Supplementary Methods

Cell Culture and Transfection

HEK293T, U2OS or HeLa cells were grown under standard conditions. Plasmid transfections were carried out with the Calcium Phosphate method for HEK293T cells and with FuGene HD (Roche) for HeLa or U2OS cells, respectively. For siRNA-mediated knockdown 5 x 10^4 U2OS or HeLa cells were transfected with the respective siRNA oligonucleotides (20 μM) using Oligofectamine (Invitrogen) according to manufacturer's instructions.

Cloning and mutagenesis

cDNAs for NPM1.1 and NPM1.2 were isolated by RT-PCR from a HeLa cDNA library. For HA- or Flag-tagging NPM1 was cloned into pcDNA3.1 (Invitrogen) or pCI (Invitrogen). For Knock-down/knock-in complementation-assays Flag-tagged versions of NPM1, NPM1^{K263R} or a C-terminal fusion of non-conjugatable SUMO2 (SUMO2GA) to NPM1 were cloned into pRTS1, a tet-inducible hygromycine-selectable, episomal vector system (Holzel et al, 2007, Holzel et al, 2005). The constructs were rendered siRNA-resistant by introduction of silent mutations in the region targeted by the NPM1-1 siRNA.

A cDNA for SENP3 was amplified by PCR from an EST clone (RZPD, Germany) and cloned into pcDNA3.1 (Invitrogen) for SV5-tagging or pCI (Invitrogen) for Flag-tagging. Flag-tagged p14^{ARF} was kindly provided by Moshe

Oren (Weizmann Institute, Israel). Site-directed mutagenesis was carried out using the QuickChange Mutagenis Kit (Stratagene) according to the manufacturer's instructions.

Antibodies used for Western blotting and immunofluorescence

The following antibodies were used for Western blotting: anti-HA (clone 16B12, Covance), anti-Flag (clone M2, Sigma-Aldrich), anti-SV5 (Invitrogen), anti-β-Tubulin (clone TUB 2.1, Sigma-Aldrich), anti-SENP5 (gift of Ed Yeh, MD Anderson Cancer Center, Houston), anti-NPM1 (clone FC-61991, Zymed), anti-PARP-1 (clone 42, BD Biosciences), anti-pRB (clone 4H1, Cell Signaling), anti-$p14^{ARF}$ (clone DCS 240, Sigma-Aldrich), anti-Histone H3 (Abcam). Anti-SENP3 antibodies were raised in rabbits by immunization with a mixture of the following peptides LPPRWSQLGTSQRPR and TQQDMPKLRRQIYK (Eurogentech SA, Belgium). Serum was affinity-purified against the antigenic peptides immobilized on Sulfolink Coupling Gel matrix (Pierce).

As primary antibodies for immunofluorescence anti-Flag (rabbit polyclonal, Sigma-Aldrich) and anti-SV5 (Invitrogen) were used, as secondary antibodies CY3-anti-rabbit raised in donkey (Jackson ImmunoResearch) and FITC-anti-mouse raised in donkey (Jackson ImmunoResearch) were used.

Knock-down/knock-in assays

To establish polyclonal cell lines expressing Flag-NPM1, Flag-$NPM1^{K263R}$ or Flag-NPM1-SUMO2 HeLa cells were transfected with the respective constructs

cloned into pRTS1. Cells were selected with 200µg/ml hygromycine for 10 days. The cell lines were treated with 1 µg/ml doxycycline to induce expression of the respective genes. 24hr after induction cells were depleted of endogenous NPM1 by transfection of siRNA oligos targeting NPM1. 72hr after the siRNA transfection cells were harvested for Western blot analysis or metabolic labelling of nascent rRNA was performed as described.

