# SUPPLEMENTARY INFORMATION

## USP36 stabilizes nucleolar Snail1 to promote ribosome biogenesis and cancer cell survival upon ribotoxic stress

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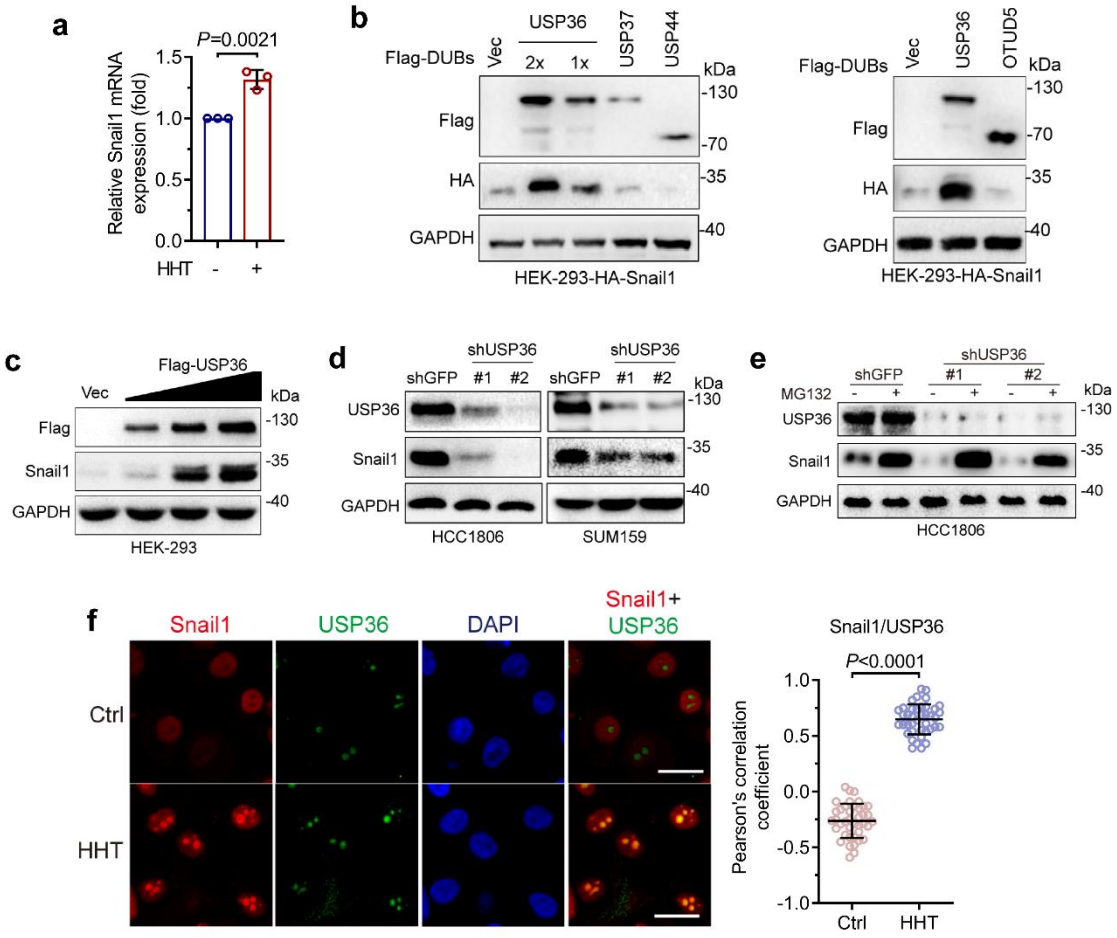
**Supplementary Figures S1-S10**

