Nucleophosmin/B23 Is a Candidate Substrate for the BRCA1-BARD1 Ubiquitin Ligase*§

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Accelerated Publication

The breast and ovarian tumor suppressor BRCA1 forms a heterodimeric RING-type ubiquitin ligase with BARD1 to catalyze untraditional Lys-6-linked polyubiquitin chains. It is not clear how the BRCA1-BARD1 ligase regulates various cellular processes such as DNA repair, cell-cycle progression, transcriptional regulation, and centrosome duplication. Here we report that BRCA1-BARD1 catalyzes the polyubiquitination of nucleolar phosphoprotein nucleophosmin/B23 (NPM). Two different mass spectrometry screens for protein ubiquitinated by BRCA1-BARD1 both identified NPM. NPM interacts with N-terminal fragments of BRCA1 and BARD1 in a manner dependent upon BRCA1-BARD1 heterodimer formation. NPM colocalizes with BRCA1 and BARD1 in mitotic cells suggesting the possibility of NPM regulation by BRCA1-BARD1 during mitosis. BRCA1-BARD1 catalyzes the ubiquitination of NPM in vitro and in vivo, and BRCA1-BARD1 co-expression in cells causes NPM stabilization rather than degradation. This is consistent with the notion that this ligase catalyzes untraditional polyubiquitin chains. Given the many overlapped functions between NPM and BRCA1, we propose that NPM is a strong candidate as a substrate of the BRCA1-BARD1 ubiquitin ligase.

The breast and ovarian cancer susceptibility gene product BRCA1 has been implicated in a variety of biological processes (1). However, to date, the only known biochemical function attributed to BRCA1 is E3 ubiquitin (Ub)† ligase activity (2–4). A Ub ligase (E3) catalyzes the formation of poly-Ub chains onto substrate proteins via isopeptide bonds utilizing Ubs that have been sequentially activated by enzymes E1 and E2 (5). The most common poly-Ub chain is linked through Lys-48 of Ub and serves as a signal for rapid degradation of substrates by the proteasome-dependent proteolysis pathway (5).

BRCA1 contains an N-terminal RING finger domain, a common motif found in Ub ligases. It acquires significant Ub ligase activity when bound to another conformationally similar RING finger protein, BARD1, as a RING heterodimer (2, 4, 6). Deleterious missense mutations in the RING finger domain of BRCA1 found in familial breast cancer abolish the Ub ligase activity of BRCA1-BARD1 (2, 3, 6). This suggests that the Ub ligase activity may well be important for the role of BRCA1 as a tumor suppressor. However, the manner in which the activity contributes to the biological function of BRCA1 remains to be determined.

We and others (7, 8) recently showed that BRCA1-BARD1 catalyzes Lys-6-linked poly-Ub chains. Unlike Lys-48-linked poly-Ub chains, the 26 S proteasome deubiquitinates Lys-6 chains without degradation in vitro (8). These observations suggest that ubiquitination mediated by BRCA1-BARD1 could signal a process other than degradation. Another key issue to connect the Ub ligase activity to the biological function of BRCA1 is the identification of its specific substrate(s). Because BRCA1 interacts with a number of proteins and functions in a variety of cellular processes, many candidate substrates for the BRCA1-BARD1 Ub ligase have been suggested including histone H2A or H2AX, the large subunit of RNA polymerase II and FANC D2 (1, 4, 9). However, the in vivo evidence for those substrates remains to be determined. In this paper we demonstrate nucleophosmin/B23 (NPM) as a novel candidate substrate for the BRCA1-BARD1 Ub ligase that connects the ligase activity with the known biological functions of BRCA1.

