Deubiquitination of γ-Tubulin by BAP1 Prevents Chromosome Instability in Breast Cancer Cells

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Abstract

Microtubule nucleation requires the γ-tubulin ring complex, and during the M-phase (mitosis) this complex accumulates at the centrosome to support mitotic spindle formation. The posttranslational modification of γ-tubulin through ubiquitination is vital for regulating microtubule nucleation and centrosome duplication. Blocking the BRCA1/BARD1-dependent ubiquitination of γ-tubulin causes centrosome amplification. In the current study, we identified BRCA1-associated protein-1 (BAP1) as a deubiquitination enzyme for γ-tubulin. BAP1 was downregulated in metastatic adenocarcinoma breast cell lines compared with noncancerous human breast epithelial cells. Furthermore, low expression of BAP1 was associated with reduced overall survival of patients with breast cancer. Reduced expression of BAP1 in breast cancer cell lines was associated with mitotic abnormalities. Importantly, rescue experiments including expression of full length but not the catalytic mutant of BAP1 reduced ubiquitination of γ-tubulin and prevented mitotic defects. Our study uncovers a new mechanism for BAP1 involved in deubiquitination of γ-tubulin, which is required to prevent abnormal mitotic spindle formation and genome instability. Cancer Res; 74(22); 6499-508. ©2014 AACR.

Introduction

Posttranslational modification of proteins by covalent attachment of ubiquitin controls many essential cellular processes by targeting the proteins for assembly into complexes, transport, and degradation (1, 2). The type of polyubiquitin chains, including lysine 48 (K48) versus lysine 63 (K63), or mono ubiquitination can induce different signaling pathways that regulate different aspects of cellular functions. The most prominent function of polyubiquitination chains through K48 linkage degrades the substrate through the mechanism of the proteasome, whereas K63-linked ubiquitin chains add new functional properties to the modified protein, except for proteosomal degradation (1, 2). Monoubiquitination or multiple monoubiquitination generally leads to transcription regulation as well as DNA repair and cell-cycle progression (1, 2). The action of ubiquitin ligation enzymes responsible for the attachment of the ubiquitin moieties to the substrate is reversed by the action of deubiquitination enzymes (DUB; ref. 3). The human genome encodes a large number of putative DUBs, and accumulating evidence indicates that most of these enzymes regulate a limited number of proteins (4). The dysregulation of DUB function is highly related to human diseases, especially cancer, with examples of hyperactivation or inactivation due to genetic or epigenetic changes. In this aspect, it is vital to identify substrate-specific DUBs to understand the signaling pathways that are dysregulated in cancer cells. Although it is becoming increasingly clear that DUBs play critical roles in various cellular pathways, physiologic roles for most of the human DUBs remain unknown.

BRCA1-associated protein-1 (BAP1) is a nuclear-localized DUB that was originally identified as a BRCA1-binding protein in a yeast two-hybrid screen (5). BAP1 is ubiquitously expressed in different human tissues, especially in the testis, placenta, and ovaries, with varying levels detected in other tissues, including human breast. In mice, BAP1 expression is upregulated in the breast during puberty and pregnancy (6). BAP1 belongs to the ubiquitin C-terminal hydrolase (UCH) family of DUBs, and the activity of BAP1 is restricted to the N-terminal UCH domain, which executes binding and cleavage of the ubiquitin isopeptide bond (5). The BAP1 gene is frequently mutated in lung, breast, melanoma, and renal cell carcinoma (5, 7) and overexpression of BAP1 in lung cancer cells reduces both tumor formation in mice and cell growth in culture. It has thus been suggested that BAP1 acts as a tumor suppressor gene (6). The tumor suppressor property of BAP1 is dependent on its nuclear localization and deubiquitin activity (8). In MCF7 breast cancer cell lines, it was shown that the binding of BAP1 to breast cancer type 1 susceptibility protein (BRCA1) enhances BRCA1-mediated growth suppression (5). However, in contrast to this result, it has been shown that growth suppression is independent of BRCA1 and that BAP1 disrupts the BRCA1/BRCA1-associated ring domain 1 (BARD1) heterodimer, but cannot reverse its autoubiquitination (6, 9, 10).
Therefore, the exact role of BAP1 in BRCA1-mediated signaling and functions remain unknown. Host cell factor C1 (HCF-1) is another binding partner for BAP1. HCF-1 is a transcriptional cofactor found in a number of important regulatory complexes and it localizes to chromatin. BAP1 was shown to regulate HCF-1 protein expression, leading to changes in gene expression involved in the G1–S transition and cell proliferation (11).