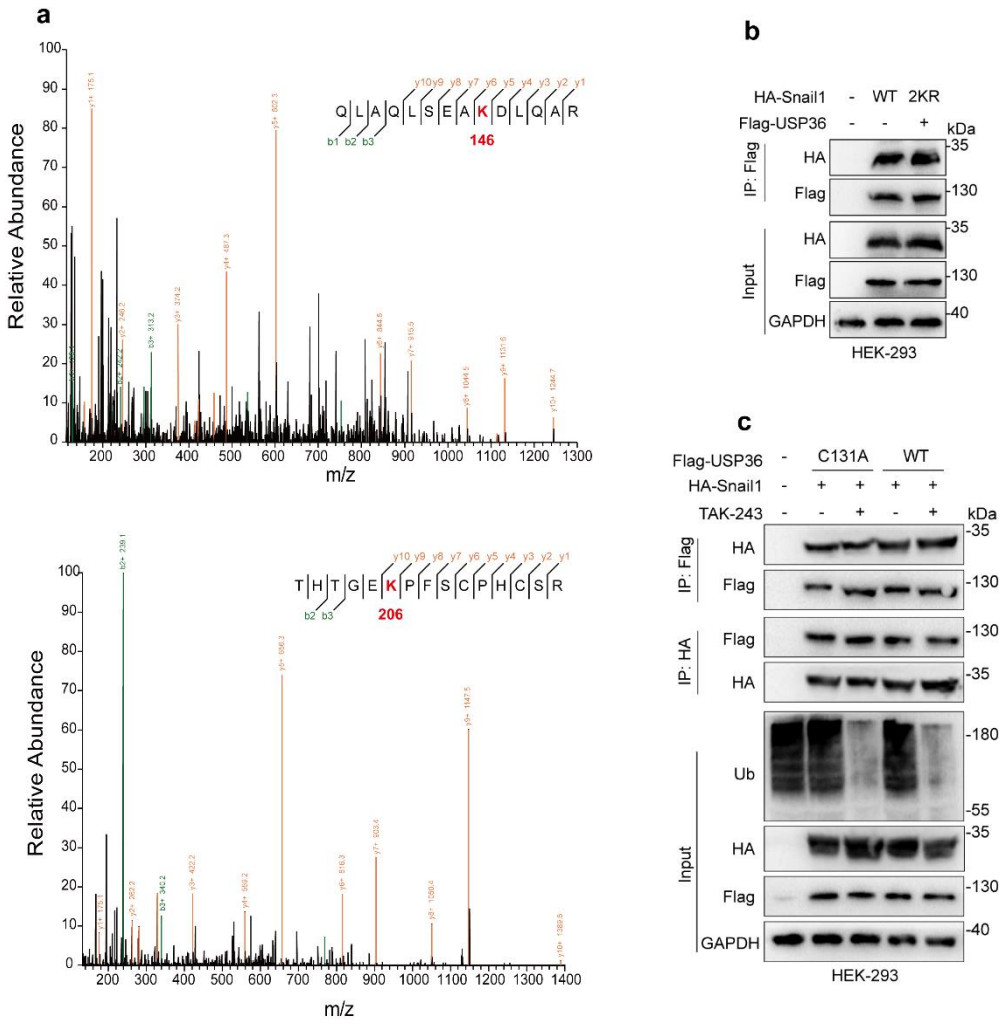
**Supplementary Table 1-4**

**Supplementary Figure S1**



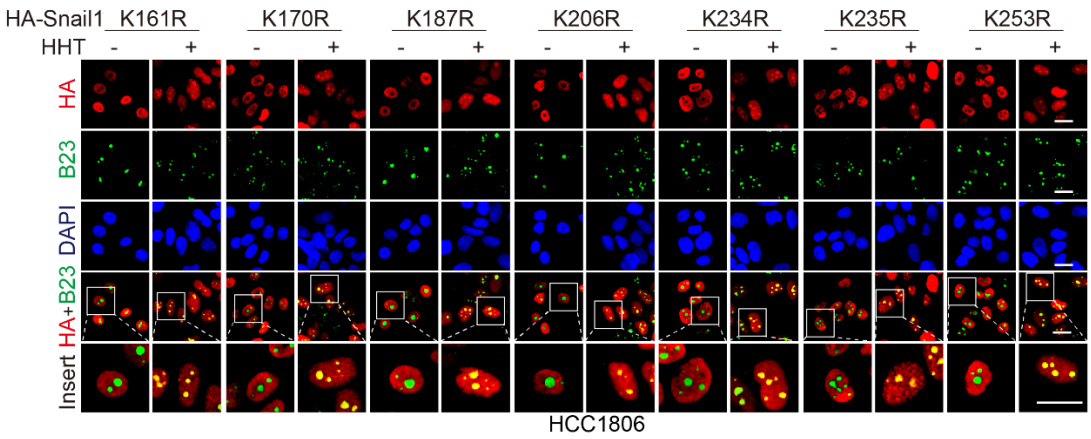

**Supplementary Figure S1. USP36 promotes Snail1 protein expression. (a)** HCC1806 cells were treated with or without HHT (20 ng/mL) for 24 h. Cells were subjected to qPCR analyses for Snail1 mRNA expression (n=3 biologically independent samples). **(b)** HEK-293 cells stably expressing HA-Snail1 were transiently transfected with a plasmid encoding a deubiquitinase as indicated or vector control (Vec) for 48 h. Cells were subjected to western blot analyses. **(c)** HEK-293 cells were co-transfected with HA-Snail1 and an increased dose of Flag-USP36 for 48 h. Cells were subjected to western blot analyses. **(d)** HCC1806 or SUM159 cells stably expressing shUSP36 (#1 or #2) or shGFP were subjected to western blot analyses. **(e)** HCC1806 cells stably expressing shUSP36 (#1 or #2) were treated with or without proteasome inhibitor MG132 (10 μM) for 6 h. Cells were subjected to western blot analyses. **(f)** HCC1806 cells were treated with or without HHT (20 ng/mL) for 24 h. Cells were subjected to immunofluorescent staining for co-localization of USP36 and Snail1. Quantification of the co-localization between Snail1 and B23 using Pearson's correlation coefficient. Quantification was carried out on 40 cells derived from three independent experiments. Scale bar, 25 μm. These experiments have been repeated for three times with similar results (b-e). Data were presented as mean ± SD and comparisons were performed with unpaired two-tailed Student's t-test (a and f).