MATERIALS AND METHODS

Antibodies, Expression Constructs, and Purified Proteins—Mouse monoclonal antibodies to HA (12C5, Roche Applied Science), Myc (9E10, BabCo), FLAG (M2, Sigma), α- and β-tubulin (DM1A+BM1B, Neomarkers), and NPM (Sigma or Zymed Laboratories Inc.) as well as rabbit polyclonal antibody to BRCA1 (Santa Cruz Biotechnology catalog number sc-642) were purchased commercially. Rabbit polyclonal antibody to BARD1 that was used for immunoblotting was a gift from Dr. Richard Baer (Columbia University). For immunofluorescence and immunoprecipitation rabbit polyclonal antibody made against the C terminus of BARD1 was generated to the synthesized peptide CVMS-FELLPLDS and affinity purified before use.

cDNA for full-length human NPM (B23.1) was amplified by polymerase chain reaction from a HeLa cell cDNA library using Pfu polymerase (Stratagene) and subcloned into pcDNA3 or pET vector in frame with appropriate N-terminal tag. Mammalian expression plasmids for BRCA1, BARD1, and quadruple repeated HA-Ub were described previously (2, 8).

Rabbit E1 (Affiniti Research Products), bovine Ub (Sigma), and FLAG-Ub (Sigma) were purchased commercially. His-UbcH5c, His-BRCA1-304, and His-BARD114-159 were described previously (2). Full-length His-FLAG-NPM was obtained by two-step purification using nickel-agarose beads followed by anti-FLAG-cross-linked agarose beads.

Cell Culture, Transfection, and Immunological Techniques—Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic agent (Invitrogen). Cells were transfected using the standard calcium phosphate glutamin; NPM, nucleolar phosphoprotein nucleophosmin/B23; LC/MS/MS, liquid chromatography-tandem mass spectrometry.

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The supernatant from 10 reactions (30 μg) of BARD1 (3 indicated antibodies. Immunoprecipitated with anti-BARD1 antibodies with (●) or without (○) competing antigen peptide and analyzed by immunoblotting using indicated antibodies. IP, immunoprecipitates; IB, immunoblotting.

precipitation method as previously described (10). Total plasmid DNA was adjusted to be equal by adding the parental pcDNA3 vector.

Immunoprecipitation and immunoblotting methods, including the detection of in vitro ubiquitinated substrates, as well as in vitro Ub ligation assay were described previously (2, 8).

Screening Using Mass Spectrometry—The Ub ligase reaction was performed using FLAG-Ub, E1, His-UbcH5c, and anti-Myc immunocomplexes immobilized on protein A-agarose beads precipitated from 293T cells expressing Myc-BRCA11–772 and BARD1 as described (8). The supernatant from 10 reactions (30 μl each) was collected and incubated with a 30-μl volume of anti-FLAG antibody cross-linked beads (Sigma). The proteins conjugated with FLAG-Ub were eluted off the beads in 30 μl of 25 mM ammonium bicarbonate containing 0.1 mg/ml of FLAG peptide and digested with 7.4 μg/ml trypsin for 20 h at 30°C.

Alternatively, proteins interacting with the complex of HA-BARD11–408 and either wild-type or mutant (I26A) FLAG-BRCA11–222 were immunoprecipitated and eluted with FLAG peptide, resolved by SDS-PAGE, and stained with Sypro Ruby (Molecular Probe). The bands of interest were excised from the gel and digested with trypsin using the In Gel Digest Kit (Millipore) according to the manufacturer’s instructions.

The peptide fragments were subjected to liquid chromatography-tandem mass spectrometric (LC/MS/MS) analysis as described (8). The acquired collision-induced dissociation spectra were analyzed by Mascot software.

Indirect Immunocytochemistry—Proliferating cells were fixed with 3% formalin for 15 min and permeabilized with 0.2% Triton X-100 for 5 min. Cells were washed with phosphate-buffered saline, blocked with 0.5% bovine serum albumin in phosphate-buffered saline, and stained with indicated antibodies. For detection of BRCA1 and NPM colocalization at spindle poles, cells were fixed with cold methanol and permeabilized with 0.1% Triton X-100 buffer as described elsewhere (11). Primary antibodies were diluted in the blocking buffer at the following concentrations: anti-NPM (0.5 μg/ml), anti-αIβ-tubulin (1 μg/ml), anti-BARD1 (3 μg/ml), and anti-BRCA1 (2 μg/ml). Fluorescein isothiocyanate or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:50 dilution. The nucleus was counterstained with 0.5 μg/ml TO-PRO-3 (Molecular Probes), cells were then mounted with fluorescent mounting medium (Bio-Rad) and examined with a confocal laser scan microscope (LSM 510, Carl Zeiss).