Yeast two-hybrid assay

cDNAs encoding the respective prey or bait proteins were cloned in frame with the GAL activation domain of pGAD vector or the GAL binding domain of the pGBD vector and directed binding assays were done according to standard procedures.

siRNA sequences

siRNA	sequence 19mer
SENP3-1	5'-caccagggcuggaaagguu-3'
SENP3-2	5'-cuggcccugucucagccau-3'
SENP3-3	5'-ggaugcugcucuacucaaatt-3'
SENP3-4	5'-aggauccuuuguagauaaa-3'
SENP5-1	5'-gaacaucguucuaauacc-3'
NPM1-1	5'-ugaugaaaaugagcaccag-3'
NPM1-2	5'-ggaagucucuuuaagaaaa-3'
control	5'-cguacgcggaauacuucga-3'

Legends to Supplementary Figures

Supplementary Figure 1. Identification of NPM1 by mass-spectrometry
(A) A subset of tryptic peptides derived from the mass-spectrometric analysis of the 37kDa SENP3-interacting protein matches with the theoretically calculated mass of tryptic peptides of NPM1 (B) The position of the matching peptides within the NPM1.1 sequence is shown.

Supplementary Figure 2. The N-terminal region of NPM1 interacts with SENP3
Fragments of NPM1, spanning the indicated domains of NPM1 were tested for interaction with SENP3. NPM1.2 is a splice variant of NPM1 lacking a C-terminal exon. HoD=homodimerization domain, NLS=nuclear localization signal, HeD=heterodimerization domain, NBD=nucleotide binding domain.

Supplementary Figure 3. Endogenous NPM1 is modified by SUMO
HEK293T cells were transfected with HA- or His-tagged SUMO1 or SUMO2, respectively, and cell lysates were prepared under denaturing conditions. His-SUMO conjugates were recovered on Ni-NTA beads and subjected to Western blotting using anti-NPM1 antibody.

Supplementary Figure 4. Expression of $p14^{ARF}$ does not affect the interaction of SENP3 with NPM1, but leads to recruitment of SUMO2 to the nucleolus
(A) HeLa cells were transfected with HA-$p14^{ARF}$ and Flag-SENP3 as indicated. Cell lysates were prepared and Flag-SENP3 was immunoprecipitated with anti-Flag agarose beads. Bound material was separated by SDS-PAGE and probed by Western blotting with the indicated antibodies. (B) HeLa cells were transfected with Flag-SUMO2 and HA-$p14^{ARF}$ or empty vector and the localization was determined by immunostaining with anti-Flag and anti-HA antibody. Nuclei are visualized by DAPI staining.

Supplementary Figure 5. Downregulation of SENP3 interferes with pre-rRNA processing

(A, B) U2OS cells were transfected at time 0h and 24h with siRNA duplexes targeting NPM1, SENP5 or SENP3 as indicated. Transfection of an unspecific oligonucleotide served as a control. 48hr after the second transfection, cells were pulse labelled with ^{32}P-orthophosphate for 1hr, and chased for 2hr. An equal amount of RNA was loaded into each lane. Ethidium bromide staining of 28S and 18S rRNA is shown. Downregulation of the respective proteins was analyzed by Western blotting. **(C)** The signal intensities of the 28S and 32S rRNA forms were quantified by phosphoimager analysis and the 28S/32S ratio was calculated.

Supplementary Figure 6. NPM1^{K263R} does not exhibit a defect in SUMO modification, nucleolar localization and pre-rRNA processing

(A) HeLa cells expressing tetracycline inducible siRNA-resistant versions of Flag-NPM1 or Flag-NPM1^{K263R} were transfected with control siRNA duplexes or two different siRNA duplexes targeting NPM1. Cells were pulse labelled with ^{32}P-orthophosphate for 1hr, and chased for 2hr. An equal amount of RNA was loaded into each lane. Ethidium bromide staining of 28S and 18S rRNA is shown. Downregulation of the respective proteins was analyzed by Western blotting. **(B)** Flag-NPM1 and Flag-NPM1^{K263R} were expressed alone or together with HA-p14^{ARF} and His-tagged SUMO2 in HEK293T cells. His-SUMO2 conjugates were recovered on Ni-NTA beads and subjected to Western blotting using anti-Flag antibody. Expression of the respective proteins was monitored by Western blotting of cell lysates. **(C)** ^{35}S-labelled NPM1 and NPM1^{K263R}, generated by in vitro transcription/translation, was incubated with recombinant E1, E2 and either SUMO1 or SUMO2 in the presence of ATP. In the control reactions SUMO was omitted. **(D)** HEK293T cells were transfected with Flag-NPM1 and Flag-NPM1^{K263R} and the localization was determined by immunostaining with anti-Flag antibody. Nuclei are visualized by DAPI staining.