**Supplementary Figure S2**



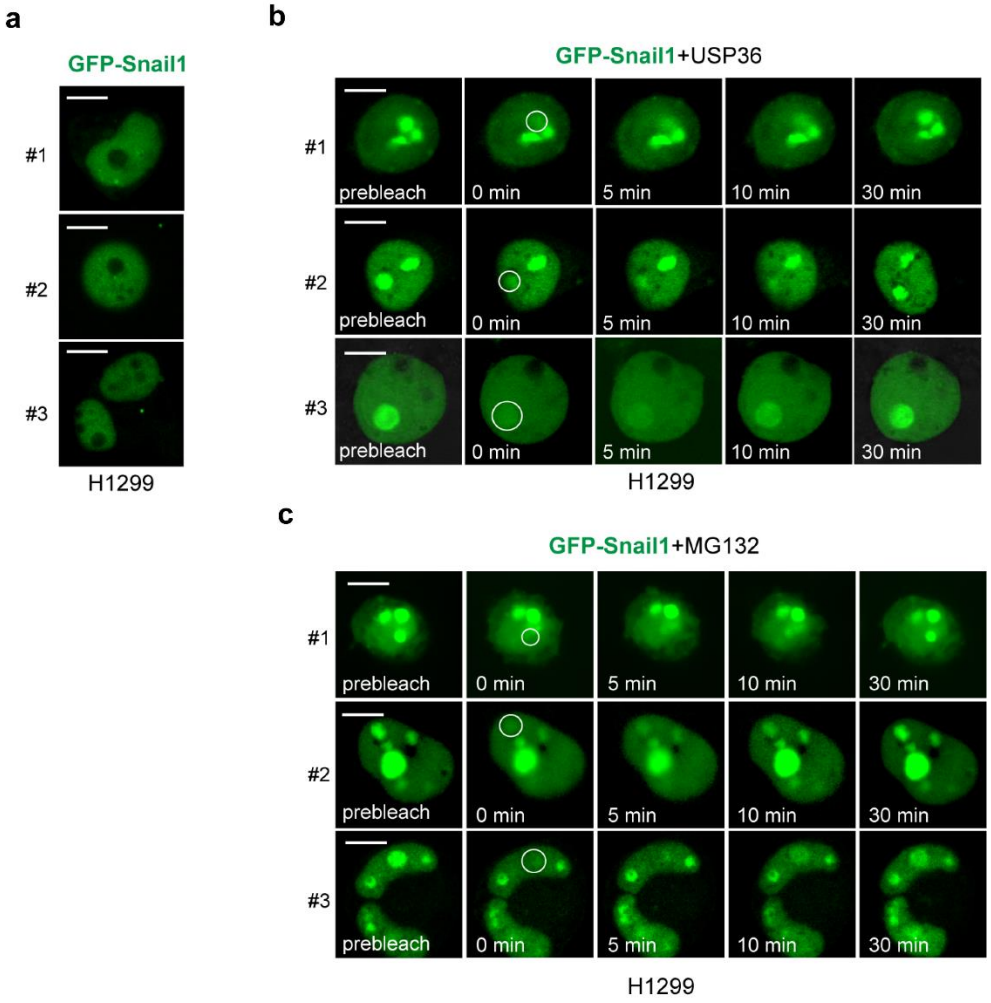

**Supplementary Figure S2. K146 and K206 of Snail1 were ubiquitinated and USP36 interacted with Snail1 regardless of the status of its ubiquitination. (a)** Representative fragmentation spectrum of the identified Snail1 protein ubiquitination sites. **(b)** HEK-293 cells stably expressing HA-Snail1 (WT or 2KR) were transiently transfected with Flag-USP36 for 48 h. Cells were treated with MG132 (10 μM) for 6 h followed by immunoprecipitation-western blot analyses. **(c)** HEK-293 cells stably expressing HA-Snail1 were transiently transfected with Flag-USP36 (WT or C131A) for 48 h. Cells were treated with or without E1 inhibitor TAK-243 (500 nM) for 8 h prior to MG132 (10 μM) treatment for 6 h followed by immunoprecipitation-western blot analyses. These experiments have been repeated for three times with similar results (b-c).

