Supplemental methods

Expression and purification of GST-hnRNP K variants

GST-hnRNP K(wt) and GST-hnRNP K(318-464) were kind gifts of K. Bomsztyk (1), GST-hnRNP K(127-237) was a kind gift of H. Leffers (2). GST-hnRNP K(1-121) was described previously (3). GST tagged proteins were expressed and purified as described in (3).

Purification of the hnRNP K fragment

The hnRNP K derived fragment was purified by conventional chromatography. Cytoplasmic extract from puromycin treated K562 cells was loaded on DEAE sepharose (GE Healthcare) in 20 mM Tris pH 8.0, 10% sucrose (buffer A), 50 mM potassium chloride and proteins were eluted with a 50-500 mM potassium chloride gradient. Fractions containing the fragment were adjusted to 100 mM potassium chloride and purified on heparin sepharose (GE Healthcare). Elution was performed with a 100-640 mM potassium chloride gradient in buffer A. Fragment containing fractions were loaded on Hydroxyapatite (Biorad) and eluted with a 0-50% gradient from buffer B (150 mM KCl, 10 mM Imidazol pH 8.0 at 20°C, 5% sucrose) to buffer C (500 mM potassium phosphate pH 8.0 at 20°C). For the last purification step Mono Q (GE Healthcare) was used and the hnRNP K fragment was eluted with a 100-500 mM potassium chloride gradient in buffer D (10 mM HEPES pH 7.5 at 15°C, 10 mM Tris, 10% sucrose).

Ubiquitin Capture Assay

Erythroid differentiation of K562 cells was induced for up to two days by addition of 1.5 mM sodium butyrate. Where indicated, the proteasome inhibitor MG132 was added at a final concentration of 0.1 or 1 µM, respectively. 200 µg cell lysate were used to detect ubiquitination with the UbiQapture-Q Kit (Enzo Life Sciences) according to manufacturer instructions. Aliquots of the input, beads and supernatant were used for Western blot analysis.

Antibodies

Antibodies from Cell Signaling (Ubiquitin), Imgenex (Tak-1), Enzo Life Sciences (Uba6) and Santa Cruz (HDM2, Uba1) were used according to manufacturer instructions.

RNA isolation and RT-PCR

RNA isolation and semiquantitative RT-PCR analysis were performed as described (4). GAPDH mRNA specific primer were described in (4). Primers for detection of HDM2 mRNA were: fw: AGGAGCAGGCAAATGTGCAA, rv: ATGGCTTTGGTCTAACCAGG.

siRNAs

Uba1: GCUAUGGUUUCUAUGGUUAdTdT

Uba6: CCAUAAGGCUCUUCAGCUUdTdT

Supplemental Figure legends

Figure S1 HDM2 is not expressed in K562 cells during erythroid differentiation.

Erythroid differentiation of K562 cells was induced with 1.5 mM sodium butyrate for the indicated time points. Upper panel: RT-PCR analysis with primers specific for HDM2 and GAPDH in MCF-7 cells and K562 cells. Lower panel: Western blot analysis of lysates from MCF-7 cells or K562 cells with HDM2 and GAPDH specific antibodies.

Figure S2 HnRNP K specific antibody #1 detects an epitope within amino acids 1-121.

Indicated amounts of GST-hnRNP K(wt), GST-hnRNP K(1-121), GST-hnRNP K(127-237) and GST-hnRNP K(318-463) were used for Western blots with antibody hnRNP K #1.

Figure S3 Purification of the hnRNP K derived cleavage product from puromycin treated K562 cells.

Fractions from each purification step were analyzed on a 12% SDS-PAGE by Coomassie or silver staining, as indicated or used for Western blots with antibody hnRNP K #1: a) DEAE sepharose, b) Heparin sepharose, c) Hydroxyapatite, d) Mono Q chromatography.

Figure S4 Caspase-3 catalyzed cleavage of recombinant hnRNP K is not affected by the r15-LOX mRNA 3'UTR DICE.

His-hnRNP K(wt) or His-hnRNP K(D334E) were incubated with recombinant Caspase-3 and 500 ng DICE or ctrl RNA as indicated at 37°C for 16 hours. Analysis in Western blots with antibodies directed against the N-terminal His-tag and Caspase-3.

Figure S5 Cleavage of endogenous hnRNP K by Caspase-3 is not affected by the DICE.

