The nucleolus is a subnuclear compartment with multiple cellular functions, including ribosome biogenesis. USP36 is a deubiquitylating enzyme that localizes to nucleoli and plays an essential role in regulating the structure and function of the organelle. However, how the localization of USP36 is regulated remains unknown. Here, we identified a short stretch of basic amino acids (RGKEKKIKKFKREKRR) that resides in the C-terminal region of USP36 and serves as a nucleolar localization signal for the protein. We found that this motif interacts with a central acidic region of nucleophosmin/B23, a major nucleolar protein involved in various nucleolar functions. Knockdown of nucleophosmin/B23 resulted in a significant reduction in the amount of USP36 in nucleoli, without affecting the cellular USP36 level. This was associated with elevated ubiquitylation levels of fibrillarin, a USP36 substrate protein in nucleoli. We conclude that nucleophosmin/B23 recruits USP36 to nucleoli, thereby serving as a platform for the regulation of nucleolar protein functions through ubiquitylation/deubiquitylation.

Ubiquitylation is reverted by deubiquitylating enzymes that deconjugate ubiquitin from target proteins (7). USP36, a deubiquitylating enzyme of the ubiquitin-specific protease (USP) family, localizes to nucleoli in mammalian cells (8–10), where it deubiquitylates nucleolar proteins such as NPM and FBL and prevents their proteasomal degradation (10). RNA interference-mediated knockdown of USP36 causes loss of the nucleolar structure and retardation in the rates of rRNA transcription and processing, suggesting a crucial role for protein deubiquitylation in nucleoli (10). However, how the localization and activity of USP36 are regulated in nucleoli remains unknown.

NPM is a multifunctional protein that localizes mainly in nucleoli, although it shuttles between the nuclei and cytoplasm and exhibits diverse functions in and out of nucleoli (11). With its endoribonuclease activity, NPM processes the 32 S rRNA precursor to the 28 S mature rRNA in nucleoli (12). It also plays roles as a molecular chaperone in preventing the aggregation of assembling rRNAs and ribosomal proteins in nucleoli (13) and in transporting assembled ribosomal subunits to the cytoplasm (14). NPM also regulates the p53 checkpoint pathway through association with Arf in nucleoli (15, 16). Finally, the biological importance of NPM is underscored by observations that overexpression or alteration of the NPM gene is frequently associated with human cancer (11).

In this study, we show that NPM recruits USP36 and regulates the level of protein ubiquitylation in nucleoli. Our results demonstrate a novel role for multifunctional NPM and provide a molecular basis for the regulation of protein ubiquitylation in nucleoli.

**MATERIALS AND METHODS**

**DNA Constructs**—Expression vectors for FLAG-tagged human wild-type USP36, USP36-(1–420), USP36-(421–800), and USP36-(801–1121), as well as those for FLAG-FBL and glutathione S-transferase (GST)-USP36-(921–1121), were constructed as described (10). cDNAs for USP36ΔBM1, USP36ΔBM2, USP36ΔBM3, USP36ΔBM4, and USP36ΔBM5 were generated using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) and cloned into the mammalian expression vector pME-FLAG. The enhanced green fluorescent protein (EGFP) fusion construct was generated by inserting chemically synthesized double-stranded oligonucleotides into pEGFP-N1 (Clontech). FLAG-tagged NPM constructs were provided by Dr. Charles J. Sherr (St. Jude Children’s Research Hospital, Memphis, TN). The His-tagged NPM construct was generated by inserting the full-length NPM cDNA into pET-26b(+) (EMD Chemicals,
Gibbstown, NJ). Small interfering RNA (siRNA) expression vectors for human NPM were constructed by inserting chemically synthesized double-stranded oligonucleotides into pSilencer 1.0-U6 (Ambion, Austin, TX). The mRNA target sites were nucleotides 819–837 (5′-GAATGGCTTCCGAT-GACT-3′) and 670–688 (5′-GGCAACAGATCTCTCAAGA-3′) from the translation initiation codon for NPM siRNAs 1 and 2, respectively.