The eukaryotic function of γ-tubulin is highly conserved and includes regulating microtubule nucleation and centrosome duplication (12). Ubiquitination of γ-tubulin at lysines 48 and 344, achieved by the activity of the heterodimeric tumor suppressor complex BRCA1/BARD1, was shown to be important for microtubule nucleation at the centrosome and centrosomal amplification (13–16). As BRCA1/BARD1 promotes ubiquitination of γ-tubulin, the DUBs responsible for reversing this process are not known.

In the current study, we identify BAP1 as a specific DUB for γ-tubulin. Deubiquitination of γ-tubulin is an important event in mitosis that regulates mitotic spindle organization and prevents genomic instability.

Materials and Methods

Cell culture

The human breast cell lines were cultured for 5 days at 37°C and 5% CO2 as follows: MCF10A cells were cultured in DMEM/F12 (GIBCO, Life Technologies), supplemented with 5% horse serum (Sigma Aldrich), 20 ng/mL EGF (Sigma Aldrich), 100 ng/mL cholera toxin (Sigma Aldrich), 0.01 mg/mL insulin, 500 ng/mL hydrocortisone (Sigma Aldrich), and 1% penicillin/streptomycin (GIBCO, Life Technologies). The breast cancer cells (MCF7, MDA-MB-231, and MDA-MB-468) were cultured in RPMI (HyClone, Thermo Scientific), supplemented with 10% FBS (Sigma Aldrich), 0.1% sodium pyruvate (Sigma Aldrich), and 0.1% penicillin/streptomycin (GIBCO, Life Technologies).

Immunofluorescence and confocal microscopy

Cells were cultured on coverslips in 6-well plates for 24 hours and then fixed in paraformaldehyde fixation (4% for 15 minutes). Next, the cells were permeabilized with 0.3% Triton X-100 solution, after which they were blocked with 1% BSA and 5% goat serum for 30 minutes to prevent nonspecific binding. Cells were then incubated with primary antibodies against BAP1 (Santa Cruz Biotechnology), α-tubulin (Sigma Aldrich), γ-tubulin (Sigma Aldrich), and UCHL1 (Cell Signaling Technology). After washing coverslips, fluorescent antibodies (Alexa 488 goat anti-mouse or Alexa 546 goat anti-rabbit; Invitrogen) were applied and then the coverslips were washed and mounted in Vectashield with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Fluorescence image were captured and processed using a confocal microscope.

Cell-cycle synchronization

Synchronized cells were obtained by double treatment with 2 mmol/L thymidine (Sigma-Aldrich) in tissue culture medium for 12 to 16 hours. Cells were washed and incubated in normal medium for 8 to 10 hours to release them from the block. Cells treated in this manner were further enriched for mitotic phase by treatment with 40 ng/mL nocodazole (Sigma Aldrich) for 12 to 16 hours. Mitotic cells were washed in PBS and released from the nocodazole block by addition of fresh medium (0–180 minutes). Cells were fixed and stained with DAPI.

Retrovirus production and transduction

Retroviral vector alone or vector encoding wild-type FLAG-HA–tagged BAP1 (plasmid 22539) carrying a puromycin-resistant gene for the selection of stably transduced cells were obtained from Addgene. Retrovirus production was performed by a research engineer at Vector Unit in Lund University (Lund, Sweden). MDA-MB-468 cells were seeded in 6-well plates with 40% confluency in RPMI medium (HyClone, Thermo Scientific) supplemented with 10% FBS, 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Aldrich). Next day, medium was changed with 1 mL medium containing hexadimethrine bromide (Sigma Aldrich; final concentration 8 mg/mL) to each well. Ten microliters of retroviral particles were added to appropriate wells and cells were incubated for 24 hours at 37°C in a humidified incubator in an atmosphere of 5%–7% CO2. Medium was changed with 1 mL RPMI containing puromycin (Sigma Aldrich). The transduction efficiency was initially evaluated and checked using Western blot analysis and immunofluorescence staining. The cells were used directly for experiments without picking clones.

