**Molecular and Cellular Pathobiology**

**Recruitment of Phosphorylated NPM1 to Sites of DNA Damage through RNF8-Dependent Ubiquitin Conjugates**

Ayaka Koike¹², Hiroyuki Nishikawa³, Wenwen Wu², Yukinori Okada¹, Ashok R. Venkitaraman⁴, and Tomohiko Ohta¹²

**Abstract**

Protein accumulation at DNA double-strand breaks (DSB) is essential for genome stability; however, the mechanisms governing these events are not fully understood. Here, we report a new role for the nucleophosmin protein NPM1 in these mechanisms. Thr199-phosphorylated NPM1 (pT199-NPM1) is recruited to nuclear DNA damage foci induced by ionizing radiation (IR). Foci formation is impaired by depletion of the E3 ubiquitin ligases RNF8 and RNF168 or the E2 Ubc13, and pT199-NPM1 binds to Lys63-linked ubiquitin polymers in vitro. Thus, phosphorylated NPM1 may interact with RNF8-dependent ubiquitin conjugates at sites of DNA damage. The interaction was found to rely on T199 phosphorylation, an acidic tract, and an adjacent ubiquitin-interacting motif–like domain. Depletion of the breast cancer suppressor BRCA1 or its partner, RAP80, enhanced IR-induced NPM1 foci and prolonged persistence of the foci, possibly implicating BRCA1 in pT199-NPM1 action and dynamics. Replacement of endogenous NPM1 with its nonphosphorylatable T199A mutant prolonged persistence of IR-induced RAD51 foci accompanied by unrepaired DNA damage. Collectively, our findings suggest that phosphorylated NPM1 is a novel component in DSB repair that is recruited by ubiquitin conjugates downstream of RNF8 and RNF168.

**Introduction**

DNA double-strand breaks (DSB), such as those induced by ionizing radiation (IR) or some anticancer agents, are among the most destructive and cytotoxic DNA lesions. Their repair in mammalian cells involves recruitment to DSBs of many different proteins that sense, signal, and repair these lesions (1). The nuclear structures that reflect assembly of effector proteins at or near the site of DSBs in homologous recombination (HR) repair pathway can be detected as IR-induced foci (IRIF). IRIF formation at the DSB sites is initiated by transient chromatin changes followed by ATM-dependent phosphorylation of the histone variant H2AX (γ-H2AX) and MDC1 (1–3). Phosphorylation of MDC1 recruits an E3 ubiquitin ligase, RNF8, which catalyzes lysine 63 (K63)-linked polyubiquitin chains at the DSB sites together with the E2 Ubc13 and RNF168 or the E2 Ubc13, and pT199-NPM1 binds to Lys63-linked ubiquitin polymers. Thus, phosphorylated NPM1 may interact with RNF8-dependent ubiquitin conjugates at sites of DNA damage. The interaction was found to rely on T199 phosphorylation, an acidic tract, and an adjacent ubiquitin-interacting motif–like domain. Depletion of the breast cancer suppressor BRCA1 or its partner, RAP80, enhanced IR-induced NPM1 foci and prolonged persistence of the foci, possibly implicating BRCA1 in pT199-NPM1 action and dynamics. Replacement of endogenous NPM1 with its nonphosphorylatable T199A mutant prolonged persistence of IR-induced RAD51 foci accompanied by unrepaired DNA damage. Collectively, our findings suggest that phosphorylated NPM1 is a novel component in DSB repair that is recruited by ubiquitin conjugates downstream of RNF8 and RNF168.

**Authors' Affiliations:** ¹Department of Translational Oncology; ²Division of Breast and Endocrine Surgery, Department of Surgery; ³Institute of Advanced Medical Science, St. Marianna University Graduate School of Medicine, Kawasaki, Japan; and ⁴The Medical Research Council Cancer Cell Unit, Hutchison/MRC Research Centre, Cambridge, United Kingdom

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**Corresponding Author:** Tomohiko Ohta, Department of Translational Oncology, St. Marianna University Graduate School of Medicine, Kawasaki 216-8511, Japan; Phone: 81-44-977-8111; Fax: 81-44-975-1400; E-mail: to@marianna-u.ac.jp.