**Supplementary Figure S3**



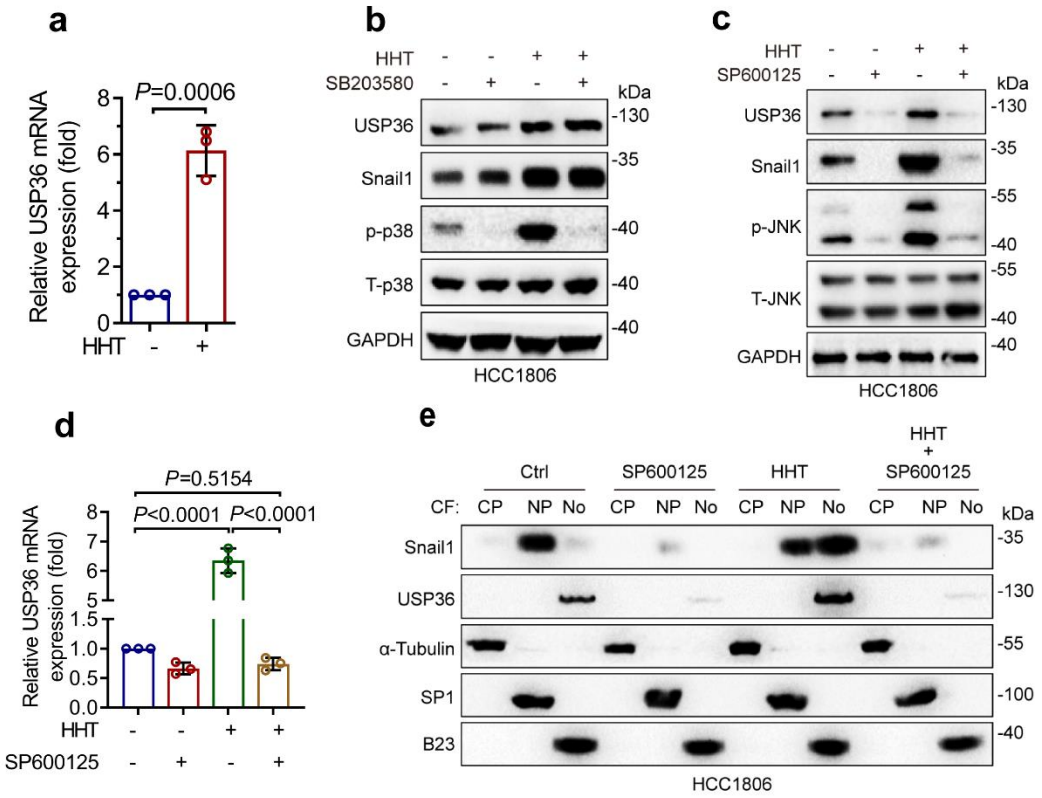

**Supplementary Figure S3. HHT promotes Snail1 accumulation in the nucleoli.** HCC1806 cells stably expressing HA-Snail1$^{K161R}$, HA-Snail1$^{K170R}$, HA-Snail1$^{K187R}$, HA-Snail1$^{K206R}$, HA-Snail1$^{K234R}$, HA-Snail1$^{K235R}$, or HA-Snail1$^{K253R}$ were treated with or without HHT (20 ng/mL) for 24 h. Cells were subjected to immunofluorescence staining. Notice that all the point mutant proteins could be accumulated in the nucleoli upon HHT treatment. This experiment has been repeated for three times with similar results. Scale bar, 25 μm.