In vitro deubiquitination assay

In vitro deubiquitination assay was carried out by immunoprecipitation of wild-type or catalytic inactive mutant of BAP1 (BAP1-C91A) using anti-FLAG M2-agarose beads (Sigma Aldrich). Beads were incubated with cell extracts for overnight under constant rotation. Before elution, beads were washed four times with TBS-T. The amount of BAP1 protein in the crude extracts was estimated by immunoblot using anti-FLAG antibody (Sigma Aldrich). The Ub-γ-tubulin was incubated in a reaction buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA pH 8.0, 2 mmol/L dithiothreitol) with eluted BAP1 at 37°C for 2 hours. Products were resolved by SDS-PAGE and analyzed by immunoblotting with ubiquitin antibody.

siRNA DUB screen

MCF10A cells were grown in 6-well plates to 30%–50% confluency and then transfected with human DUBs siRNA library (Thermo Scientific) at 2 μmol/L using interferin (Polyplus Transfection). After 72 hours, cells were lysed by radioimmunoprecipitation buffer. Lysates were subjected to SDS-PAGE followed by immunoblotting with indicated antibodies.

BAP1 mutagenesis

Active-site (C91A) mutation was introduced into BAP1-FLAG by using QuickChange Site-Directed Mutagenesis (Stratagene) and the following primers: C91A-BAP1-forward, 5′-CACCACGTATAACCACTCTGCTGAACATCTAGGCCTTGCGTGAG-3′ and reverse, 5′-CTCAGCAAAGCATGAGTCACAGCGTGTCG-3′.

Live cell imaging

Cell divisions were followed live by real-time scanning laser microscopy. Cells expressing GFP-tagged H2B protein were...
seeded in 4-well or 8-well chamber slides (Lab-Tek, Thermo Scientific) in phenol red–free DMEM (HyClone, Thermo Scientific) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Aldrich). Cells were allowed to attach overnight in a humidified incubator (37°C, 5% CO₂) and analyzed the next day using a Zeiss LSM 710 confocal scanning microscope (Carl Zeiss) in a temperature (37°C) and atmosphere (21% O₂, 5% CO₂) controlled chamber, on a heated stage insert (37°C). Live imaging of division stages were followed using 488 nm laser excitation, 493–598 emission filters, and captured with an EC Plan-Neofluar 40×/1.30 oil DIC M27 objective (Carl Zeiss) scanning every 30 seconds during the division time.

Quantitative PCR

The cells were rinsed in cold PBS and total RNA extracted using the Perfect Pure RNA tissue Kit (5 PRIME) according to the manufacturer’s instruction. The purity of RNA was analyzed and quantified by a NanoDrop spectrophotometer (Saveen Werner) and used for cDNA synthesis according to the manufacturer’s instruction (QPCR cDNA Synthesis Kit, Stratagene). PCR runs were performed in the Mx3005P real-time thermocycler (Stratagene) with the following program: 2 minutes in 50°C, 10 minutes in 95°C followed by 40 three-step cycles consisting of 95°C for 20 seconds and 60°C for 30 seconds and 72°C for 1 minute. The following primers were used:

- BAPI Forward 5’-GACCCAGGCCTTTACCC-3’; BAPI Reverse 5’-AGTCCCTCATGCAGCAGG-3’
- GAPDH Forward 5’-GCCAACCACTGCTTAGC-3’; GAPDH Reverse 5’-GGCATGGACTGTGGTCATGAG-3’

Statistical analysis

Data are presented as mean ± SD or ±SEM. Statistical comparisons were assessed by ANOVA or by Student t test (P < 0.05).