Cytoplasmic extract from non-treated K562 cells was incubated with recombinant Caspase-3 in the presence or absence of 500 ng DICE or ctrl RNA at 37°C for 16 hours and analyzed with Caspase-3, PARP, hnRNP K and GAPDH specific antibodies.

Figure S6 hnRNP K is not ubiquitinated during erythroid differentiation.

K562 cells induced for erythroid differentiation with 1.5 mM sodium butyrate for up to two days were treated with the proteasome inhibitor MG132 as indicated. Cell lysates were used for a Ubiquitin capture assay. Aliquots of the input, beads and supernatant fractions were analyzed with antibodies directed against Ubiquitin, hnRNP K, Tak-1 and XIAP.

Figure S7 HnRNP K is not stabilized by Uba1 and Uba6 knock down in puromycin treated K562 cells.

Upper panel: Schematic representation of the experiment. K562 cells were either mock transfected, transfected with a non-specific siRNA (ctrl) or with Uba1 and Uba6 specific siRNAs, singly or in combination. 24 hours post transfection 5 µg/ml puromycin was added. Cells were harvested at the time points indicated. Lower panel: Lysate from the experiments shown above was analyzed with Uba1, Uba6, hnRNP K and GAPDH specific antibodies.

Figure S8 HnRNP K is not stabilized by Uba1 and Uba6 knock down in K562 cells induced for erythroid differentiation.

Upper panel: Schematic representation of the experiment. K562 cells were either mock transfected, transfected with a non-specific siRNA (ctrl) or with Uba1 and Uba6 specific siRNAs, singly or in combination. 24 hours post transfection erythroid differentiation was induced with 1.5 mM sodium butyrate (NaB). Cells were harvested at the time points indicated. Lower panel: Cell lysate from the experiments shown above was analyzed with Uba1, Uba6, hnRNP K and GAPDH specific antibodies.

Supplemental references

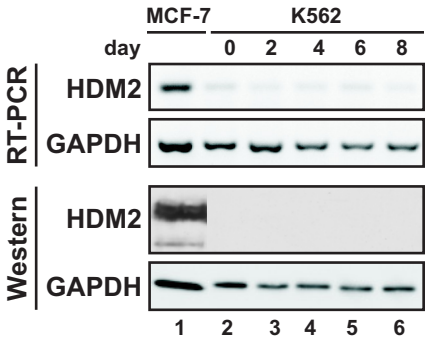
1. Van Seuningen I, Ostrowski J, Bustelo XR, Sleath PR, Bomsztyk K. The K Protein Domain That Recruits the Interleukin 1-responsive K Protein Kinase Lies Adjacent to a Cluster of c-Src and Vav SH3-binding Sites. *J Biol Chem* 1995; **270:** 26976-26985.

2. Dejgaard K, Leffers H. Characterisation of the Nucleic-Acid-Binding Activity of KH Domains Different Properties of Different Domains. *Eur J Biochem* 1996; **241:** 425-431.

3. Adolph D, Flach N, Mueller K, Ostareck DH, Ostareck-Lederer A. Deciphering the Cross Talk between hnRNP K and c-Src: the c-Src Activation Domain in hnRNP K Is Distinct from a Second Interaction Site. *Mol Cell Biol* 2007; **27:** 1758-1770.

4. Naarmann IS, Harnisch C, Flach N, Kremmer E, Kühn H, Ostareck DH*, et al.* mRNA Silencing in Human Erythroid Cell Maturation. *J Biol Chem* 2008; **283:** 18461-18472.

Supplemental Figure S1



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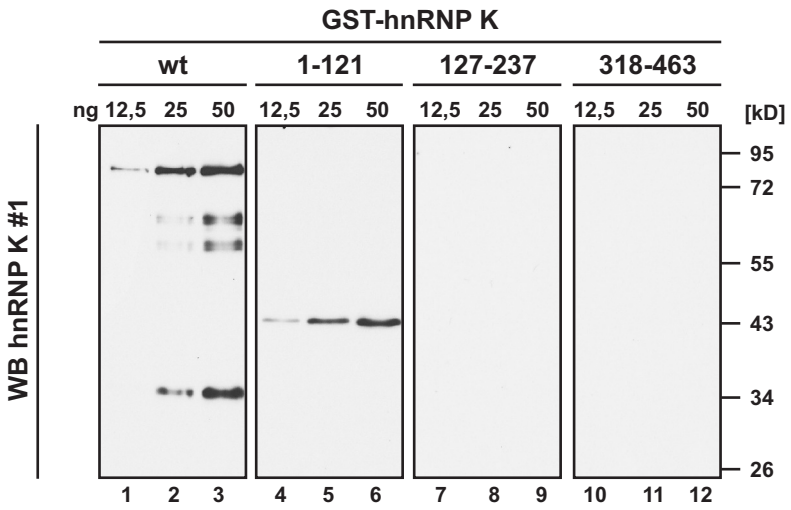