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(22). In this study, we show for the first time that only a fraction of the total cellular pool of NPM1, phosphorylated on residue Thr199 (pT199-NPM1), is recruited to DSBs and plays a critical role in the RNF8-dependent DNA repair pathway.

Materials and Methods

Antibodies, plasmids, and purified proteins

Antibodies used are shown in Supplementary Table S1. Full-length human NPM1 cDNA (14) was subcloned into pcDNA4 vector or pGEX vector in frame with the COOH-terminal Myc tag or the NH₂-terminal glutathione S-transferase (GST) tag, respectively. Mutants were generated by site-directed mutagenesis and restriction enzyme digestion. To generate siRNA-insensitive NPM1-Myc constructs, the underlined silent mutations (CGAGGCTCACCAATTAAA) were introduced into the siRNA target sequence in the NPM1 coding region. For Venus-fusion proteins, BARD1 and NPM1 were cloned in frame with the COOH terminus of p3FLAG-CMV10-Venus-N (1–172) and to the NH₂ terminus of pcDNA3HA-Venus-C (173–238) [gifts from Drs. Mutsuhiro Takekawa (Department of Molecular Cell Signaling, Institute of Medical Sciences, The University of Tokyo, Minato-ku, Tokyo, Japan) and Atsushi Miyawaki (Laboratory for Cell Function and Dynamics, Advanced Technology Development Group, Brain Science Institute, RIKEN, Wako-city, Saitama, Japan)], respectively. GST-fused recombinant proteins were purified as described (23). Ubiquitin and SUMO polymers were purchased commercially (Boston Biochem).

Cell culture

Cells were cultured in DMEM as previously described (23–25). For IR, cells were exposed to X-ray irradiation (5 Gy) and cultured for the indicated time before analyses. To analyze the effect of proteasome inhibitors on IRIFs, 50 μmol/L MG132 (Calbiochem) or the vehicle DMSO was added to cells 90 minutes after IR, and cells were incubated for an additional 4.5 hours. For radiosensitivity, cells were irradiated and cultured in fresh medium for 4 days. Cell viabilities were analyzed in triplicate by using Cell Titer-Blue (Promega).

siRNA and transfection

siRNA oligonucleotides targeting NPM1 (M-015737-00), BRC1 (M-003461-01), RAP80 (M-006995-01), RNF168 (1: D-007152-02 and 2: D-007152-03), and nontargeting control (D-001206-14) were purchased (Dharmacon). For RNF8 and Ubc13 siRNA, oligonucleotides used in a previous report (5), which target the following sequences, were synthesized (sense strand): RNF8 (5′-AGAAUGAGCUCCAAUGAUUU-3′) and Ubc13 (5′-GCACAGUUCGCUAUUGAUUU-3′). RNA duplexes (final concentration 50 nmol/L) were transfected into the cells by using Lipofectamine (2000). For the NPM1 depletion and mutant add-back experiment, U2OS cells were first transfected with pcDNA4 plasmids encoding siRNA-insensitive wild-type or T199A mutant NPM1-Myc. Cells stably expressing the proteins were selected with 40 μg/mL zeocin and maintained in medium containing 20 μg/mL zeocin. Cells were then transfected twice with siRNA oligonucleotides targeting the NPM1 mRNA sequence 5′-CGAAGGGCAGUCCAUUAA-3′ or with nontargeting siRNA (Dharmacon, D-001210-05), and analyzed 48 hours after the second transfection. For Venus-IRIF studies HEK-293T cells were transfected by using the standard calcium phosphate precipitation method and irradiated (5 Gy) 12 hours after transfection.

Western blot analysis

Western blots were performed as described (25).

Immunofluorescence microscopy

Indirect immunofluorescence labeling was performed as described (14, 22), with the appropriate concentration of the antibodies (Supplementary Table S1).

GST pull-down

GST pull-down assay was performed as described (14, 23), with GST fusion protein (10 μg) mixed with K63-linked ubiquitin polymers (3 μg) containing two to seven molecules of ubiquitin (Ub2–7).