Results

Identification of BAP1 in regulating γ-tubulin deubiquitination

In the current study, we aimed to identify the specific DUB responsible for deubiquitination of γ-tubulin using the non-cancerous human breast epithelial cell line MCF10A. MCF10A cells were used because γ-tubulin can bind and undergo ubiquitination via BRCA1 in breast cells (15). Indeed, we found that BRCA1 was colocalized with γ-tubulin during prophase in MCF10A cells, but not during metaphase, anaphase, or telophase (Supplementary Fig. S1). Screening a siRNA library of DUBs, we identified BAPI and UCHL1, by observing an additional band corresponding to the possible deubiquitination of γ-tubulin (Fig. 1A and B). Double depletion of BAPI and UCHL1 resulted in ubiquitination of γ-tubulin to the same extent as single depletion (Fig. 1B, C).
last lane). In addition, we found that BAP1 but not UCHL1 created a connection network with γ-tubulin and BRCA1 (Supplementary Fig. S2). Deconjugation of ubiquitin from γ-tubulin was also confirmed by siRNA oligonucleotides against BAP1 (Fig. 1C).

**Downregulation of BAP1 leads to increased γ-tubulin ubiquitination**

Initially, we investigated the total levels of BAP1 in human breast cancer cells (MCF-7, MDA-MB-231, and MDA-MB-468) and noncancerous human breast epithelial cells (MCF10A). BAP1 was significantly downregulated at the mRNA (Fig. 2A) and protein (Fig. 2B) levels in metastatic adenocarcinoma of the breast cell line MDA-MB-231, whereas another metastatic adenocarcinoma cell line MDA-MB-468 expressed even less BAP1 compared with nonmetastatic MCF7 or MCF10A cells. The subcellular distribution of endogenous BAP1 by cellular fractionation and confocal microscopy showed both cytoplasmic and nuclear localization of BAP1 in MCF10A cells (Supplementary Fig. S3). To investigate whether BAP1 expression is altered in breast cancer, we studied the association of BAP1 levels with breast cancer patient outcomes. Kaplan–Meier survival analysis using a microarray dataset from 2,978 patients with breast cancer revealed that high BAP1 expression was significantly associated with better overall survival \( (P = 3.4 \times 10^{-7}) \) (Fig. 2C).

Furthermore, high BAP1 expression in tissue samples from basal and luminal subtype tumors were significantly associated with prolonged progression-free survival (Fig. 2D–F). Together, *in vitro* data and patient tumor data suggest that BAP1 expression is inversely correlated with tumor cell aggressiveness.

Next, we hypothesized that loss of BAP1 in MDA-MB-468 leads to an increase in γ-tubulin ubiquitination. Notably,
Figure 3. Downregulation of BAP1 in MDA-MB-468 cells leads to increased γ-tubulin ubiquitination. A, the levels of γ-tubulin in total cell lysates of MCF10A, MCF7, MDA-MB-231, and MDA-MB-468 cells were determined by Western blotting using anti-γ-tubulin and -actin antibodies. The arrowhead points to a shift in size of γ-tubulin in cell lysates from MDA-MB-468 cells. B, immunoprecipitation of γ-tubulin in total cell lysates from MDA-MB-468 cells and a Western blot using antibodies against γ-tubulin and ubiquitin. The arrowhead points to a shift in size of γ-tubulin corresponding to ubiquitinated γ-tubulin. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. C, immunoprecipitation of ubiquitin in total cell lysates from MDA-MB-468 cells and a Western blot using antibodies against γ-tubulin and actin. The arrowhead points to a shift in size of γ-tubulin corresponding to ubiquitinated γ-tubulin. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. D, immunoprecipitation of γ-tubulin in total cell lysates from MDA-MB-468 cells under denaturing conditions (8 mol/L urea) and a Western blot using antibodies against γ-tubulin and ubiquitin. The arrowhead points to a shift in size of γ-tubulin corresponding to ubiquitinated γ-tubulin. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation.