**Cell Culture and DNA Transfection**—HeLa and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Where described, cells were treated with 20 μM MG132 for 8 h prior to preparation of the lysate. For ectopic expression of proteins, expression vectors were transfected into cells for 48 h using FuGENE 6 transfection reagent (Roche Diagnostics). For RNA interference, siRNA expression vectors were transfected twice at 48-h intervals.

**Immunoprecipitation and Immunoblotting**—Cell lysates were prepared by solubilizing cells in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 10 mM N-ethylmaleimide, 50 mM NaF, 30 mM sodium pyrophosphate, 1 mM Na3VO4, and protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A)) and collecting the supernatant after centrifugation. Immunoprecipitation and immunoblotting of the lysates were performed using standard procedures. For immunoprecipitation, anti-NPM (3 μg; Zymed Laboratories Inc., South San Francisco, CA), anti-FLAG (1 μg; clone M2, Sigma), and anti-GFP (1 μg; Invitrogen) antibodies were used. The primary antibodies used for immunoblotting were anti-USP36 (1:100), anti-NPM (2 μg/ml), anti-FLAG (4 μg/ml), anti-GST (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin (1:20,000; Sigma), and anti-ubiquitin (5 μg/ml; clone FK2, MBL, Nagoya, Japan). The secondary antibodies used were peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (GE Healthcare). Immunoblots were detected using ECL reagent (GE Healthcare).

**GST Pulldown**—His-tagged NPM protein was expressed in Escherichia coli and purified using the nickel-nitrilotriacetic acid buffer kit (EMD Chemicals). GST fusion protein (~5 μg) was also expressed in E. coli, coupled to glutathione-Sepharose beads (10 μl; GE Healthcare), and incubated with cell lysates or purified NPM (~5 μg) for 16 h at 4°C. After the beads were washed with lysis buffer, bound proteins were subjected to immunoblotting.

**Immunocytochemistry**—Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min on ice, permeabilized with 0.2% Triton X-100 for 5 min, and stained consecutively with primary and secondary antibodies. The primary antibodies used were rabbit anti-FLAG (0.4 μg/ml), mouse anti-nucleolus (1:20; clone MAB1277, Millipore), mouse anti-NPM (10 μg/ml), and rabbit anti-USP36 (1:1000). The secondary antibodies used were Alexa 488- and Alexa 594-conjugated anti-mouse IgG and anti-rabbit IgG (1:1000; Invitrogen). To stain nuclei, cells were incubated with TO-PRO-3 iodide (642/661) (50 μM; Invitrogen) during incubation with secondary antibodies. Images were captured with a laser scanning confocal microscope (Axiovert 200M, Carl Zeiss, Oberkochen, Germany).

**RESULTS**

**Nucleolar Localization Signal (NoLS) in USP36**—We have shown recently that the C-terminal third of USP36 (USP36-(801–1121)) (Fig. 1A) is required for the nucleolar localization of USP36 (10). Because this region contains five clusters of basic amino acids (BM1–5) (Fig. 1A), we examined whether BM1–5 are required for the USP36 localization. We deleted the BM sequences individually from FLAG-tagged USP36 and expressed the mutants in HeLa cells. Each mutant protein was expressed at levels similar to those of wild-type USP36 as assessed by immunoblotting of the lysates of transfected cells (see Fig. 3C, middle panel) (data not shown). Immunofluorescence staining of transfected cells with anti-FLAG antibody showed that the mutants lacking each of BM1–4 (supplemental Fig. S1), as well as wild-type USP36 (Fig. 1, B–B′), distributed normally in nucleoli. By contrast, deletion of BM5 (1075RGKEKKIKFKREKRR1091) abolished the nucleolar localization of USP36 and caused its leakage to the nucleoplasm (Fig. 1, C–C′), suggesting that BM5 is required for the nucleolar localization of USP36, whereas BM1–4 are dispensable.