Surface plasmon resonance analysis

Analyses were performed using a BIAcore 3000 instrument (GE Healthcare). Purified GST-tagged NPM1 peptides or ubiquitin polymers were immobilized (700–1,000 resonance units) on the surface of each CM5 sensor chip using a GST Capture kit or an amine coupling kit, respectively (GE Healthcare). The analyte (either GST-NPM1 peptide, ubiquitin polymer, or SUMO polymer) was injected at different concentrations at a flow rate of 20 μL per minute in 20 mmol/L NaCl/20 mmol/L NaPO₄ (pH 5.0) containing 0.08% surfactant P20 (GE Healthcare). The absence of detectable nonspecific absorption was confirmed in this condition. An equivalent volume of each protein sample was injected over a surface with no protein immobilized or with GST protein on immobilized anti-GST antibody to serve as a blank sensorgram for subtraction of the bulk refractive index background. For NPM1 fragments that do not oligomerize, kinetic analyses were performed with a range of analyte concentrations (0, 100, 200, 400, and 800 nmol/L). Dissociation constants (Kd) were derived by using BIAevaluation version 3.1 software and a 1:1 Langmuir model.

Comet assay

Neutral comet assays were performed using Trevigen’s CometAssay kit (4250-050-K, Trevigen). Mean tail moment was analyzed by the TriTek CometScore Freeware program with 100 cells per sample.

Results

NPM1 phosphorylated on residue Thr199 forms IRIF

Although several lines of evidence support the notion that NPM1 could have a role in the DSB repair pathway, NPM1 has not been observed at sites of DNA damage. However, because NPM1 is an abundant protein, only a small fraction of the total cellular pool of NPM1 protein may participate in the...
pathway. Supporting this idea, we found that phosphorylated NPM1, pT199-NPM1, is a candidate for this specific function. HeLa and U2OS cells were irradiated. pT199-NPM1 foci became clear within 1 hour to several hours after IR treatment and colocalized with γ-H2AX foci, conjugated ubiquitin foci, and BRCA1 foci (Fig. 1A; Supplementary Fig. S1), supporting the idea that pT199A-NPM1 is recruited to DSB sites. pT199-NPM1-IRIF formation was detected in all cell lines tested, including HeLa, U2OS, MCF7, T47D, 293T, and MCF10A normal human mammary epithelial cells. Inhibition of NPM1 expression with siRNA significantly decreased the amount of foci recognized by anti–pT199-NPM1 immunostaining (Fig. 1B; Supplementary Fig. S1E), ensuring that the foci are indeed composed of phosphorylated NPM1. We also examined the kinetics of steady-state level of pT199-NPM1 after IR in each cellular fraction by immunoblot (Supplementary Fig. S2). pT199-NPM1 in the cytoplasm and the nuclear extract was dramatically reduced within 1 hour after IR. On the contrary, pT199-NPM1 in the chromatin fraction was increased 30 minutes after IR, supporting the idea that a part of pT199-NPM1 is incorporated at the DSB sites.

We previously found that NPM1 interacts with BRCA1-BARD1 (14). To examine whether NPM1 interacts with this complex at sites of DNA damage, we cotransfected plasmids encoding fusions of NPM1 to the COOH terminus of the fluorescent protein Venus (NPM1-VC) and BARD1 to the NH2 terminus of Venus (VN-BARD1). Successful assembly of the Venus protein as detected by fluorescence would indicate specific interaction of NPM1 with BARD1 (Fig. 1C). Whereas untreated cells only exhibited weak fluorescence in the nucleolus, nuclear fluorescent foci became apparent after IR and colocalized with BRCA1 foci or γ-H2AX foci (Fig. 1C and D). This suggests that NPM1 interacts with the BRCA1-BARD1 complex at sites of DNA damage. Fluorescent Venus-IRIF formation significantly decreased when VN-BARD1 was substituted with nonfused VN or when NPM1-VC was substituted with MEK-VC (Fig. 1D; Supplementary Fig. S3).