Although total levels of γ-tubulin were similar in the different cell lines, a slow migrating band above the γ-tubulin band could be observed only in the MDA-MB-468 cell line (Fig. 3A). Endogenous immunoprecipitation by using antibodies against γ-tubulin (Fig. 3B) or ubiquitin (Fig. 3C) showed a physical association of ubiquitin with γ-tubulin. To confirm the posttranslational modification of γ-tubulin, we used urea-treated denatured cell extracts for γ-tubulin immunoprecipitation and detected ubiquitin by immunoblotting (Fig. 3D).

Interaction between BAP1 and γ-tubulin occurs during metaphase, anaphase, and telophase

To further investigate the specific cell-cycle stage where the interaction between BAP1 or UCHL1 and γ-tubulin occurs, cells were synchronized in G1, S, and G2–M cell-cycle phase. Endogenous immunoprecipitation of γ-tubulin in MCF10A cells precipitated BAP1 during mitosis and UCHL1 during G1 cell-cycle phase (Supplementary Fig. S4A). In addition, we found that BAP1 at the M-phase and UCHL1 at the G1 phase of cell cycle reduce the levels of ubiquitin-associated tubulin compared with S-phase (Supplementary Fig. S4A) in MCF10A cells. The interaction between BAP1 and γ-tubulin at the M phase was also confirmed in two other breast cancer cell lines, including CAMA and T47D (Supplementary Fig. S5A-B). As expected, downregulation of BAP1 in these cell lines elevated the levels of γ-tubulin ubiquitination (Supplementary Fig. S5C). This suggests that BAP1 and UCHL1 both act as DUBs for γ-tubulin but in different phases of the cell cycle. Furthermore, interphase- and mitosis-synchronized cells confirmed the interaction between BAP1 and γ-tubulin at the M-phase (Fig. 4A). As the levels of BAP1 are low in MDA-MB-468 cells (Fig. 2A and 2B), we virally infected these cells with BAP1 (Supplementary Fig. S6). Consistent with the MCF10A data, immunoprecipitation of overexpressed BAP1 in MDA-MB-468 cells precipitated γ-tubulin during the process of mitosis (Fig. 4B) and in vitro pull-down experiments using γ-tubulin and BAP1 recombinant proteins showed a direct interaction between these two proteins (Fig. 4C). Furthermore, immunofluorescence staining and confocal microscopy experiments showed colocalization of endogenous γ-tubulin and BAP1 during metaphase, anaphase, and telophase but not during prophase in MCF10A cells (Fig. 4D). Overexpression of BAP1 in MDA-MB-468 cells also resulted in a colocalization between exogenous BAP1 and γ-tubulin during metaphase, anaphase, and telophase (Fig. 4E). Consistent with the confocal microscopy results, endogenous BAP1 (Fig. 4F) or overexpressed BAP1 (Fig. 4G) was efficiently coimmunoprecipitated with γ-tubulin during metaphase (M1), anaphase (M2), and telophase (M3) but not during prophase (M0) in cell extracts prepared from different stages of mitosis. Consistent with these results, γ-tubulin ubiquitination was only detected during prophase (M0) and not during metaphase (M1), anaphase (M2), and telophase (M3) of cell cycle (Fig. 4H). As expected, we could not find any interaction between UCHL1 and γ-tubulin in MCF10A cell extracts prepared from different stages of mitosis (Supplementary Fig. S5B). These results together suggest that a direct interaction between BAP1 and γ-tubulin as well as deubiquitination of γ-tubulin occurs during metaphase, anaphase, and telophase.

