide (PI) in 100 µl HEPES buffer (10 mM HEPES, 140 mM NaCl, 2.5mM CaCl2, pH 7.4) at 4°C for 15 min. Following incubation, an additional 300 µl of HEPES buffer was added. Cells were analyzed by flow cytometry.

Cell growth and cell viability assays

NCI-H226 cells (0.5×10^5 cells/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 days. At each time point, cells were stained with 0.8 mM 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 towards 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) (Figure 1) and are highly conserved in BAP1 orthologs (Figure 2A).

To test how these cancer-associated mutations affect deubiquitinating activity of BAP1, we measured activity using the *in vitro* DUB substrate Ub-AMC (13). Wild-type BAP1 was able to hydrolyze Ub-AMC, while the active site point mutant (C91A) had no activity and the cancer-associated BAP1 mutants (A95D and G178V) were much less active (Figure 2B). Immunoblot analysis of *E. coli* cell lysates showed equivalent amounts of wild-type or mutant BAP1 protein expressed (Figure 2C). The specific activity of BAP1-expressing *E. coli* lysates was determined (Figure 2C). BAP1 G178V showed low activity while 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 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 Figure 2D, purified BAP1 was able to hydrolyze Ub-AMC and this was inhibited by the addition of saturating amounts of Ub-VS. Whereas wild-type 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) (3) which are conserved in BAP1 orthologs (Figure 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 (Figure 3B, panels 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 (Figure 3B, panels C and E) generated a signal similar to that of the dGFP vector alone.

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 Figure 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 (Figure 3C, panels 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 (Figure 3C, panels 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 (Figure 3C panels G-I), suggesting that only NLS2 is required for nuclear localization of BAP1.

A homozygous 8bp 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 1–393 of BAP1 could efficiently be targeted to the nucleus, an HA-BAP1-GFP construct was created that expressed residues 1–393 (BAP1 (1–393), Figure 1) and its localization in HeLa cells was assessed. As predicted, BAP1 (1–393) was restricted to the cytoplasm (Figure 3C, panels J-L). This confirms that the C-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 impact 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 (Figure 3C, panels 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* (Figure 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 wild-type BAP1. Immunoblot analysis of *E. coli* cell lysates showed equivalent levels of expression of wild-type and mutant BAP1 (Figure 3D). Together, our data show for the first time that BAP1 has a functional classical NLS and that a cancer-associated truncation mutant lacks nuclear localization but not deubiquitinating activity, while 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 non-small cell lung cancer (NSCLC) cell line that harbors a deletion at the *BAP1* locus (3). We confirmed that this cell line does not express BAP1 protein (Figure 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 vector alone or vector encoding wild-type BAP1 and after 48 h the levels of BAP1 protein were analyzed by immunoblotting. Using the lentivirus expression system, BAP1 was

expressed at levels comparable to endogenous WT BAP1 protein found in HeLa and 293 FT cells (Figure 4A). We estimate that expression of BAP 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 vector alone or vector encoding wild-type or mutant BAP1. Immunoblot analysis showed equivalent amounts of mutant or wild-type BAP1 48 h after infection (Figure 4B). After growth in selective medium the number of surviving colonies was quantified using crystal violet staining and the data represented graphically (Figure 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 wild-type BAP1 resulted in strong growth suppression (almost 100%), while the expression of active site or nuclear localization mutants had very slight or no effect (0–30%) (Figure 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 approximately ten-fold higher (Figure 2B and Figure 2C). Correspondingly, the BAP1 G178V mutant showed some growth suppression of NCI-H226 cells (approximately 30%), compared to the C91A (0%) and A95D (5%) mutants (Figure 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 demonstrated approximately 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 over-expression of WT BAP1 resulted in approximately 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 demonstrate 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 wild-type 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 wild-type *BRCA1* (17) as well as *BRCA1*-reconstituted HCC1937 cells (HCC1937-BRCA1). It is prudent to note that although HCC1937 cells lack wild-type BRCA1 they express a truncated form of the protein that lacks the functional BRCT motifs. Several lines of evidence show that re-expressed 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 cells infected with vector alone-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 (Figure 4D). Over-expression of BAP1 had a growth suppressive effect (approximately 60%) on HCC1937 as well as on HCC1937-BRCA1 cells (approximately 80%), Figure 4D (lanes 2 and 4), demonstrating that BAP1-mediated growth suppression does not require wild-type 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 vector alone, wild-type 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 parameters: tumor growth rate (mm^3/day), Figure 5A, and final tumor volume (mm^3), Figure 5B. We found that expression of WT BAP1 significantly abolished tumorigenicity of NCI-H226 cells while 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 (Figure 5C), which reached final volumes of 567 mm^3, 426 mm^3 and 338 mm^3 respectively (p-values are 0.0006, 0.0001 and 0.016 respectively, when compared to WT BAP1). The other p-values demonstrated that there was not a significant difference between the other tumors: VC vs. C19A=0.1143; VC vs. NLS2-Ala=0.1206; C91A vs. 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, 1) flow cytometry analysis was used to investigate the effect of BAP1 on alterations in DNA content, cell cycle distribution, and apoptosis and 2) a growth curve of BAP1-expressing cells vs. control cells was done.