Inhibition of conjugated ubiquitin-IRIF abolishes pT199-NPM1-IRIF formation

The colocalization of pT199-NPM1-IRIF with repair proteins prompted us to test whether inhibition of RNF8- and RNF168-dependent ubiquitination at DSB sites affects the foci formation. We first used the 26S proteasome inhibitor MG132. Consistent with a previous report (4), inhibition of the 26S proteasome trapped conjugated ubiquitinated products in the cytosol and impaired ubiquitin-IRIF (Fig. 2A). Importantly, this was accompanied by the disappearance of pT199-NPM1-IRIF, whereas γ-H2AX-IRIF remained intact (Fig. 2A; Supplementary Fig. S4A). To further determine the role of RNF8-26S mediated ubiquitination in pT199-NPM1-IRIF formation, we inhibited the expression of RNF8 or Ubc13 with siRNA knockdown (Fig. 2B). Depletion of RNF8 and Ubc13 resulted in inhibition of BRCA1-IRIF, conjugated ubiquitin-IRIF, and pT199-NPM1-IRIF, whereas γ-H2AX-IRIF remained intact (Fig. 2C; Supplementary Fig. S4B). Depletion of RNF168 also inhibited pT199-NPM1-IRIF formation (Supplementary Fig. S4C–E). These results suggest that pT199-NPM1-IRIF formation is involved in the DSB-induced RNF8 cascade and that it is an event downstream of RNF8-Ubc13– and RNF168-Ubc13–induced ubiquitination.

BRCA1-IRIF are not required for pT199-NPM1-IRIF formation

To further clarify the step in the cascade at which pT199-NPM1 is recruited, we next inhibited BRCA1-IRIF by siRNA knockdown of either BRCA1 or RAP80. Individual siRNA treatment successfully inhibited expression of BRCA1 and RAP80 (Fig. 3A), resulting in depletion of BRCA1-IRIF formation in both cases (Fig. 3B). However, neither BRCA1 nor RAP80 depletion inhibited pT199-NPM1-IRIF formation (Fig. 3C). During these experiments, we noticed that pT199-NPM1-IRIF formation was enhanced by BRCA1 or RAP80 depletion. To further examine the effect, the kinetics of foci formation were monitored following IR treatment. Six hours after IR exposure, BRCA1- and RAP80-depleted cells exhibited higher numbers of pT199-NPM1-IRIF per cell than control cells (Fig. 3C). The number continued to increase until 12 hours post-IR, at which time foci in control cells were hardly detectable (Fig. 3C and D). These results indicate that pT199-NPM1 is recruited to the DSB sites independently of the RAP80-Abraxas-BRCA1 complex; however, the complex is required for normal turnover of NPM1 at the sites.

NPM1 interacts with K63-linked ubiquitin conjugates in vitro

We next tested the possibility that pT199-NPM1 binds directly to the ubiquitin conjugates in a manner similar to RAP80 (10). Notably, phosphomimetic T199D mutant of GST-NPM1, but not the wild-type or T199A, interacted with K63-linked polyubiquitin (Ub2−7), preferring polymers containing at least three ubiquitin molecules (Fig. 4A). Further, surface plasmon resonance (SPR) analyses showed that T199D-NPM1 directly interacted with K63-linked tetraubiquitin and its steady-state response was higher than wild-type (Fig. 4B). Importantly, substitution of K63-linked tetraubiquitin with K48-linked tetraubiquitin significantly decreased the response (Fig. 4B).

To determine the site responsible for interaction with the ubiquitin conjugates, we constructed several NPM1 fragments that contained T199D (Fig. 5A). Human NPM1 is composed of several domains, including an N terminus and core domain that is responsible for oligomer formation and three acidic tracts (A1, A2, and A3; Fig. 5B; refs. 15, 26, 27). Whereas fragments composed of residues 152 to 259 or 1 to 212 of NPM1-T199D interacted with the K63-linked ubiquitin polymer, the fragment composed of residues 189 to 259 that lacks the third acidic tract (A3; 161–188) did not (Fig. 5A). The results suggest an interaction between the ubiquitin conjugates and the region of NPM1 located between residues 152 and 212, which include the A3 domain and the T199 phosphorylation site (Fig. 5B). The results also indicate that the interaction does not require oligomerization of NPM1.