**Supplementary Figure S4**



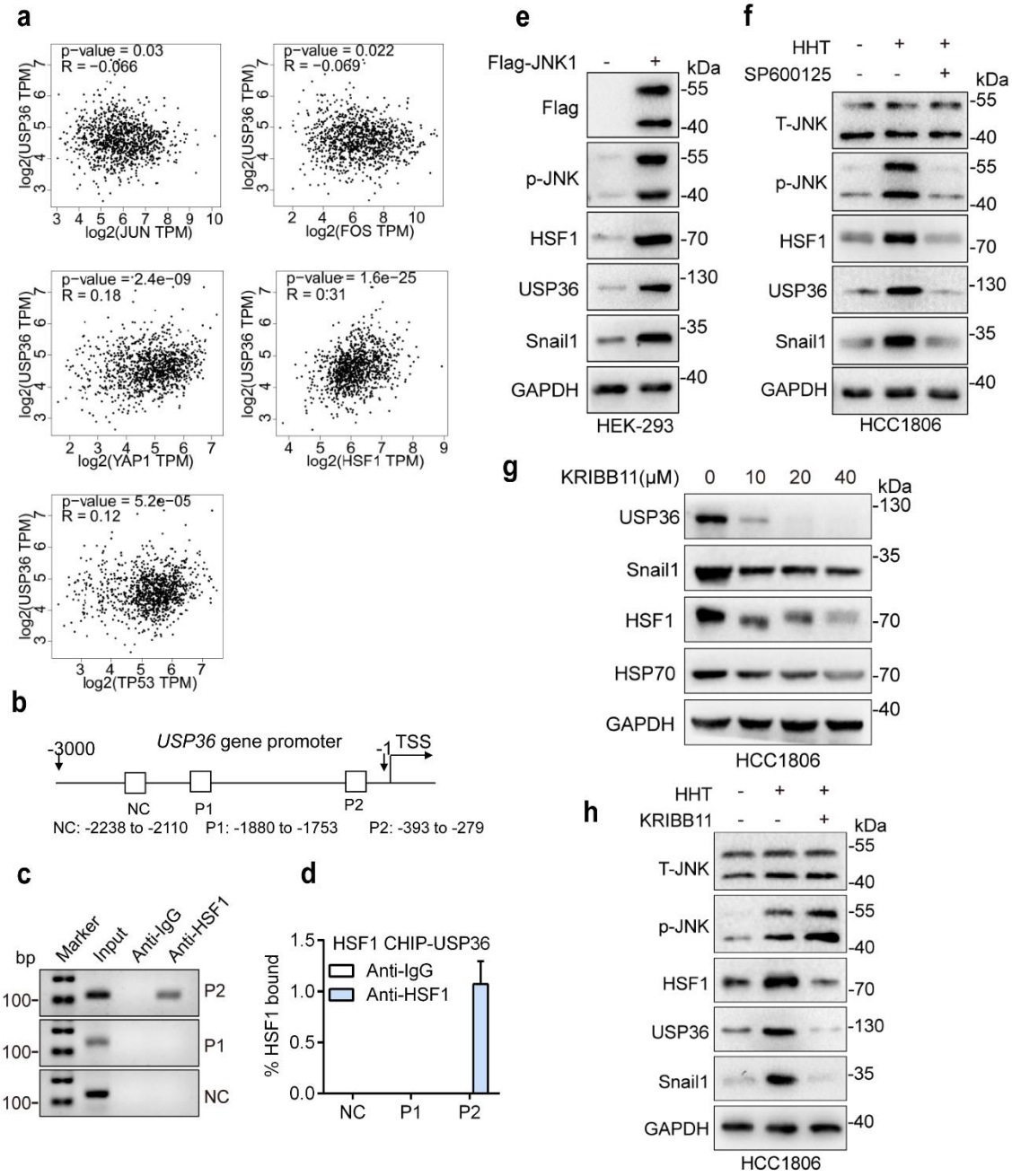

**Supplementary Figure S4. Snail1 protein translocates into nucleolus by diffusion in a USP36-independent manner. (a)** H1299 cells stably expressing Snail1-GFP were image-captured. **(b)** H1299 cells stably expressing Snail1-GFP were transiently transfected with USP36 for 48 h. Cells were image-captured before and during recovery after the bleaching of a part of the nucleolus (the bleached areas are outlined). **(c)** H1299 cells stably expressing Snail1-GFP were treated with MG132 (10 μM) for 6 h. Cells were image-captured before and during recovery after the bleaching of a part of the nucleolus (the bleached areas are outlined). These experiments have been repeated for three times with similar results (a-c). Scale bar, 25 μm.