Supplementary Figure 7. Flag-NPM1-SUMO2 retains the ability to interact with NPM1 and binding partners of NPM1

HeLa cells expressing Flag-NPM1 or Flag-NPM1-SUMO2 from a tetracycline inducible promoter were induced as indicated. Cell lysates were prepared and the respective proteins were immunopurified on anti-Flag agarose beads. Bound material was separated by SDS-PAGE and probed by Western blotting with the indicated antibodies. In the upper panel exogenous Flag-NPM1 and endogenous NPM1 are marked by asterisks and arrowheads, respectively.

Supplementary Figure 1

A

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
25 - 32	1023.5694	1022.5621	1022.4821	0.0800	1	K.ADKDYHFK.V	
33 - 45	1568.8178	1567.8105	1567.7226	0.0879	0	K.VDNDENEHQLSLR.T	
46 - 54	803.5278	802.5205	802.4549	0.0656	0	R.TVSLGAGAK.D	
81 - 101	2227.4176	2226.4104	2226.2083	0.2021	0	K.MSVQPTVSLGGFEITPPVVLR.L	
81 - 101	2243.3749	2242.3676	2242.2032	0.1644	0	K.MSVQPTVSLGGFEITPPVVLR.L	Oxidation (M)
207 - 221	1603.8540	1602.8467	1601.7757	1.0710	1	K.SNQNGKDSKPSSTPR.S	
213 - 221	974.5955	973.5882	973.4829	0.1053	0	K.DSKPSSTPR.S	
222 - 229	910.5399	909.5326	909.4556	0.0770	1	R.SKGQESFK.K	
240 - 248	931.5392	930.5320	930.4658	0.0661	0	K.GPSSVEDIK.A	
251 - 257	806.4749	805.4676	805.4004	0.0673	0	K.MQASIEK.G	
278 - 291	1819.9913	1818.9840	1818.8359	0.1481	0	R.MTDQEAIQDLWQWR.K	
278 - 291	1835.9628	1834.9555	1834.8308	0.1247	0	R.MTDQEAIQDLWQWR.K	Oxidation (M)