Expression of WT BAP1 resulted in a cell cycle defect compared to cells expressing vector alone or mutant BAP1 (C91A or NLS2-Ala). There were approximately 20% fewer cells in G0/G1 compared to the other cell lines, a slight accumulation of cells in S-phase and approximately 10% increase in the sub-G0/G1 population (indicative of apoptosis) (Figure 6A).

Cells were analyzed for binding to AnnexinV at 48 h and 120 h after Blasticidin selection. AnnexinV-positive/PI-negative cells are indicative of early apoptosis, while AnnexinV-positive/PI-positive cells are indicative of late apoptosis and/or necrosis. At 48 h, WT BAP1-expressing cells showed a significant increase (43%) in early apoptosis compared to VC (9%), C91A (18%) and NLS2-Ala (26%) cells. By 120 h, most WT BAP1-expressing cells were in late apoptosis/necrosis (55%). Control and mutant cell lines showed similar profiles at 48 h (data not shown) and 120 h (Figure 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 to 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 whether BAP1 affects the growth of NCI-H226 cells in the absence of antibiotic selection. NCI-H226 cells were infected with control and wild-type 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, while WT-BAP1-expressing cells hardly grew (Figure 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 (SCLC), renal cell carcinomas (RCC), over 90% of non-small-cell lung cancer (NSCLC) cell lines and more than 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, since 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 wild-type BAP1 re-expression 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 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 herpesvirus-associated ubiquitin-specific protease (HAUSP, or ubiquitin-specific protease 7) (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 *et al.*, (10) have shown that BAP1 does not appear to function in the deubiquitination of the BRCA1/BARD1 complex. Secondly, we have shown that BAP1-mediated growth suppression is independent of wild-type BRCA1 (Figure 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 appears 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 (Figure 2B and Figure 2C) but are also defective in tumor suppressor activity (Figure 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 classical NLS (Figure 3C) and that localization to the nucleus is required for BAP1-mediated tumor suppression (Figure 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 classical 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.

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 (Figure 6A). Our data also imply that BAP1-mediated tumor suppression results from an increase in cell death by apoptosis. (Figure 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 in order to determine the specific mechanism of BAP1-mediated growth inhibition. For example, other studies have described cases of alternative (non-apoptotic) programs of cell death which also exhibit features characteristic of necrosis (28). 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 un-repaired DNA damage and eventual induction of cell death.

In summary, we have provided strong evidence that BAP1 fulfills three major criteria for a genuine tumor suppressor: i) it is mutated in cancer, ii) these cancer-associated mutations inactivate a key growth suppressive activity of the protein, and iii) restoration of the protein expression in mutant tumor cells antagonizes tumor growth. We have shown that BAP1 has a functional classical 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 appears 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 anti-cancer drugs.