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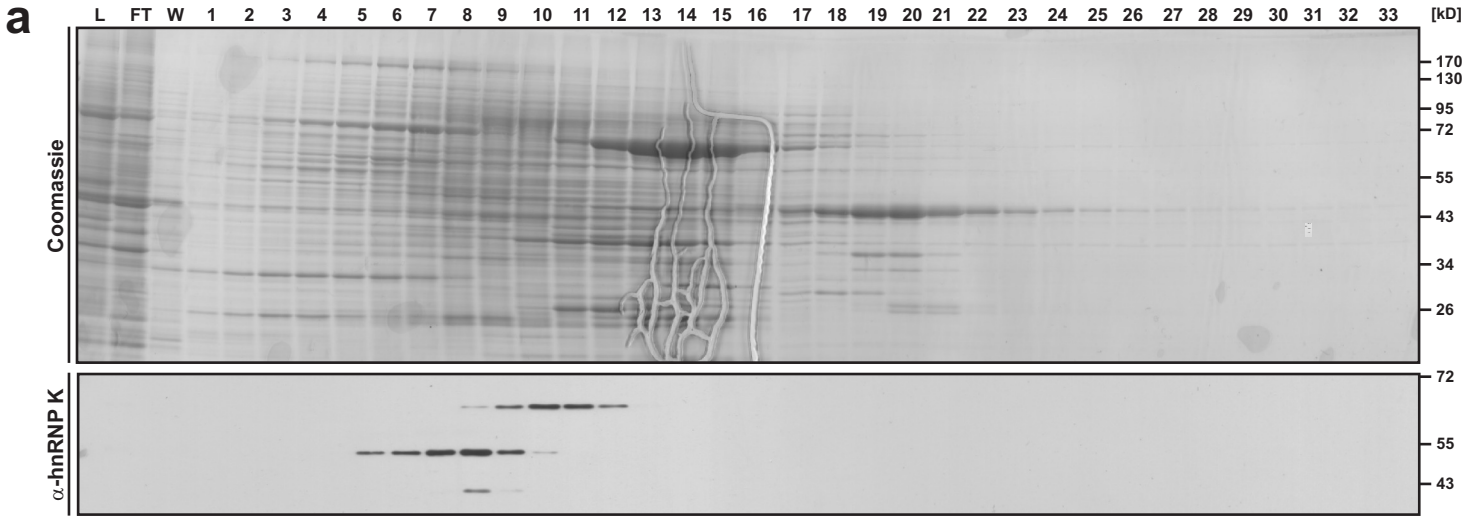