B

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  1 MEDSMDMDMS PLRPQNYLFG CELKADKDYH FKVDNDENEH QLSLRTVSLG
 51 AGAKDELHIV EAEAMNYEGS PIKVTLATLK MSVQPTVSLG GFEITPPVVL
101 RLKCGSGPVH ISGQHLVAVE EDAESEDEEE EDVKLLSISG KRSAPGGGSK
151 VPQKKVKLAA DEDDDDDDEE DDDEDDDDDD FDDEEAEEKA PVKKSIRDTP
201 AKNAQKSNQN GKDSKPSSTP RSKGQESFKK QEKTPKTPKG PSSVEDIKAK
251 MQASIEKGGS LPKVEAKFIN YVKNCFRMTD QEAIQDLWQW RKSL
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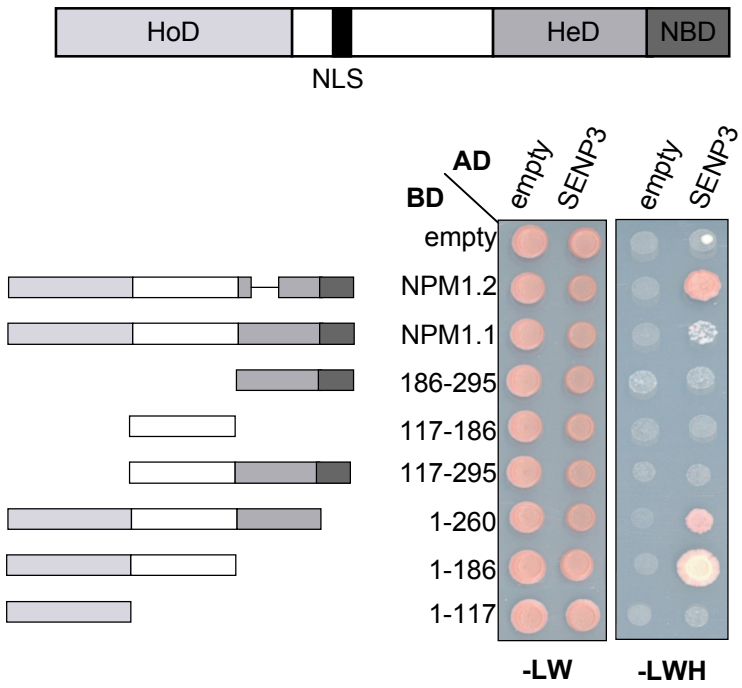
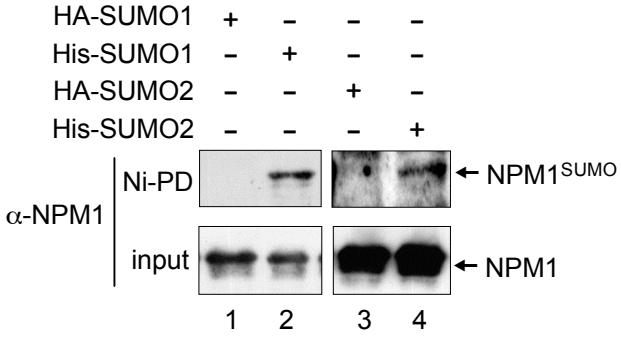
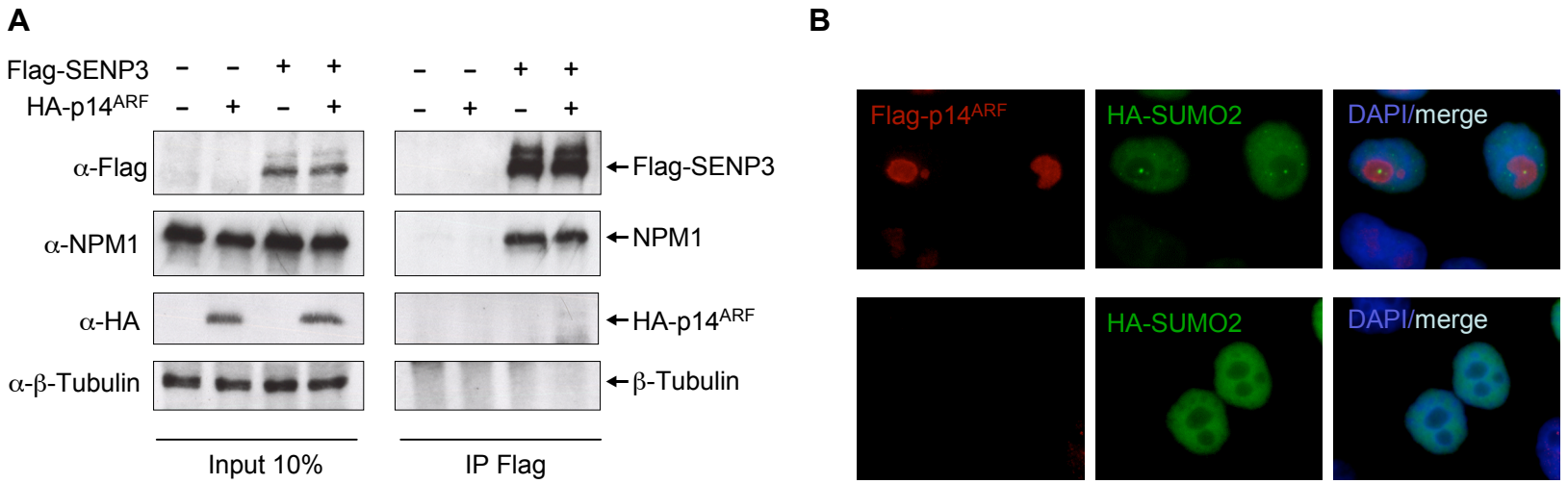
Supplementary Figure 2