RESULTS

Identification of NPM in BRCA1-BARD1 Immunocomplexes—To determine the biological significance of Ub ligase activity of BRCA1-BARD1, we searched for candidate substrates using two different approaches. First, hypothesizing that Ub ligase immunocomplexes may contain substrates, we performed in vitro polyubiquitination of BRCA1-BARD1 immunocomplexes and analyzed the polyubiquitinated products by nanoscale capillary LC/MS/MS analysis (Fig. 1A). Of the proteins identified, NPM exhibited the highest possibility with 17 peptides displaying a probability based Mowse score of 126. Second, presuming that substrate proteins would associate with a Ub ligase transiently and that this association could be stabilized by the loss of ligase activity, we compared the proteins associated with wild-type BRCA1-BARD1 ligase and catalytically inactive BRCA1 (I26A)-BARD1 ligase. The I26A mutation in BRCA1 retains the BARD1 interaction site, but it disrupts the interaction with UbcH5c resulting in a catalytically dead BRCA1-BARD1 heterodimer enzyme (6). A protein of ~38–40 kDa was present in the mutant ligase complex at a higher level and was subjected to LC/MS/MS (Fig. 1B). Four peptides were obtained from the band, and all matched with NPM. Thus, two different screening approaches identified the same protein, NPM.

The interaction between BRCA1-BARD1 and NPM in vivo was confirmed by transient transfection followed by immunoprecipitation (IP)-Western analysis (Fig. 1C). 293T cells were cotransfected with Myc-BRCA11–772, HA-BARD1, and FLAG-NPM. In vivo association of NPM with the BRCA1-BARD1 complex was demonstrated by reciprocal immunoprecipitation-Western analysis (lane 4). Omitting either BRCA1 or BARD1 from the transfection abolished the interaction with NPM (lanes 1 and 2). This suggests that the NPM interaction is dependent on BRCA1-BARD1 heterodimer formation. N-terminal fragments of BRCA11–222 and BARD11–320 were also capable of interacting with NPM (Fig. 1B and data not shown). To obtain evidence for endogenous BRCA1-BARD1-NPM association under physiological conditions, 293T cell lysate was immunoprecipitated with antibodies to BARD1, and precipitates were analyzed by Western blotting (Fig. 1D). BARD1 (lane 1, upper panel) and NPM (lane 1, lower panel) were readily detected in the BRCA1 immunocomplexes and were specifically competed by BARD1 antigen peptide (lane 2). Thus, NPM appears to physically interact with and may represent a substrate of the BRCA1-BARD1 ligase complex.

Colocalization of NPM with BRCA1-BARD1 during Mitosis—To further verify the in vivo interaction of NPM with
BRCA1-BARD1, the subcellular localization of these proteins was examined (Fig. 2). With formalin fixation it was observed that NPM predominantly localized to nucleoli in interphase cells as reported previously, while BARD1 was absent (Fig. 2A, three cells at the left of each panel). However, in mitotic cells, NPM colocalized with BARD1 most intensely over the chromosomal surfaces and the perichromosomal cytoplasm (Fig. 2A, a cell at the right of each panel). It has been individually reported that both BRCA1 (11) and NPM (12) localize to the spindle poles when using methanol fixation. Therefore, we next examined the colocalization of BRCA1 and NPM under these reported conditions (11). As expected, BRCA1 localized to the spindle poles during mitosis (Fig. 2B) and colocalized with NPM (Fig. 2C). This suggests a cell cycle-dependent BRCA1-BARD1-NPM interaction and potential regulation of NPM by BRCA1-BARD1 during mitosis.