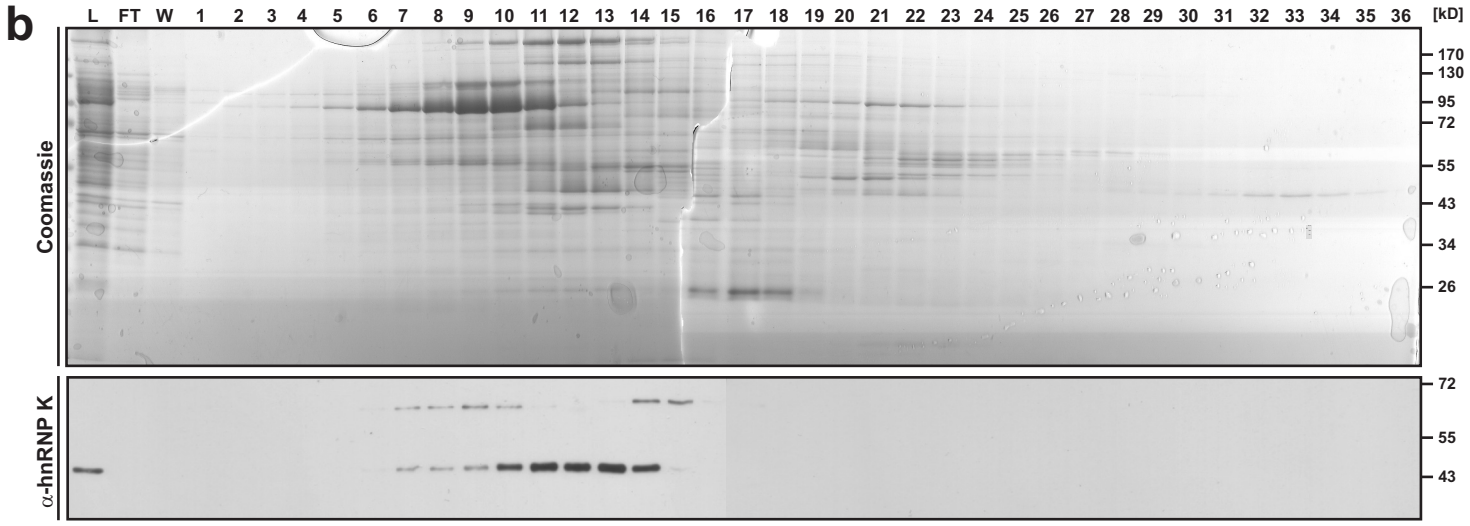

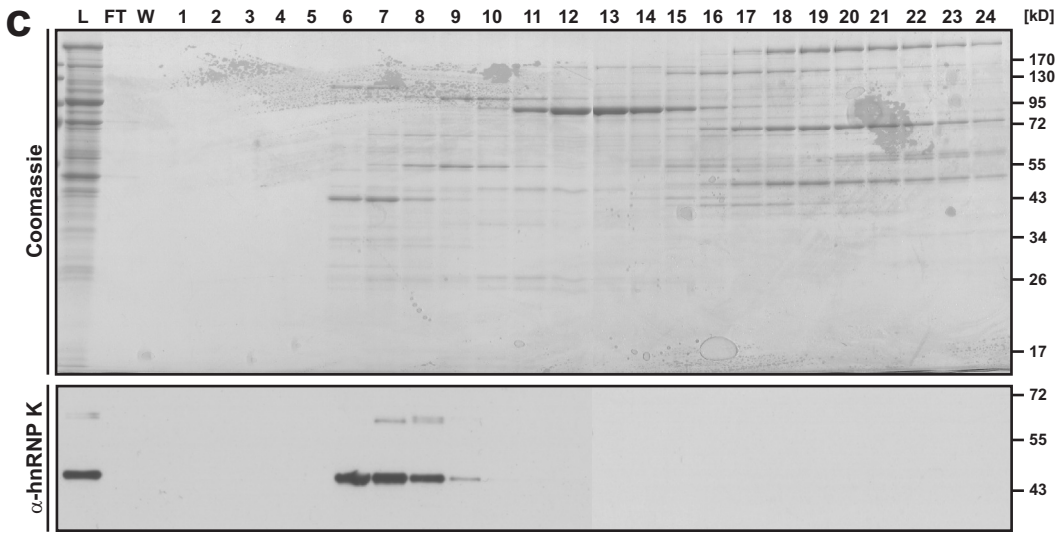

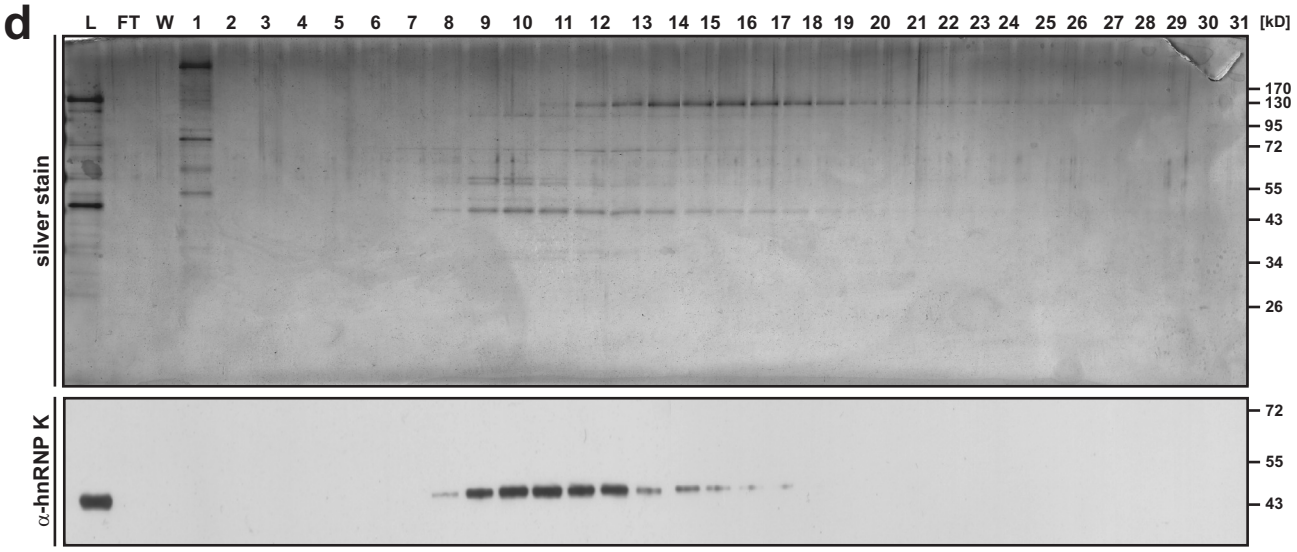
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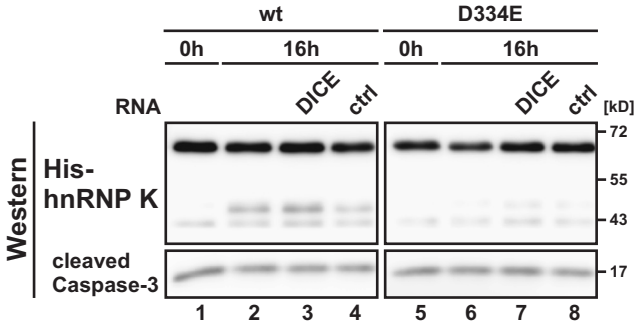
Supplemental Figure S4



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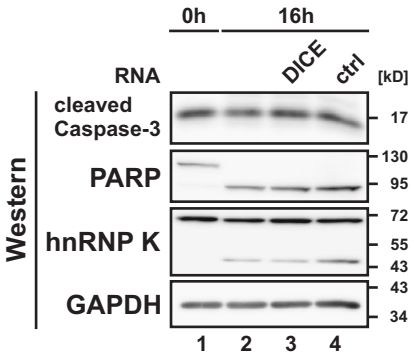