We noticed that there is a region relatively similar to a UIM motif (UIM-like; UIML) that is adjacent to or overlaps...
Figure 1. IRIF formation of NPM1. A, HeLa cells were untreated (−) or treated with 5 Gy IR, incubated for 6 h, and immunostained with anti–pT199-NPM1 and anti–conjugated-ubiquitin (FK2) antibodies. The nucleus was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). B, U2OS or HeLa cells transfected with control (siCTR) or NPM1-targeting (siNPM1) siRNA oligonucleotides were immunoblotted with NPM1 antibody (left panels) and analyzed for IRIF with anti–pT199-NPM1 and anti–γH2AX antibodies (right panels). C and D, NPM1 interacts with BARD1 at sites of DNA damage. C, the scheme illustrates the principle used to detect the interaction between NPM1-BARD1 and the Venus fluorescent protein. HEK-293T cells transfected with NPM1-VC (Venus-C) and VN (Venus-N)-BARD1 were left untreated (−) or irradiated (IR) and examined for Venus-IRIF and BRCA1 immunostaining. D, HEK-293T cells treated as in C or with nonfused VN (bottom) were examined for colocalization of Venus-IRIF with BRCA1- or γ-H2AX-IRIF.
with the A3 tract in the ubiquitin-binding region (Fig. 5B and D). We therefore investigated whether this region interacts with ubiquitin conjugates. GST-tagged fragments of T199D-NPM1, A3+UIML (152–212), or UIML (181–212) were immobilized onto a biosensor chip (Supplementary Fig. S5A), and K63-linked tetraubiquitin was injected as an analyte. Whereas the A3+UIML fragment interacted with tetraubiquitin, the UIML fragment did not (Fig. 5C), consistent with the pull-down assays that required the A3 tract for interaction.

Using the A3+UIML fragment of NPM1, which contains only a single CDK-phosphorylation site, T199 (Fig. 5B), we next tested if phosphorylation of T199, instead of phosphomimic mutant T199D, indeed contributes to the binding. Supporting the phosphorylation-dependent interaction, CDK2/cyclin A–phosphorylated pT199-A3+UIML (Supplementary Fig. S5B) bound to the ubiquitin polymer with significantly higher affinity than the unphosphorylated A3+UIML (Fig. 5C; Supplementary Fig. S5C). The dissociation constant of binding of K63-linked tetraubiquitin to pT199-A3+UIML (K_\text{D}; 0.35 \mu\text{mol/L}) was 4.4-fold greater than that to unphosphorylated A3+UIML (1.55 \mu\text{mol/L}). Importantly, substitution of the K63-linked tetraubiquitin with K48-linked tetraubiquitin or monoubiquitin abolished the binding to pT199-A3+UIML (Supplementary Fig. S5C), again supporting the linkage-specific interaction. SUMO polymers also did not interact with the NPM1 fragments (Supplementary Fig. S5D).

We next evaluated the contribution of each residue within the UIML to K63-linked ubiquitin polymer binding. Together with T199D, point mutations of residues in UIML were introduced in the GST-NPM1-A3+UIML fragment (Supplementary Fig. S5A), and dissociation constants (K_\text{D}) were determined (Fig. 5D; Supplementary Fig. S5E; Supplementary Table S2). Some mutations of the residue conserved in UIM motif, including e182-5A (1.57 \mu\text{mol/L}), A190G (1.35 \mu\text{mol/L}), and D198A (2.12 \mu\text{mol/L}), significantly decreased the affinity, whereas S195D (0.27 \mu\text{mol/L}), a mutation of another conserved residue, did not. A186G (0.31 \mu\text{mol/L}), which retains the hydrophobic status, did not affect the affinity. Some mutations of the residue that are not conserved in UIM motif, including E187A (1.03 \mu\text{mol/L}), E188A (1.13 \mu\text{mol/L}), P191A (1.25 \mu\text{mol/L}), and V192D (2.05 \mu\text{mol/L}), also decreased the affinity.