Acknowledgments

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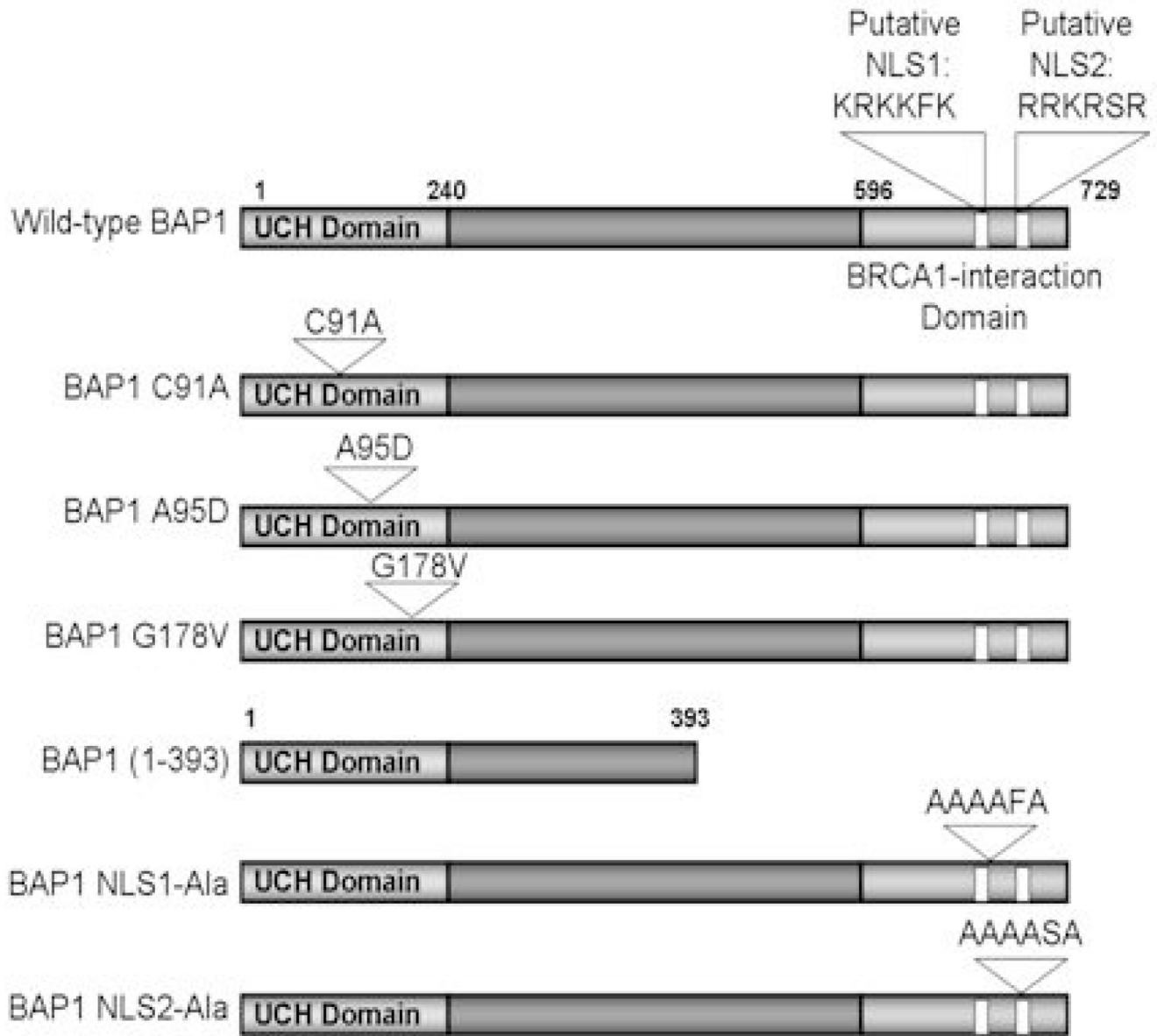
Figure 1.
Depiction of BAP1 constructs used in this study. UCH (ubiquitin C-terminal hydrolase), NLS (nuclear localization signal).