We next fused the BM5 sequence to the N terminus of EGFP (Fig. 2A) and expressed the fusion protein in HeLa cells (see Fig. 3D, middle panel). Fluorescence imaging showed that whereas EGFP is scattered in the nucleoplasm and cytoplasm (Fig. 2,
USP36 Binds to NPM via the NoLS—We reported previously the interaction between ectopically expressed USP36 and NPM (10). To elucidate its physiological relevance, the interaction of endogenous proteins was examined. Anti-NPM (but not control) antibody co-immunoprecipitated endogenous USP36 from untransfected HeLa cells, suggesting that these proteins associate with each other under normal conditions (Fig. 3A).

To examine the possibility that USP36 localizes to nucleoli via interaction with NPM, we mapped the USP36-binding site in NPM. We next examined the USP36-binding site in NPM by examining the interaction of truncated NPM mutants (Fig. 4A) (15) with USP36 in GST pulldown experiments. The C-terminal portion of USP36 containing BM5 ( residues 921–1121) was bacterially expressed and purified as a GST fusion protein. GST-USP36-(921–1121) coupled to glutathione beads was incubated with lysates of COS-7 cells transfected with EGFP or BM5-EGFP were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-NPM (upper panel) and anti-FLAG (middle panel) antibodies. Total lysate was immunoblotted with anti-NPM antibody (lower panel). Asterisks in B indicate the positions of the USP36 mutants. WT, wild-type. D, lysates of COS-7 cells transfected with EGFP or BM5-EGFP were immunoprecipitated with anti-FLAG antibody (lower panel). Total lysate was immunoblotted with anti-NPM antibody (lower panel).

3D). The results in Fig. 3 indicate that BM5 also serves as an NPM-binding site.

USP36 Binds to the Central Region of NPM—We next mapped the USP36-binding site in NPM by examining the interaction of truncated NPM mutants (Fig. 4A) (15) with USP36 in GST pulldown experiments. The C-terminal portion of USP36 containing BM5 (residues 921–1121) was bacterially expressed and purified as a GST fusion protein. GST-USP36-(921–1121) coupled to glutathione beads was incubated with lysates of COS-7 cells transfected with FLAG-tagged NPM mutants, and bound NPM proteins were detected by immunoblotting with anti-FLAG antibody. The binding ability of the C1
and C2 mutants to GST-USP36-(921–1121) was not significantly reduced compared with that of wild-type NPM (Fig. 4B). By contrast, C3 and N3, both of which lack the central region containing the acidic clusters, almost completely lost the binding ability (Fig. 4B). GST did not pull down wild-type NPM by itself (data not shown). These results suggest that NPM binds to USP36 via the central region and that the N-terminal region is also involved in the interaction in some way.

To confirm that USP36 interacts with NPM directly, His6/Ni2+ affinity beads (Fig. 4B) were incubated with GST or GST-USP36-(921–1121) coupled to glutathione beads, and bound NPM proteins were immunoblotted (IB) with anti-FLAG antibody (upper panel). Amounts of GST-USP36-(921–1121) used were assessed by anti-GST immunoblotting of the pulldown samples (middle panel). Inputs of the NPM mutants were assessed by anti-FLAG immunoblotting of the total lysate used for the pulldown assay (lower panel). Asterisks indicate the positions of the NPM mutants. WT, wild-type. C, His-tagged NPM was purified from transformed E. coli and stained with Coomassie Brilliant Blue (CBB). D, purified NPM was incubated with GST or GST-USP36-(921–1121) coupled to glutathione beads, and bound NPM was immunoblotted with anti-NPM antibody (upper panel). Amounts of GST proteins (asterisks) were assessed by Coomassie Brilliant Blue staining (lower panel).