In vitro binding experiments indicate that the A3 tract, UIM domain, and phosphorylated T199 are all required for high-affinity interaction with K63-linked ubiquitin polymers, and that molecular mechanism for ubiquitin interaction of NPM1-UIML could be different from conventional UIM.

**Substitution of endogenous NPM1 with the T199A mutant causes failure to repair DNA damage**

To observe phenotypes in response to DNA damage specifically due to loss of pT199-NPM1, we expressed an siRNA-insensitive, wild-type, or nonphosphorylable T199A mutant of NPM1 in NPM1-depleted cells and studied IRIF formation. The desired, engineered expression of NPM1 was confirmed by Western blot (Fig. 6A, lanes 3 and 5). The wild-type cells...
exhibited pT199-NPM1-IRIF, whereas T199A cells did not (Fig. 6A; Supplementary Fig. S6), supporting the presumption that the exogenous wild-type NPM1 but not its T199A mutant can replace endogenous NPM1 at the sites of DNA damage. Using these cells, we tested whether inhibiting NPM1 T199 phosphorylation affects RAD51-IRIF, a marker for HR repair acting downstream of the RNF8-RAP80-BRCA1 cascade. The number of RAD51-IRIF in T199A was approximately the same as that in wild-type cells until 6 hours after IR exposure (Fig. 6B, top). However, a dramatic difference was observed 12 to 18 hours post-IR. Whereas the number of RAD51-IRIF in wild-type cells decreased to approximately half the level observed at 6 hours post-IR, the foci in T199A cells continued to increase until 18 hours post-IR (Fig. 6B, top). U2OS and HeLa cells simply transfected with NPM1-specific siRNA also exhibited kinetics similar to that of T199A cells (Fig. 6B, middle and bottom). Similar results were observed for BRCA1-IRIF (Supplementary Fig. S7). These results suggest that phosphorylated NPM1 is dispensable for recruiting BRCA1 and RAD51 to DSB sites but is required for the normal process of the repair after recruitment of these proteins.

The persistent retention of RAD51- and BRCA1-IRIF in T199A cells raised the question of whether DNA damage is left unrepaird. Consistent with this possibility, the number of γH2AX-IRIF was higher in T199A cells than that in wild-type cells (Supplementary Fig. S8A). We further performed neutral comet assay to directly measure DSBs (Fig. 6C; Supplementary Fig. S8B). Immediately after irradiation, approximately the same amount of DNA fragments were generated in wild-type and T199A cells, an observation that was noted until 6 hours post-IR. However, T199A cells exhibited an increased amount of DNA fragments at 12 and 24 hours post-IR, whereas wild-type cells repaired a majority of the damage by that time. These results suggest that retention of phosphorylated NPM1 to sites of DNA damage is required for DSB repair. The increased DNA damage may reflect newly generated DSBs due to continuous DNA replication, which can convert IR-induced single-strand breaks to DSBs. In

Figure 3. BRCA1 or RAP80 depletion leads to enhanced formation and prolonged persistence of pT199-NPM1-IRIF. A and B, HeLa cells transfected with control, BRCA1-specific, or RAP80-specific siRNA were immunoblotted with the indicated antibodies (A) and examined for IRIF with anti-BRCA1 and anti-γH2AX antibodies (B). C, cells were then examined for pT199-NPM1-IRIF. The time course of pT199-NPM1-IRIF formation per cell is shown at the indicated time after 5 Gy IR. Means of 100 cells with SD at each time point are shown. D, representative data of cells 12 h post-IR are shown with pT199-IRIF (red) and DAPI (blue) stains.
addition, the different amounts of DNA damage could be due to different proportions of S-phase cells between wild-type and T199A cells. However, cell cycle analyses showed approximately the same proportions of the S-phase fraction in wild-type and T199A cells before and after IR (Supplementary Fig. S9). On the other hand, T199A cells after IR showed a higher proportion of apoptotic cells, which may reflect a part of the increased DNA fragments observed in T199A cells.