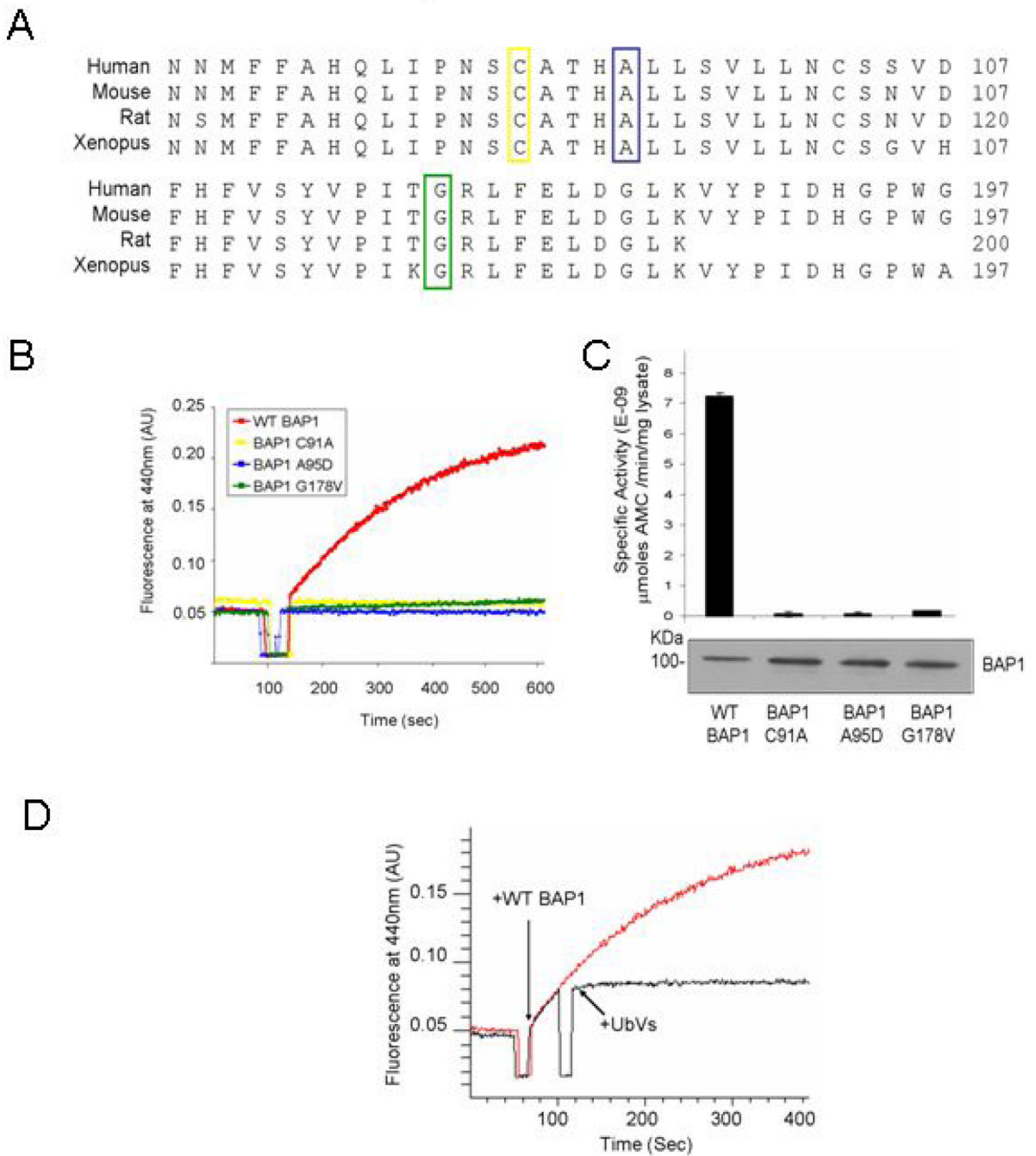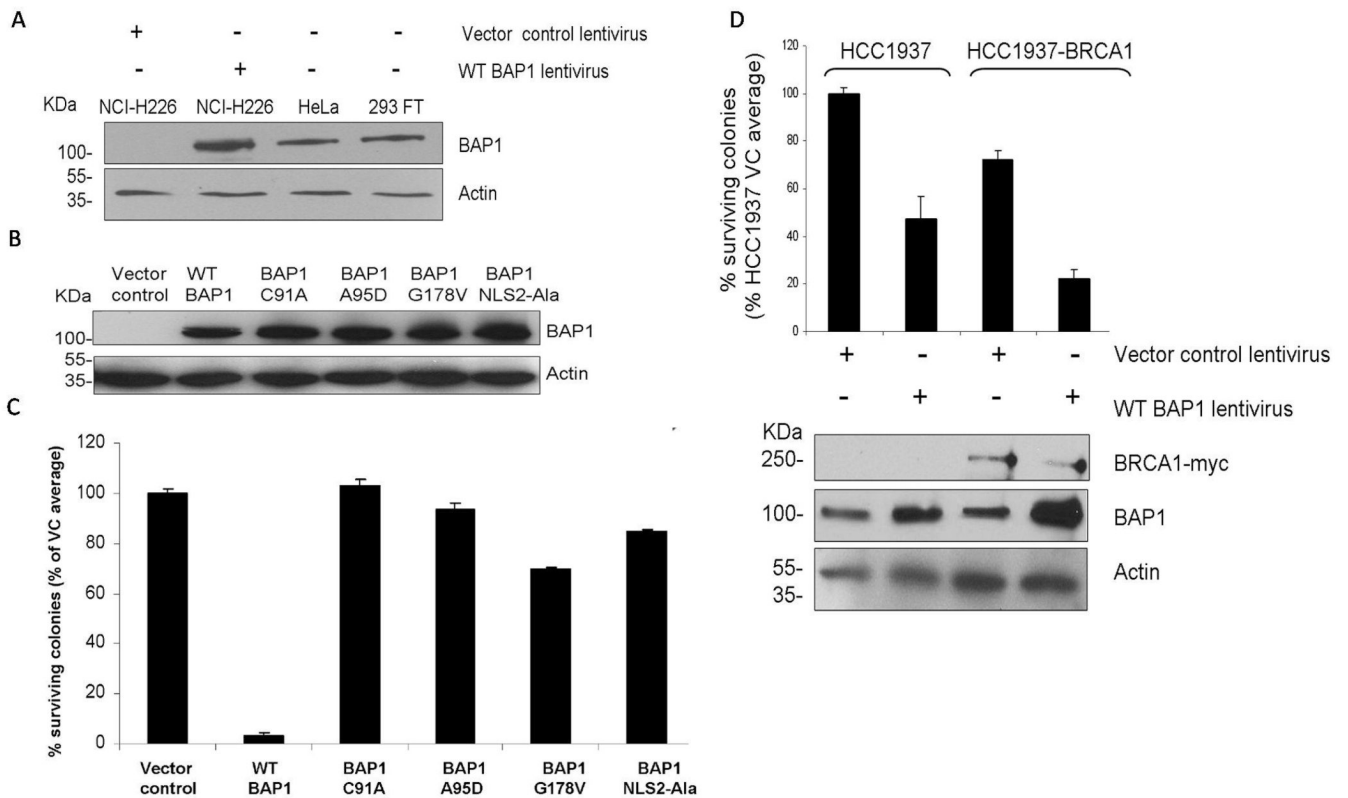
Figure 2.
Cancer-associated mutations in *BAP1* result in protein lacking deubiquitinating activity. (*A*) Alignment of BAP1 N-terminal region from different species showing that the catalytic cysteine 91 (yellow box) and residues mutated in cancer cell lines alanine 95 (blue box) and glycine 178 (green box) are conserved. 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*) Deubiquitinating activity assay. Wild-type (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 C-terminal 7-amido-4-