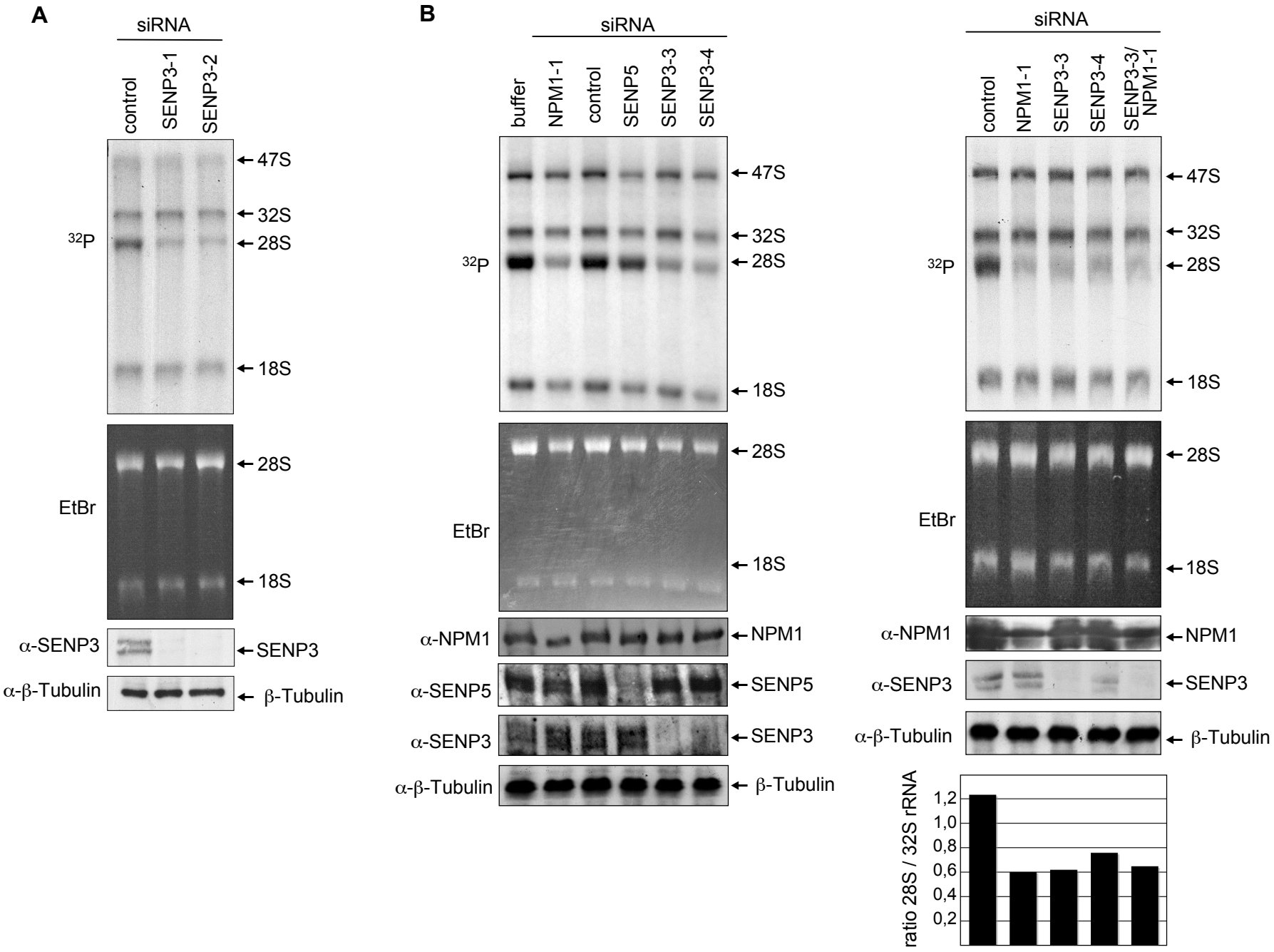
Supplementary Figure 3