**Supplementary Figure S5**



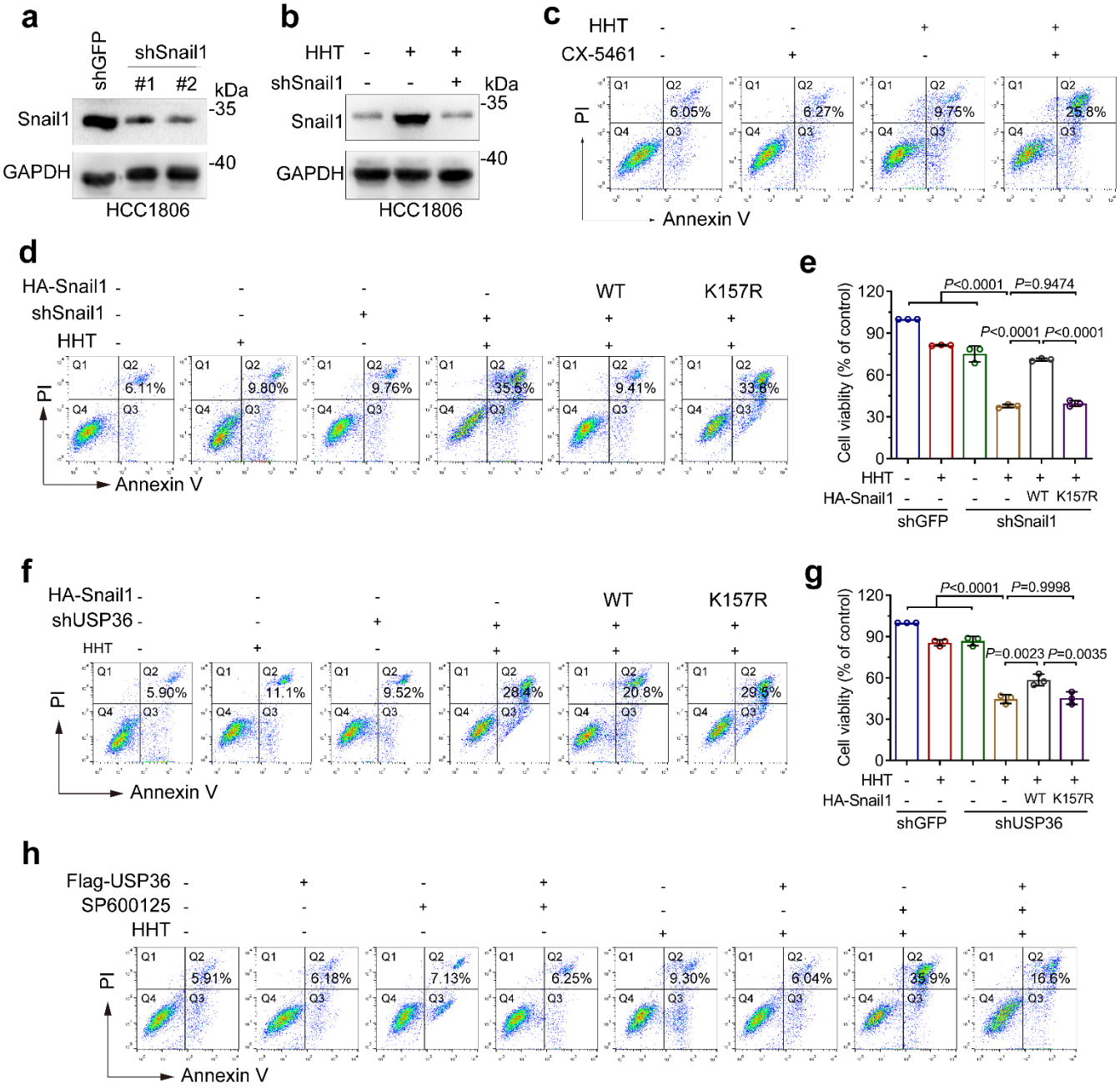

**Supplementary Figure S5. Ribotoxic stress promotes USP36 expression via activating the JNK signaling. (a)** HCC1806 cells were treated with or without HHT (20 ng/mL and hereafter) for 24 h. Cells were subjected to qPCR analyses for USP36 mRNA expression (n=3 biologically independent samples). **(b)** HCC1806 cells were treated with or without HHT in the presence or absence of a p38 inhibitor SB203580 (10 μM) for 24 h. Cells were subjected to western blot analyses. T-p38: total p38; p-p38: phospho-p38 (Thr180/Tyr182). **(c-d)** HCC1806 cells were treated with or without HHT in the presence or absence of a JNK inhibitor SP600125 (20 μM and hereafter) for 24 h. Cells were subjected to western blot analyses (c) or qPCR analyses (d, n=3 biologically independent samples). T-JNK: total JNK; p-JNK: phospho-JNK (Thr183/Tyr185). **(e)** HCC1806 cells were treated with or without HHT in the presence or absence of SP600125 for 24 h followed by cell fractionation procedure and western blot analyses. CP: Cytoplasm; NP: Nucleoplasm; No: Nucleolus. These experiments have been repeated for three times with similar results (b-c and e). Data were presented as mean ± SD and comparisons were performed with unpaired two-tailed Student's t-test (a) or one-way ANOVA with Tukey's test (d).