The significant increase in unrepaired DNA damage observed in T199A cells prompted us to test whether it increases the sensitivity of cells to IR (Fig. 6D). The viability of T199A cells after IR was lower than that of wild-type or control cells at all IR doses examined, although the effect was relatively mild. On the other hand, NPM1 depletion did not dramatically affect radiosensitivity.

Discussion

In this study, we show that NPM1 is recruited to DSBs in a manner dependent on RNF8/RNF168-mediated ubiquitination. Unlike BRCA2 and RAD51, the recruitment of NPM1 is not through the BRCA1 complex. Instead, our data suggest that NPM1 is recruited through direct interaction with K63-linked ubiquitin conjugates. In addition to RAP80, several other proteins are also recruited through interaction with K63-linked ubiquitin conjugates at DSB sites. These include RNF168, which possesses a motif interacting with the ubiquitin domain (7, 8, 28), and RAD18, another E3 ligase that binds to ubiquitin through its zinc finger domain (29). Components of the RAP80-BRCA1 complex (called the BRCA1 A complex), including Abraxas, BRE, BRCC36, and NBA1, were also shown to directly interact with ubiquitin conjugates in vitro through MPN, Uev, MPN+, and VWA domains, respectively (30). Therefore, the complex formation of repair proteins at DSB sites depends on ubiquitin conjugates in multifaceted ways. Our results suggest that NPM1 is one such protein.

It is intriguing that NPM1 recruitment is through a newly identified UIML motif in cooperation with an NH2-terminal acidic tract (A3) and COOH-terminal T199 phosphorylation. The UIM motif is an 18- to 21-residue sequence composed of highly conserved alanine and serine residues flanked by conserved acidic patches (Fig. 5D; refs. 31–33). A single UIM is capable of binding to a single ubiquitin molecule, although the affinity is relatively low in general (34). In the case of RAP80, tandem UIMs greatly increase the affinity to K63-linked diubiquitin because each UIM interacts with each ubiquitin molecule (35, 36). However, the UIM motif found in NPM1 is significantly different from the classic UIM in that it possesses an additional two residues between the NH2-terminal acidic domain and the central alanine and serine residues. Indeed, neither UIML or A3+UIML interacted with single ubiquitins. This may indicate that this motif interacts with ubiquitin conjugates in a manner different from UIMs. In addition to the UIML domain, the acidic A3 tract may help to increase the affinity by interacting with another ubiquitin molecule in the K63-linked chain. Although the UIML domain possesses a COOH-terminal acidic residue (D198), our results indicate that phosphorylation of T199 is required for the full affinity of interaction with ubiquitin conjugates, probably by enhancing the acidity of the COOH-terminal patch of the motif. To our knowledge, this is the first example for a ubiquitin-binding module composed of phosphorylation.

The recruitment of NPM1 by the K63-linked ubiquitin conjugates, which also directly recruits the RAP80-Abraxas-BRCA1 complex (10), together with NPM1-BARD1 interaction at the sites of DNA damage, raises the possibility that both NPM1 and BRCA1-BARD1 are recruited together in the same ubiquitin-containing complex. This is interesting from the context that NPM1 interacts with BRCA1-BARD1 and serves as an efficient substrate for the E3 activity (Supplementary Fig. S10; ref. 14). The reciprocal requirement of BRCA1 and pT199-NPM1 for normal turnover of each protein.

Figure 4. Phosphomimic T199D mutant of NPM1 interacts with K63-linked ubiquitin conjugates. A, a mixture of K63-linked ubiquitin polymers (Ub2–7) was incubated with wild-type (WT) GST-NPM1 (B23.1), T199D mutant, or T199A mutant (B23.2). Immunoblot was performed with anti-ubiquitin antibody (P4D1) after GST pull-down (left). The Ub2–7 input (15%) was also loaded. The GST-tagged proteins used in the reactions were stained with Coommasie brilliant blue (CBB, right). *, GST-NPM1 peptides. B, binding curves of wild-type or the T199D mutant of GST-NPM1 obtained by SPR analyses with K63- or K48-linked tetraubiquitin peptides. The concentrations of analyte injected (GST-NPM1) were 50, 100, 200, and 400 nmol/L.
at the sites of DNA damage could be ascribed to the functional interaction of the two proteins.