**NPM Ubiquitination by BRCA1-BARD1**—To determine whether NPM is a substrate of the BRCA1-BARD1 Ub ligase, FLAG-NPM was co-expressed in 293T cells with HA-tagged Ub, Myc-BRCA1<sup>1-772</sup>, and BARD1 (Fig. 3A). Cells were collected 36 h after transfection and boiled in 1% SDS containing buffers to dissociate non-covalently bound proteins, and FLAG-NPM was immunoprecipitated. Immunoblotting of NPM precipitates using anti-HA antibody demonstrated a ladder characteristic of polyubiquitinated NPM (lane 3, upper panel). Immunoblotting of the same sample with anti-FLAG antibody also exhibited a band migrating slower than FLAG-NPM (lane 3, lower panel). Omission of FLAG-NPM, HA-Ub, Myc-BRCA1<sup>1-772</sup>, or BARD1 all abolished the NPM ladders supporting the idea of BRCA1-BARD1-dependent NPM ubiquitination. In a parallel *in vivo* ligase assay, another RING type E3 ligase, MDM2, promoted efficient ubiquitination of its known substrate, tumor suppressor p53, but exhibited no detectable activity toward NPM (Fig. 3B, lanes 3 and 6). Conversely, BRCA1-BARD1 did not cause noticeable p53 ubiquitination (lanes 2). To eliminate the possibility that NPM ubiquitination was caused by misfolding due to ectopic overexpression, we examined whether endogenous NPM is also ubiquitinated by BRCA1-BARD1 (Fig. 3C). HA-tagged Ub was co-expressed in 293T cells with Myc-BRCA1<sup>1-772</sup> and BARD1. Endogenous NPM was immunoprecipitated, and NPM ubiquitination was analyzed by immunoblot using anti-HA antibody. Polyubiquitinated NPM was readily detected when wild-type ligase was added (lane 4), whereas it was abolished when the I26A mutant was substituted in BRCA1-BARD1 (Fig. 3C). HA-tagged Ub was co-expressed in 293T cells with Myc-BRCA1<sup>1-772</sup> and BARD1. Endogenous NPM was immunoprecipitated, and NPM ubiquitination was analyzed by immunoblot using anti-HA antibody. Polyubiquitinated NPM was readily detected when wild-type ligase was added (lane 4), whereas it was abolished when the I26A mutation was substituted in BRCA1 (lane 5), demonstrating a dependence upon the Ub ligase activity of BRCA1-BARD1. It was possible that the observed *in vivo* NPM polyubiquitination could be an indirect effect of BRCA1-BARD1 through another endogenous E3 ligase. We therefore determined the type of poly-Ub chains incorporated into the NPM polyubiquitinated protein by using an HA-tagged Ub that has a single Lys.
Proteasomal Degradation

BRCA1-BARD1 catalyzes untransformed NPM ubiquitination. This demonstrates BRCA1-Ub Lys-6 or HA-Ub Lys-29, but not HA-Ub Lys-11, HA-Ub Lys-48, or HA-Ub Lys-63, was co-expressed (supplemental Fig. 1). This is consistent with our previous observation that BRCA1-BARD1 preferentially utilizes HA-Ub Lys-6 or HA-Ub Lys-29 for BRCA1 autoubiquitination (8). Hence, the results suggest that the in vivo NPM polyubiquitination observed is directly catalyzed by BRCA1-BARD1.

To further test whether BRCA1-BARD1 directly catalyzes NPM polyubiquitination, we performed *in vitro* NPM ubiquitination using a fully recombinant system (Fig. 3D). Recombinant His-FLAG-NPM purified from bacteria incubated with purified Ub, E1, E2/His-UbcH5c, His-BRCA113–304, and His-BARD114–180 resulted in two slowly migrating products detected by anti-FLAG immunoblot (lane 7). Omission of substrate NPM, Ub, E2/His-UbcH5c, BRCA1, or BARD1 all abolished NPM ubiquitination. This demonstrates BRCA1-BARD1-dependent NPM ubiquitination in a purified system.