γ-Tubulin is a substrate for the deubiquitinating enzyme BAP1

To analyze γ-tubulin ubiquitination, we compared the overall levels of ubiquitin-associated tubulin in BAP1- or empty expression plasmid-infected MDA-MB-468 cells. As control cells showed ubiquitination of γ-tubulin, BAP1-overexpressing cells significantly reduced this effect (Fig. 5A). Furthermore, we enriched and immunoprecipitated endogenous γ-tubulin from MDA-MB-468 cells and incubated that with recombinant BAP1.
protein. The reaction was carried out for the different time points in the presence or absence of DUB inhibitor N-ethylmaleimide (NEM). This resulted in an increase of γ-tubulin–ubiquitin conjugates, as measured by ubiquitin immunoblotting (Fig. 5B). Next, we constructed a catalytic inactive mutant of BAP1 by mutation of cysteine a.a. 91 to alanine in the UCH domain of BAP1 (BAP1-C91A) and incubated purified wild-type (BAP1-Wt) or BAP1-C91A with γ-tubulin isolated from MDA-MB-468 cells. Wild-type, but not the catalytic inactive mutant of BAP1-C91A, resulted in significantly decreased γ-tubulin ubiquitination in vitro (Fig. 5C). These data together suggest that BAP1 is a specific DUB for γ-tubulin.

**BAP1 is necessary for proper mitotic spindle organization and chromosome abnormalities**

As an interaction was observed between BAP1 and γ-tubulin during mitosis (see Fig. 4), we next investigated whether BAP1 expression can affect proper mitosis. The number of cells during mitosis (see Fig. 4), we next investigated whether BAP1 expression can affect proper mitosis. The number of cells harboring mitotic abnormalities observed in control MDA-MB-468 cells (Fig. 6A–C) was reduced when the cells were synchronized MCF10A cells and immunoblotting using anti-BAP1 and γ-tubulin antibodies. MCF10A cells were synchronized by double thymidine block release and collected in mitosis by mitotic shake-off. Both asynchronous and synchronous cell extracts were used for immunoprecipitation. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. B, immunoprecipitation of HA-tagged BAP1 from synchronized MDA-MB-468 cells stably transduced with viral vectors encoding BAP1 and γ-tubulin antibodies. BAP1-MDA-MB-468 cells were synchronized by double thymidine block release and collected in mitosis by mitotic shake-off. Both asynchronous and synchronous cell extracts were used for immunoprecipitation. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. C, purified GST or GST–γ-tubulin (0.5 μg) was incubated in vitro with His-BAP1 (0.5 μg) for 30 minutes, followed by a GST pull-down assay using glutathione-agarose beads. The complexes were recovered from the beads and analyzed by means of immunoblotting against BAP1 for detection of His-BAP1 and GST for detection of GST–γ-tubulin. The arrowhead indicates the band corresponding to γ-tubulin. D, confocal plane of MCF10A cells stained for γ-tubulin (green), BAP1 (red), and DAPI (blue) as indicated. DAPI staining shows different stages of mitosis including prophase, metaphase, anaphase, and telophase. Bars, 10 μm.

E, confocal plane of MDA-MB-468 cells stably transduced with viral vectors encoding HA-tagged BAP1 (BAP1-MDA-MB-468) and stained for γ-tubulin (green), HA (red), and DAPI (blue) as indicated. DAPI staining shows different stages of mitosis including prophase, metaphase, anaphase, and telophase. Bars, 10 μm.