methylcoumarin (Ub-AMC; 80 nM) was added to crude extracts (10 µg) and hydrolysis of Ub-AMC was monitored by measuring the increase in fluorescence at 440 nm (λex= 340 nm). C91A (yellow line) is the active site point mutant, while A95D (blue line) and G178V (green line) are two mutants associated with lung cancers. (*C*) Specific activities were calculated from the slope and expressed as µmoles AMC/min/mg lysate. (mean ± standard deviation (SD), n=3). 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 ubiquitin vinyl sulfone (Ub-VS). BAP1 was purified using Ni-NTA chromatography. Enzyme activity was measured by adding 0.1 µg purified BAP1 to 80 nM Ub-AMC. Hydrolysis of Ub-AMC (red line) was measured as described in (*B*). Ub-VS was able to label WT BAP1 and inhibit the hydrolysis of Ub-AMC (black line).

Figure 3.
NLS2 is required for nuclear localization of BAP1. (*A*) Alignment of C-terminal region of
BAP1 orthologs 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 nuclear localization
signals (NLSs) of BAP1 can localize GFP to the nucleus. Wild-type (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 µg) was transfected into HeLa cells (3×10^5
cells per well of six-well plate). After 24 h, DNA was stained with 5 ng/µl bis-benzimide
(Hoechst 33258) and the localization of GFP was assessed by direct fluorescent microscopy.
The scale bar is 20 µm. (*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 non-small cell lung carcinoma cell line NCI-H1466; C91A, active-site point mutant; A95D and G178V, point mutants found in cancer cell lines. The scale bar is 10 µm. (*D*) BAP1 mutants that affect nuclear localization retain deubiquitinating activity. *E. coli* cell lysates (10 µg/µl) 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 µmoles AMC/min/mg lysate. Immunoblot analysis of *E. coli* cell lysates expressing wild-type or mutant BAP1 shows similar levels of expression (5 µg of cell lysate per lane; mean ± SD, n =3.)