FIGURE 4. USP36 binds to the central region of NPM. A, shown are the schematic structures of NPM and its mutants used in this study. OligoD, oligomerization domain; NLS, nuclear localization signal; HeteroD, heterodimerization domain; NBD, nucleic acid-binding domain. The USP36-binding ability of each NPM mutant (results in B) is summarized on the right. B, lysates of COS-7 cells transfected with FLAG-tagged NPM mutants were incubated with GST-USP36-(921–1121) coupled to glutathione beads, and bound NPM proteins were immunoblotted (IB) with anti-FLAG antibody (upper panel). Amounts of GST-USP36-(921–1121) used were assessed by anti-GST immunoblotting of the pulldown samples (middle panel). Inputs of the NPM mutants were assessed by anti-FLAG immunoblotting of the total lysate used for the pulldown assay (lower panel). Asterisks indicate the positions of the NPM mutants. WT, wild-type. C, His-tagged NPM was purified from transformed E. coli and stained with Coomassie Brilliant Blue (CBB). D, purified NPM was incubated with GST or GST-USP36-(921–1121) coupled to glutathione beads, and bound NPM was immunoblotted with anti-NPM antibody (upper panel). Amounts of GST proteins (asterisks) were assessed by Coomassie Brilliant Blue staining (lower panel).

and C2 mutants to GST-USP36-(921–1121) was not significantly reduced compared with that of wild-type NPM (Fig. 4B). By contrast, C3 and N3, both of which lack the central region containing the acidic clusters, almost completely lost the binding ability (Fig. 4B). USP36 binding of the N2 mutant was also affected considerably (Fig. 4B). GST did not pull down wild-type NPM by itself (data not shown). These results suggest that NPM binds to USP36 via the central region and that the N-terminal region is also involved in the interaction in some way.

To confirm that USP36 interacts with NPM directly, His-tagged wild-type NPM was bacterially expressed, purified using Ni2+ affinity beads (Fig. 4C), and incubated with GST-USP36-(921–1121) in the GST pulldown assay. Immunoblotting of the pulldown precipitates with anti-NPM antibody showed that purified NPM bound to USP36 but not to control GST (Fig. 4D), demonstrating direct interaction between USP36 and NPM.

NPM Is Required for the Nucleolar Localization of USP36—The above results suggested that USP36 localizes to nucleoli via interaction with NPM. To test this possibility, we examined the effect of RNA interference-mediated knockdown of NPM on USP36 localization in HeLa cells. Immunoblotting showed that, upon the transfection of two independent siRNAs for NPM (siRNAs 1 and 2), the level of endogenous NPM protein was significantly reduced (Fig. 5A). The level of USP36 was not affected by NPM depletion (Fig. 5A), suggesting that the interaction does not stabilize USP36 protein. However, immunofluorescence staining showed that, in cells transfected with NPM siRNA 1 or 2 and exhibiting mostly undetectable levels of nucleolar NPM, USP36 staining was drastically reduced in nucleoli (Fig. 5, C–C′, asterisks) (data not shown). This effect was observed in >90% of ~300 NPM-depleted cells examined...
and was not due merely to uneven antibody staining because nuclear staining with TO-PRO-3 was normal in these cells (Fig. 5, C–C’, blue).

We also examined the localization of ectopically expressed USP36 in NPM knockdown cells. HeLa cells were transfected with FLAG-USP36 together with NPM siRNA 1 or 2 and double-stained with anti-FLAG and anti-NPM antibodies. In NPM-depleted cells, FLAG-USP36 was leaked to the nucleoplasm from nucleoli (Fig. 5, D–D’, asterisks) (data not shown) similarly to FLAG-USP36ΔBM5 in normal cells (Fig. 1, C–C’). In mock-transfected cells, FLAG-USP36 correctly localized to nucleoli (data not shown). Collectively, these results suggest that NPM is required for the nucleolar retention of USP36.