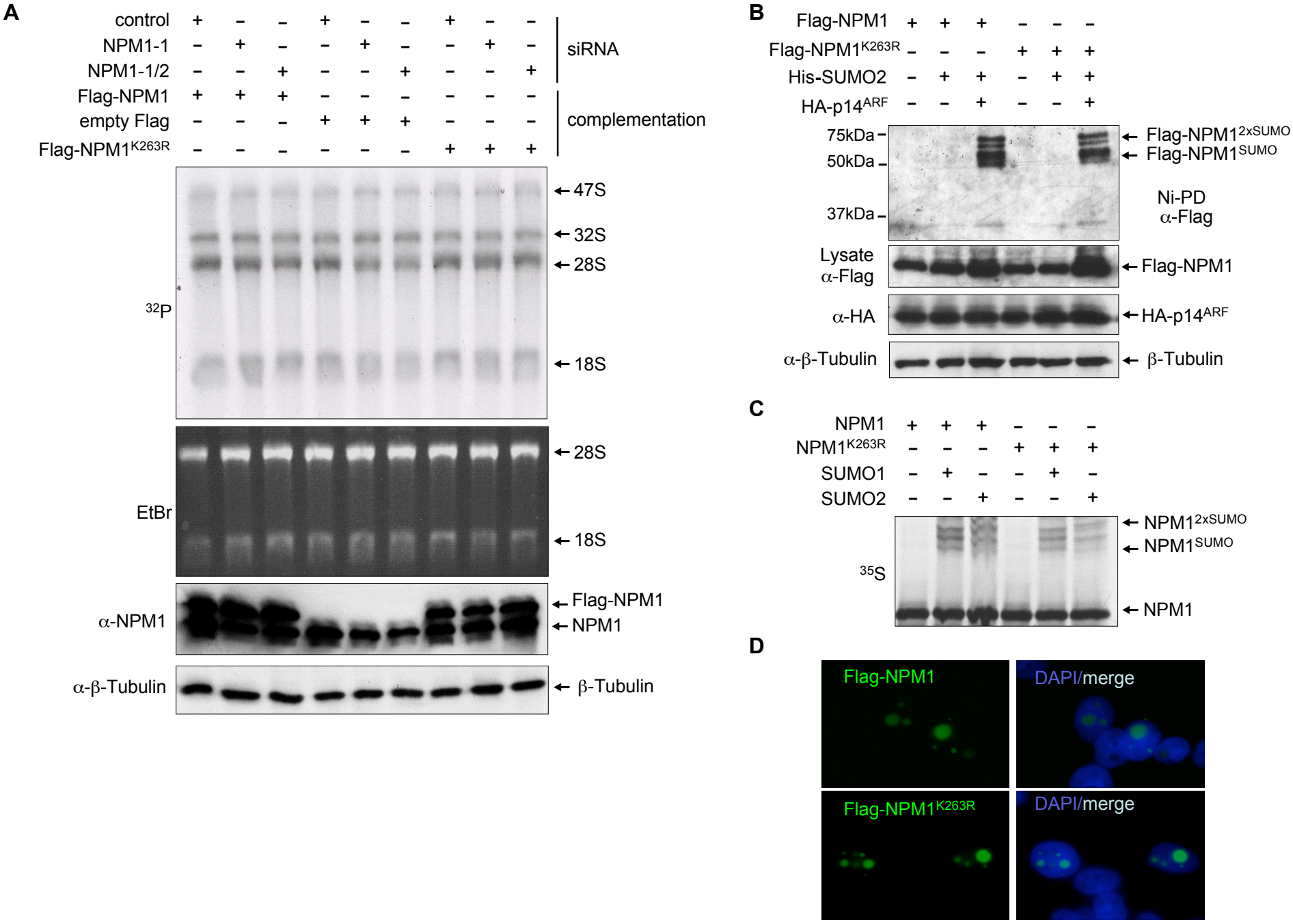
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7