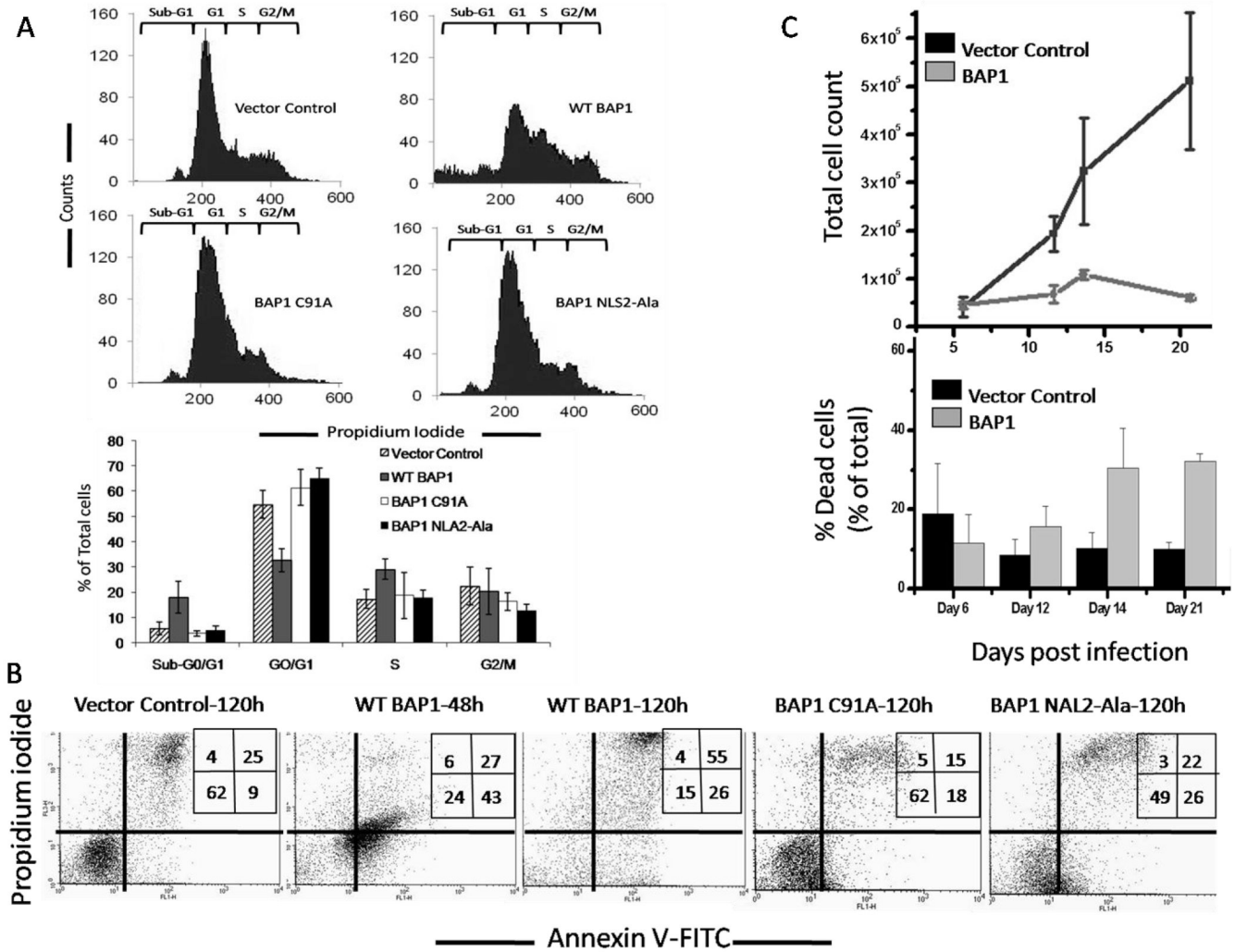
Figure 4.
Deubiquitinating activity and nuclear localization are required for BAP1-mediated growth suppression of NCI-H226 cells *in vitro*. (*A*) Immunoblot analysis of lentivirus-expressed BAP1 in NCI-H226 cells compared to endogenous WT BAP1 in cultured cell lines: NCI-H226 cells (BAP1-null) expressing vector alone (lane 1) or WT BAP1 (lane 2), HeLa cells (lane 3) and 293FT cells (lane 4) (5 µg total cell lysate per lane). (*B*) NCI-H226 cells (1×10^5) were seeded in triplicate in six-well plates. After 24 h cells were infected with lentiviruses carrying vector alone (VC), or vector encoding wild-type 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 µg cell lysate per lane). (*C*) The number of surviving colonies was quantified after incubation in selective medium (until all non-infected cells were dead), using crystal violet according to the ViraPower kit protocol. Results are represented graphically as a percentage of the vector alone (VC) mean (mean ± SD. n = 3). (*D*). BAP1-mediated growth suppression is independent of BRCA1. Human breast carcinoma cells HCC1937 and HCC1937-BRCA1 cells (HCC1937 cells expressing Myc epitope-tagged wild-type BRCA1) were seeded in triplicate in six-well plates (1×10^5 per well). After 24 h cells were infected with lentiviruses carrying vector alone (VC) or vector encoding wild-type BAP1 (WT). An additional control well was not infected with any virus. Immunoblot analysis was performed 48 h after infection (10 µg cell lysate per lane). After incubation in selective medium (until all non-infected 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. Data are mean ± SD. n = 3.