**NPM Ubiquitination by BRCA1-BARD1 Is Not a Signal for Proteasomal Degradation**—BRCA1-BARD1 catalyzes untraditional Lys-6-linked poly-Ub chain formations (7, 8). The discovery of NPM as a substrate prompted us to test whether BRCA1-BARD1-mediated NPM ubiquitination targets NPM for degradation by measuring its stability *in vivo*. The steady state level of FLAG-NPM expressed in 293T cells was increased rather than decreased by co-expression of BRCA1-BARD1 in a dose-dependent manner (supplemental Fig. 2A). Pulse-chase analysis also supports that BRCA1-BARD1 stabilizes NPM (supplemental Fig. 2B). These findings suggest that BRCA1-BARD1-mediated NPM ubiquitination affects the function of NPM through a non-proteolytic mechanism.

**DISCUSSION**

We show in this study that the acidic nuclear phosphoprotein NPM is a substrate ubiquitinated by the BRCA1-BARD1 E3 ligase *in vitro* and *in vivo*. Although the implication between the functions of BRCA1 and NPM has not been previously proposed, most of NPM functions, interestingly, overlap with or implicate BRCA1. Located mainly in the nucleolus, its primary function is thought to be ribosomal biogenesis (13, 14). However, many other functions of NPM have been reported including up-regulation by genotoxic stress (15, 16) and a role as an anti-apoptosis inhibitor (17). Similar to the function of its related family member nucleoplasmin, NPM also possesses histone chaperone activity in chromatin remodeling (18). Ubiquitination of NPM by BRCA1-BARD1 may alter its histone chaperone activity and contribute to the chromatin remodeling function of BRCA1 or transcriptional regulation by BRCA1.

In addition to these functions, NPM also regulates centrosome duplication. NPM interacts with the centrosome and protects it from duplication in G1 phase (19). Dissociation of NPM from the centrosome allows its duplication process during S phase, while daughter centrioles develop into two mature centrosomes (spindle poles) (19). In mitosis, NPM reassociates with the centrosome (12). This reassociation is thought to be important to protect the cell from centrosome hyper-amplification that causes chromosome instability and aneuploidy. It is known that targeted disruption of the *Bracl* gene in mouse also resulted in centrosome hyper-amplification and genomics instability (20). As shown in this report, NPM colocalized with BRCA1 and BARD1 during mitosis (Fig. 2). BRCA1 expression has been reported to be cell cycle dependent, with the greatest expression occurring in G2 and M phases (Ref. 21 and data not shown). Because BRCA1 and BARD1 stabilize each other (2), it is likely that the Ub ligase activity of BRCA1-BARD1 is accelerated at these stages, and NPM is exposed to the boosted activity when the nucleolus is broken down during mitosis. Ubiquitination of NPM by BRCA1-BARD1 could be important for the redistribution of NPM to the spindle poles. The defect of the Ub ligase activity of BRCA1 may result in a reduced amount of NPM at the centrosome during G1 phase and, consequently, centrosome hyper-amplification.

The ubiquitination of NPM by BRCA1-BARD1 does not cause its degradation (supplemental Fig. 1). Instead, ubiquitination stabilized NPM protein. The significance of NPM stabilization, however, is not clear at present because NPM is an abundant protein. One notion is that BRCA1-BARD1 may allow long-term stabilization of NPM in a specific subcellular compartment. For example, NPM has characteristics typical of molecular chaperones including anti-aggregation activity and promoting the renaturation of denatured proteins (22). NPM may act as a chaperone to prevent aggregation of proteins in condensated environments, such as the nucleolus, the chromosome, or the centrosome. The ubiquitination of NPM by BRCA1 may affect such a function.

In another interesting twist, it recently was reported that tumor suppressor ARF also causes ubiquitination of NPM (14). However, the ubiquitination by ARF resulted in NPM degradation suggesting that a Ub ligase other than BRCA1-BARD1 mediates this pathway. One possibility is that Lys-6-linked ubiquitination by BRCA1-BARD1 may protect NPM from a Lys-48-linking ligase by competing for the site(s) of ubiquitination. Thus, it is possible that distinct ubiquitinations of NPM may affect such a function.

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