Failure to detect the nucleoplasmic leakage of endogenous USP36 in NPM knockdown cells (Fig. 5, C–C’) was probably due to a low level of endogenous USP36, which did not allow its immunofluorescent detection when dispersed in the nucleoplasm.

**DISCUSSION**

Basic amino acid stretches of ~30 residues have been identified as NoLSs for a variety of nucleolar proteins (17). No consensus sequence has been identified in these NoLSs, and they are believed to serve as binding sites for specific residual nucleolar elements (i.e. ribosomal DNAs, rRNAs, or proteins) but not for a common nucleolus-targeting machinery. Weber et al. (18) and Horke et al. (19) pointed out, however, that NoLSs of ~10 proteins harbor a single or multiple copies of the (K/R)(K/R) sequence, where K/R represents any amino acid. The NoLS of USP36, identified in this study and referred to as BM5 (1076RGKEKKIKKKFKKKFRKKRR1091) (Fig. 1), contains three partially overlapping (K/R)(K/R)X(K/R) sequences: KKIK, KKFK, and KREK. Therefore, each of these sequences may individually serve as an NoLS when fused to EGFP, although somewhat less efficiently (data not shown). Triplication of the (K/R)(K/R)X(K/R) sequence in the NLS might enhance the affinity of USP36 for nucleoli. Although BM1–4 also consist of 4–9 K/R residues (Fig. 1), they were all dispensable for the nucleolar localization of USP36 (supplemental Fig. S1). Thus, BM1–4 may not be exposed to the surface of the USP36 molecule and not be able to interact with nucleolar elements.

The NoLS of USP36 interacted with NPM (Fig. 3). Previous studies showed that the tumor suppressor Arf (16) and the Rex protein of human T-cell leukemia virus-1 (20) harbor NoLSs containing the (K/R)(K/R)X(K/R) motif and bind NPM via the NoLSs. This tetrapeptide motif therefore might constitute the NPM-binding core for at least several proteins. The central region of NPM containing two acidic clusters was essential for USP36 binding of NPM (Fig. 4), suggesting that an electrostatic interaction between the NoLS of USP36 and the acidic regions in NPM mediates the interaction of these proteins. Together, the requirement of NPM for the nucleolar localization of USP36 (Fig. 5) indicates that NPM recruits USP36 to nucleoli.

NPM forms a homo-oligomer in vitro (21) and composes a large complex of 2–5 MDa in the cell (15). In addition, NPM interacts with various proteins (11). Based on these observations, it was proposed recently that NPM serves as a hub that mediates the retention of multiple proteins in nucleoli (17). The nucleolar localization of such NPM-binding proteins regulates their activities positively or negatively. When nucleolar localization of USP36 was perturbed by NPM depletion, ubiquitylation levels of its substrate protein FBL were elevated (Fig. 6). These results suggest that nucleolar retention facilitates, but...
does not sequester, the catalytic activity of USP36. Our previous study suggests that NPM itself is also stabilized by USP36-mediated deubiquitylation (10). Therefore, once recruited to nucleoli by NPM, USP36 may in turn regulate the function of NPM as well.

Recent reports showed that NPM also binds to desumoylating enzymes SENP3 and SENP5, which deconjugate SUMO (small ubiquitin-like modifier) proteins from target proteins (22, 23). NPM is required for the nucleolar localization of SENP3/5 and desumoylation of nucleolar proteins (23). Because a proteome analysis suggests that USP36 deubiquitylates multiple proteins in nucleoli (10), the present study, in combination with others (22, 23), demonstrates that NPM is a key regulator of nucleolar functions that serves as a platform for protein deubiquitylation and desumoylation and controls the levels of post-translational modification of various nucleolar proteins. Finally, as NPM is deeply implicated in human cancer (11), it is of interest in future studies to elucidate how this NPM function relates to cellular transformation.

Acknowledgment—We thank C. Sherr for generously providing the NPM expression constructs.