F, MCF10A cells were synchronized by double thymidine block. Cells in mitotic phase were obtained by nocodazole treatment (M0) and released from nocodazole after 45 minutes (M1), 90 minutes (M2), and 180 minutes (M3). Cell extracts were immunoprecipitated with BAP1 antibody and analyzed by Western blotting using antibodies against γ-tubulin, ubiquitin, and actin. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. G, MDA-MB-468 cells stably transduced with viral vectors encoding HA-tagged BAP1 (BAP1-MDA-MB-468) were synchronized by a double thymidine block. Cells in mitotic phase were obtained by nocodazole treatment (M0) and released from nocodazole after 45 minutes (M1), 90 minutes (M2), and 180 minutes (M3). Cell extracts were immunoprecipitated with BAP1 antibody and analyzed by Western blotting using antibodies against γ-tubulin, actin, and BAP1. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. H, MDA-MB-468 cells stably transduced with viral vectors encoding HA-tagged BAP1 (BAP1-MDA-MB-468) were synchronized by a double thymidine block. Cells in mitotic phase were obtained by nocodazole treatment (M0) and released from nocodazole after 45 minutes (M1), 90 minutes (M2), and 180 minutes (M3). Cell extracts were immunoprecipitated with γ-tubulin antibody and analyzed by Western blotting using antibodies against γ-tubulin, ubiquitin, and actin. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation.
stably expressing BAP1 (Fig. 6D). These abnormalities include chromosomes lagging during metaphase, uncondensed mitotic chromosomes, anaphase bridges, telophase bridges, and tripolar mitotic spindles, which are signs of chromosome aberrations (Fig. 6A–6D). Figure 6E and F summarizes the percentage of these abnormalities comparing BAP1- with empty expression plasmid-infected cells. Importantly, BAP1-C91A–expressing MDA-MB-468 cells did not show any differences in the percentage of different mitotic abnormalities compared with empty expression plasmid-transfected cells (Fig. 6E–G). The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. BAP1 is a specific DUB for γ-tubulin. A, immunoprecipitation of γ-tubulin in total cell lysates from MDA-MB-468 cells transduced with viral vectors encoding HA-tagged BAP1 (BAP1-MDA-MB-468, right lane) or the control (empty expression plasmid, mid lane) and a Western blot using antibodies against γ-tubulin. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation. B, γ-tubulin isolated from MDA-MB-468 cells was incubated with His-BAP1 for the indicated time in the presence or absence of NEM. The samples were analyzed by Western blotting using anti-γ-tubulin and -ubiquitin antibodies. C, BAP1 or a catalytically inactive mutant of BAP1 were purified from MDA-MB-468 cells transiently transfected with wild-type Flag-tagged BAP1 (Flag-BAP1) or Flag-BAP1-C91A and incubated with endogenous γ-tubulin immunoprecipitated from MDA-MB-468 cells. The reaction mixture was incubated at 37°C for 1 hour and examined by Western blotting using anti-γ-tubulin antibody. Densitometric analysis shows that 34% (comparing lane 2 and 3) and 36% (comparing lane 2 and 4) of total ubiquitin (lane 1) is modified via ubiquitination. The lysate (bottom) shows an equal amount of protein used for immunoprecipitation.

The mitotic defects observed in MDA-MB-468 cells with low levels of BAP1 might be indicative of a premature exit from mitosis. This was tested by performing live-cell microscopy using control and BAP1 stably expressing MDA-MB-468 cells, which were transfected with the histone marker GFP-H2B. Control (Fig. 7C; Supplementary Video S1), but not BAP1-expressing cells, showed defects in chromosome condensation and lagging of chromosomes that never correctly progressed to the metaphase plate (Fig. 7D, top; Supplementary Video S2), incorrect chromosome segregation resulting in the formation of anaphase bridges (Fig. 7D, middle; Supplementary Video S3), and finally skipped metaphase and subsequent aberrant anaphase (Fig. 7D, bottom; Supplementary Video S4). These results together suggest that low expression of BAP1 in breast cancer cells contributes to chromosome instability.
Discussion

γ-Tubulin is a member of the tubulin superfamily and is required for nucleating the polymerization of microtubules. γ-Tubulin is mainly located at the centrosome, and the amount of γ-tubulin at the centrosome increases dramatically at the beginning of mitosis and decreases at the end of mitosis (12). Posttranslational modification of γ-tubulin by ubiquitination is mediated via the BRCA1/BARD1 complex, which was shown to regulate the centrosome number and aster formation (15). During mitosis, BRCA1 localizes to centrosomes (17, 18) and the BRCA1/BARD1 complex localizes to centrosomes throughout the cell cycle, however, at very low levels during mitosis (19). Furthermore, disruption of the BRCA1 gene in mice led to centrosome amplification and aneuploidy (20). Loss of BRCA1 activity, as occurs in breast cancer cells, results in unrestrained centrosomes. This leads to hyperactive and overduplicated centrosomes often observed in breast cancer cells (15).