T199 phosphorylation of NPM1 is mediated by the cyclin-dependent kinases CDK1 and CDK2. The phosphorylation levels are highest during mitosis, but they are also expressed in the S-phase of the normal cell cycle (37–39). In response to IR treatment, pT199-NPM1 disappeared from soluble fractions likely due to inhibition of CDK by checkpoint activity. However, pT199-NPM1 instead appeared in the chromatin fraction. This suggests that pT199-NPM1 resides

Figure 5. Domains and modification required for the interaction of NPM1 with K63-linked ubiquitin conjugates. A, the interaction of K63-linked Ub2,7 with truncated mutants of GST-NPM1-T199D was examined as in Fig. 4A. B, domain structure of NPM1 and the sequence of residues 152 to 212 that contain A3 and UIML domains. NES, NLS, NuLS, and P represent nuclear exporting signal, nuclear localization signal, nucleolar localization signal, and phosphorylation site, respectively. C, SPR analyses of K63-linked tetraubiquitin peptides binding to immobilized A3+UIML or UIML fragment of GST-NPM1-T199D (left), and to immobilized phosphorylated (pT199) or unphosphorylated A3+UIML fragment of wild-type GST-NPM1 (right). D, the dissociation constants of binding of K63-linked tetraubiquitin to GST-NPM1 (A3+UIML) containing the indicated mutation. The corresponding sequence alignment of the UIML motif of NPM1 is indicated at the bottom together with the conventional UIM motif. ϕ, e, and x represent hydrophobic, acidic, and any amino acid residue, respectively.
in S-phase nucleoplasm before DNA damage, and assembly of K63-linked ubiquitin conjugates at DSB sites triggers recruitment of pT199-NPM1 from the nucleoplasm. Phosphorylation contributes to NPM1 dissociation from nucleolar components (39) in addition to its role in binding to ubiquitin conjugates.

We showed that NPM1 is dispensable for recruitment of RAD51 but required for DNA damage repair, presumably at
a late stage of the repair cascade. One possible feature of NPM1 that may contribute in this process is its histone chaperone activity. Structural and biochemical analyses revealed that NPM1 is a decameric histone chaperone that interacts with core histones (26, 27, 40, 41). NPM1 may deliver histones to DNA in collaboration with ATP-utilizing chromatin remodelling factors. From this perspective, it could be interesting that some histone chaperones and chromatin assembly factors, such as Spt6, Rsf1, Caf1a, and Npm2, possess multiple acidic tracts and possible CDK-phosphorylation sites.

In general, NPM1 inhibits apoptosis and enhances cell proliferation (15). Compatibly, NPM1−/− embryos show high levels of apoptosis, accompanied by stabilization of p53 (42, 43). However, this effect can be explained by two mechanisms: (a) a response to DNA damage caused by the absence of NPM1 and (b) the direct function of NPM1 to inhibit the ARF-MDM2-p53 pathway (15, 43). Our results suggest that the pT199-NPM1−dependent HR repair is involved in the first mechanism. A previous report (44) and our results showed that depletion of total NPM1 had no effect on survival after IR, probably reflecting the multiple functions of NPM1 in addition to DSB repair. Although overexpression of NPM1 inhibits ARF, depletion of NPM1 also inhibits ARF because NPM1 binds to and protects ARF from degradation (43, 45, 46).

However, selective inhibition of pT199-NPM1 resulted in increase of radiosensitivity, although the effect was modest. The difference may come from selective inhibition of DSB repair and conserved apoptotic pathway in T199A cells. Dual roles of NPM1 on DSB repair and apoptosis may affect tumorigenesis and chemosensitivity of a variety of cancers, including leukemia and breast cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


Recruitment of Phosphorylated NPM1 to Sites of DNA Damage through RNF8-Dependent Ubiquitin Conjugates

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