REFERENCES
1. Boisvert, F. M., van Koningsbruggen, S., Navascues, J., and Lamond, A. I. (2007) Nat. Rev. Mol. Cell Biol. 8, 574–585
2. Chen, M., Rockel, T., Steinweger, G., Hemmerich, P., Risch, J., and von Mikecz, A. (2002) Mol. Biol. Cell 13, 3576–3587
3. Itahana, K., Bhat, K. P., Jin, A., Itahana, Y., Hawke, D., Kobayashi, R., and Zhang, Y. (2003) Mol. Cell 12, 1151–1164
4. Stavreva, D. A., Kawasaki, M., Dundr, M., Koberna, K., Müller, W. G., Tsujimura-Takahashi, T., Komatsu, W., Hayano, T., Isobe, T., Raska, I., Misteli, T., Takahashi, N., and McNally, J. G. (2006) Mol. Cell. Biol. 26, 5131–5145
5. Lam, Y. W., Lamond, A. I., Mann, M., and Andersen, J. S. (2007) Curr. Biol. 17, 749–760
6. Kuo, M. L., den Besten, W., Bertwistle, D., Roussel, M. F., and Sherr, C. J. (2004) Genes Dev. 18, 1862–1874
7. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) Cell 123, 773–786
8. Anderssen, J. S., Lam, Y. W., Leung, A. K., Ong, S. E., Lyon, C. E., Lamond, A. I., and Mann, M. (2005) Nature 433, 77–83
9. Catic, A., Fiebiger, E., Korbel, G. A., Blom, D., Galardy, P. J., and Ploegh, H. L. (2007) PLoS ONE 2, e679
10. Endo, A., Matsumoto, M., Inada, T., Yamamoto, A., Nakayama, K. I., Kitamura, N., and Komada, M. (2009) J. Cell Sci. 122, 678–686
11. Grisendi, S., Mecucci, C., Falini, B., and Pandolfi, P. P. (2006) Nat. Rev. Cancer 6, 493–505
12. Savkur, R. S., and Olson, M. O. (1998) Nucleic Acids Res. 26, 4508–4515
13. Szepesi, A., and Olson, M. O. (1999) Protein Sci. 8, 905–912
14. Maggi, L. B., Jr., Kuchenuether, M., Dadey, D. Y., Schwoppe, R. M., Grisendi, S., Townsend, R. R., Pandolfi, P. P., and Weber, J. D. (2008) Mol. Cell. Biol. 28, 7050–7065
15. Bertwistle, D., Sugimoto, M., and Sherr, C. J. (2004) Mol. Cell. Biol. 24, 985–996
16. Korgaonkar, C., Hagen, J., Tompkins, V., Frazier, A. A., Allamargot, C., Quelle, F. W., and Quelle, D. E. (2005) Mol. Cell. Biol. 25, 1258–1271
17. Emmott, E., and Hiscox, J. A. (2009) EMBO Rep. 10, 231–238
18. Weber, J. D., Kuo, M. L., Bothner, B., DiGiammarino, E. L., Kriwacki, R. W., Roussel, M. F., and Sherr, C. J. (2000) Mol. Cell. Biol. 20, 2517–2528
19. Horke, S., Reumann, K., Schweizer, M., Will, H., and Heise, T. (2004) J. Biol. Chem. 279, 26563–26570
20. Adachi, Y., Copeland, T. D., Hatanaka, M., and Oroszlan, S. (1993) J. Biol. Chem. 268, 13930–13934
21. Herrera, J. E., Correa, J. J., Jones, A. E., and Olson, M. O. (1996) Biochemistry 35, 2668–2673
22. Haindl, M., Harasim, T., Eick, D., and Muller, S. (2008) EMBO Rep. 9, 273–279
23. Yun, C., Wang, Y., Mukhopadhyay, D., Backlund, P., Kolli, N., Yergey, A., Wilkinson, K. D., and Dasso, M. (2008) J. Cell Biol. 183, 589–595