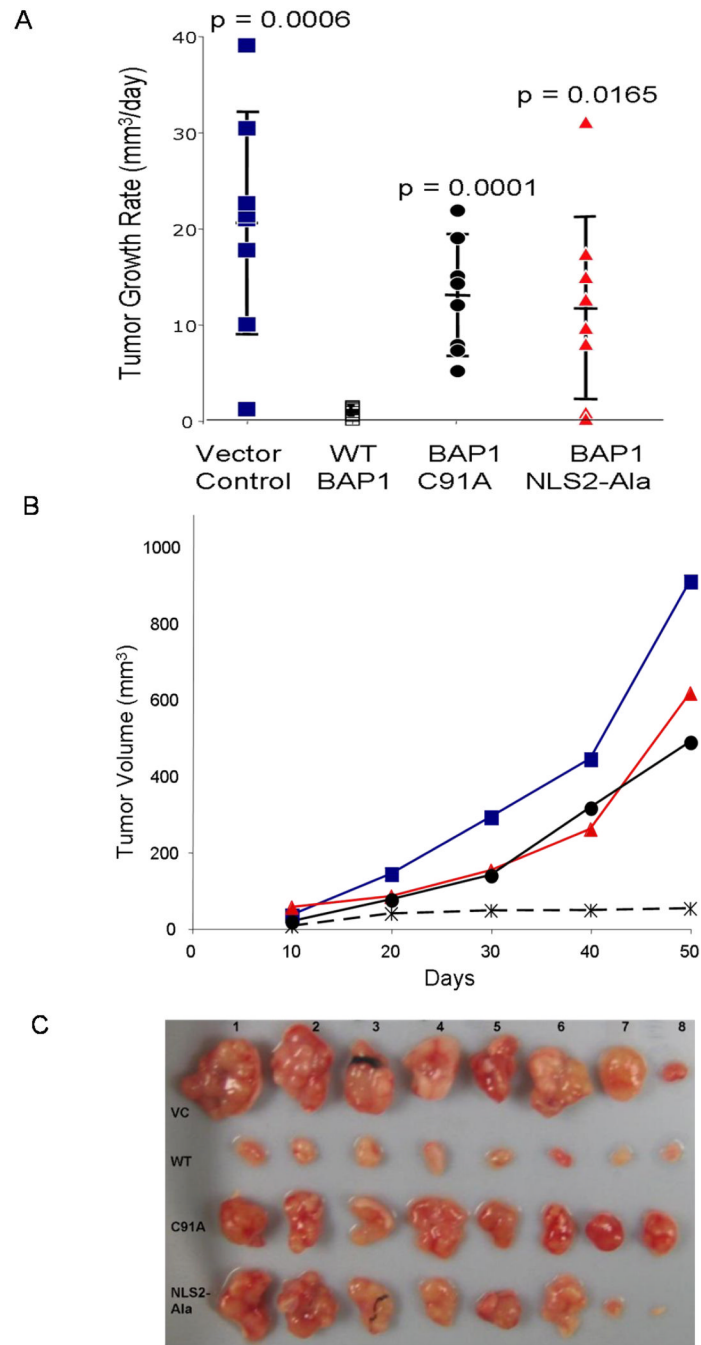
Figure 5.
BAP1-mediated suppression of NCI-H226 cell tumorigenicity *in vivo* requires deubiquitinating activity and nuclear localization. Athymic *nu/nu* female mice (5–6 weeks old) were injected subcutaneously with 1×10^6 NCI-H226 cells carrying vector alone (VC), or vector encoding wild-type BAP1 (WT), active-site point mutant BAP1 (C91A), or mutant BAP1 that does not localize to the nucleus (NLS2-Ala). (*A*) Tumor growth rates represented as mm^3/day $\pm$ SD. (*B*) The mean tumor volume was plotted as a function of time using the formula (Length x width^2 x ½). P-values shown are compared to WT BAP1 tumors. Other p-values are as follows: VC vs. C19A=0.1143; VC vs. NLS2-Ala=0.1206; C91A vs. NLS2-Ala=0.6844. n = 8. (*C*) Representative tumors excised from mice. Numbers at top represent mouse number.

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 vector alone (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 non-infected cells were dead) then analyzed by three parameters: (*A*) cells were fixed and treated with propidium iodide to study alterations in DNA content and cell cycle distribution. Gates are as follows: M1=Sub-G_0/G_1; M2=G_0/G_1; M3=S-phase; M4=G_2/M. Graph represents relative % of total cell counts in respective gates (*B*) cells were treated with AnnexinV-FITC and propidium iodide and analyzed by flow cytometry at the indicated time points. Quadrants are as follows: lower left=viable cells; lower right=early apoptotic; upper 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.