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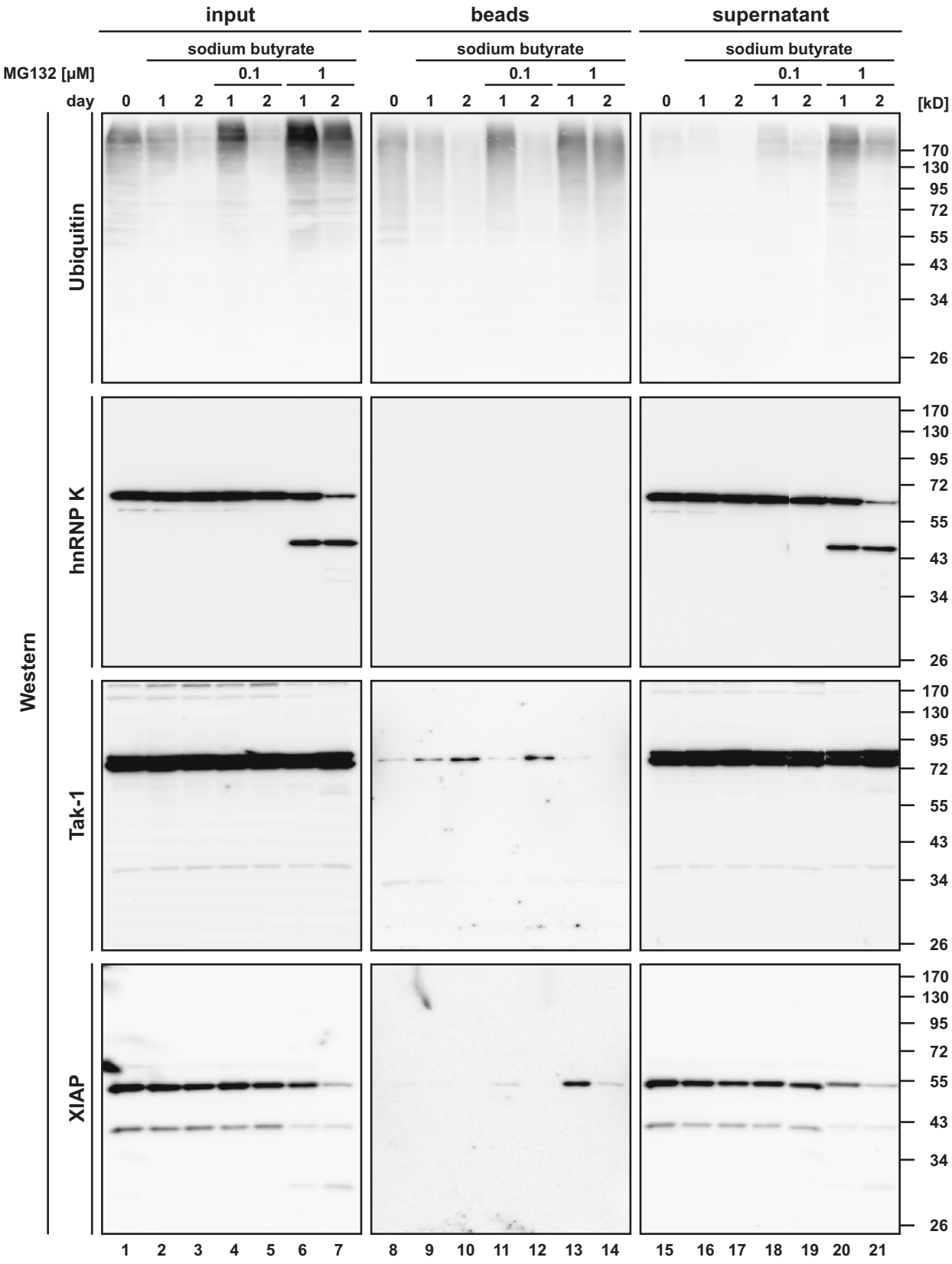

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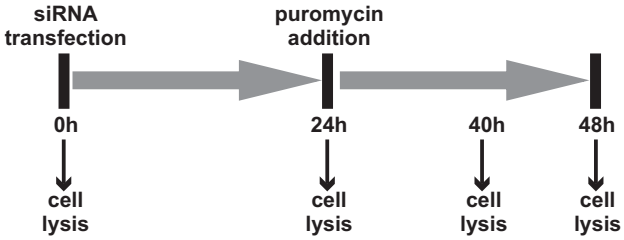

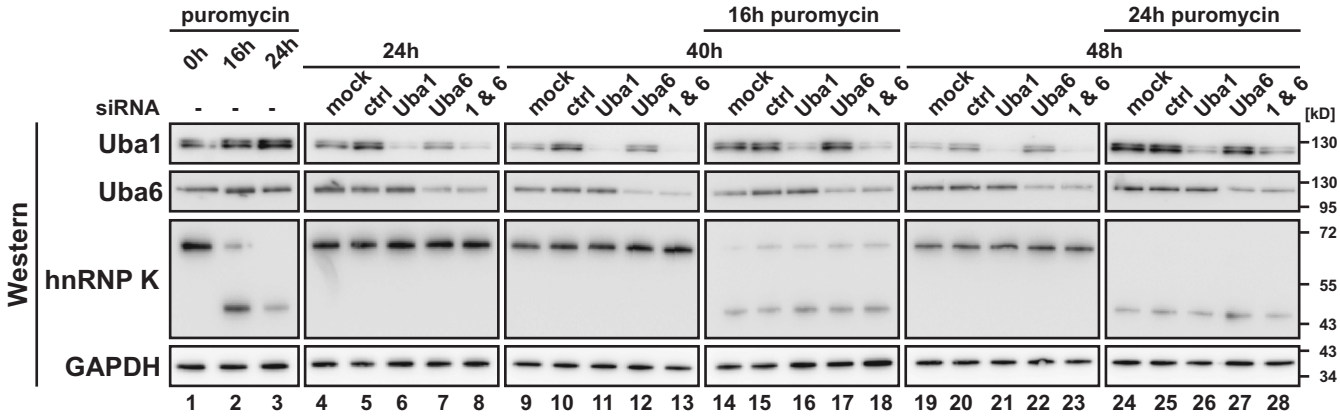

Supplemental Figure S8