In the current study, we aimed to identify the enzyme(s) responsible for reversing ubiquitination of γ-tubulin in breast epithelial cells. We screened a siRNA library for DUBs and identified BAP1 and UCHL1. Genetic studies have uncovered that BAP1 mutations are related to different tumor types,
including malignant mesothelioma (21, 22), melanocytic tumor, uveal and cutaneous melanoma (7, 23), as well as lung cancer (24, 25) and renal carcinoma (26). Restoration of BAP1 inhibits cell growth of non–small lung cell carcinoma cell line NCI-H226 that lacks BAP1 by inducing cell death (6). In MCF7 breast cancer cell lines, it was shown that the binding of BAP1 to BRCA1 enhances BRCA1-mediated growth suppression, whereas other studies demonstrated that growth suppression of cells is independent of BRCA1; instead, BAP1 disrupts the BRCA1/BARD1 heterodimer but cannot reverse its autoubiquitination (5, 6, 9, 10). Therefore, the exact role of BAP1 in breast cancer is unknown.

To investigate whether BAP1 expression in breast cancer has a prognostic implication, we performed Kaplan–Meier survival analysis using a microarray dataset from 2,978 patients with breast cancer. High BAP1 expression was associated with better overall survival, and more specifically, high BAP1 expression in tissue samples from basal and luminal subtype tumors was significantly associated with prolonged progression-free survival. The regulatory mechanism that mediates the downregulation of BAP1 in metastatic breast cancer cells needs to be assessed in future studies. In MDA-MB-468 cells, we observed γ-tubulin ubiquitination in nonsynchronized cells, whereas overexpression of BAP1 in these cells significantly reduced the levels of ubiquitin conjugates to γ-tubulin. In addition, we found that the association between BAP1 and γ-tubulin occurs during M-phase, including metaphase, anaphase, and telophase, but not during prophase of the cell cycle. The expression of BAP1 but not the catalytic inactive mutant of BAP1– or empty expression plasmid-infected cells reduced the percentage of mitotic abnormalities and showed normal bipolar microtubule organization.

The MT nucleation rate usually increases at the entry into mitosis. The centrosomal concentration of γ-tubulin reached at metaphase and centrosome size increase through anaphase, whereas nucleation remains high through telophase, implying the presence of additional regulatory processes (27). Furthermore, during mitosis there is a sudden increase of the γ-tubulin content in the centrosome (28), and mitotic centrosomes exhibit enhanced MT nucleation capacity (29). Therefore, the γ-tubulin content in the centrosome regulates MT nucleation function (14). Previous studies found that ubiquitination of γ-tubulin by BRCA1/BARD1 during the S and G2 phases (13, 14) has a significant role in regulating MT nucleation, which further prevents aberrant reduplication of the centrosomes (15). This could imply that γ-tubulin ubiquitination directly inhibits centrosome microtubule nucleation function. In addition, during early mitosis, it has been shown that Aurora A kinase (AURKA) localizes to the centrosome and mediates microtubule nucleation activity through phosphorylation and inactivation of BRCA1 (14).

Deubiquitination enzymes and E3 ligases are often found in a complex together. These interactions allowing the E3 ligase to regulate the target and its deubiquitination enzyme simultaneously or deubiquitination enzyme/E3 ligase interactions confer target specificity, which is strictly dependent on the E3 ligase for recognition of the substrate (4). The current study
suggests that the complex interaction between BAP1, BRCA1, and γ-tubulin is essential for deubiquitination of γ-tubulin to allow proper mitotic progression. In summary, our present findings together with previous observations suggest that in the M-phase when BRCA1 becomes inactive through AURKA phosphorylation, BAP1 colocalizes with γ-tubulin and removes ubiquitin from γ-tubulin. Removal of ubiquitin from γ-tubulin by BAP1 might induce recruitment of unmodified γ-tubulin to the centrosome. Deubiquitination of γ-tubulin by BAP1 can further control the metaphase to anaphase transition and mitotic spindle organization as well as preventing genomic instability by inhibiting aberrations in mitotic spindles. Our findings may have important implications for tumor progression, as chromosomal instability and aneuploidy are associated with spindle defects in several tumor types, including breast carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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