**Supplementary Figure S6**



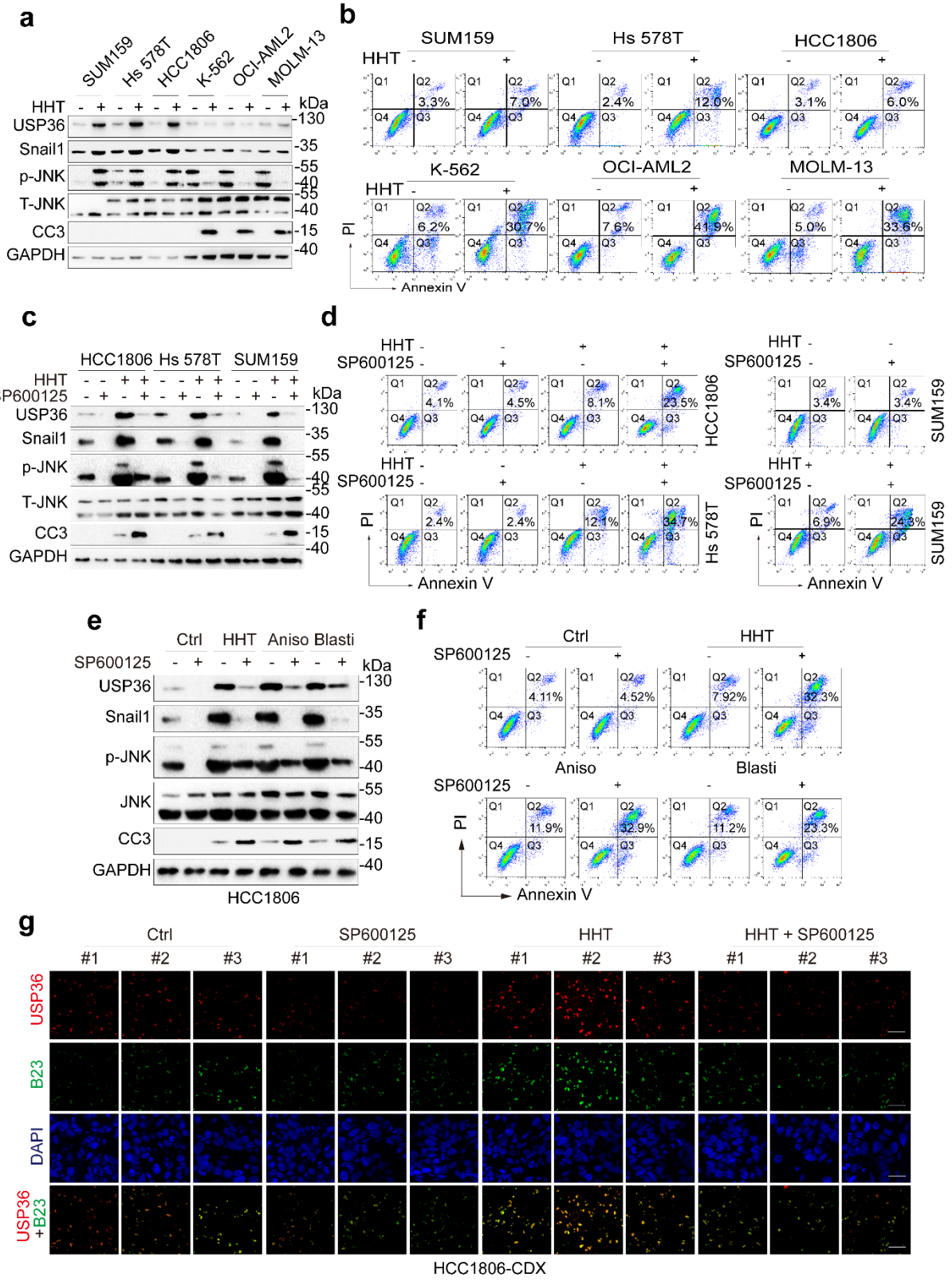
**Supplementary Figure S6. Ribotoxic stress activates the JNK-HSF1 signaling to promote USP36 transcription. (a)** The GEPIA 2 database (http://gepia2.cancer-pku.cn) was used to analyze the clinical relevance between JUN, FOS, TP53, YAP1, or HSF1, and USP36 in mRNA levels in breast cancers. **(b-d)** A schematic presentation depicts two putative HSF1-binding elements (P1 and P2) on the *USP36* gene promoter (b). Chromatin immunoprecipitation (ChIP) analyses using a specific HSF1 antibody or a control IgG were performed in HCC1806 cells. Specific primers for P1, P2, or negative control (NC) were used. Data derived from reverse transcription-PCR (RT-PCR) (c) or qPCR analyses (d) were shown. TSS: Transcription Start Site. **(e)** HEK-293 cells were transiently transfected with Flag-JNK1 for 48 h. Cells were subjected to western blot analyses. **(f)** HCC1806 cells were treated with or without HHT (20 ng/mL and hereafter) in the presence or absence of SP600125 (10 μM) for 24 h. Cells were subjected to western blot analyses. **(g)** HCC1806 cells were treated with an HSF1 inhibitor KRIBB11 (0, 10, 20, or 40 μM) for 24 h. Cells were subjected to western blot analyses. **(h)** HCC1806 cells were treated with or without HHT in the presence or absence of KRIBB11 (20 μM) for 24 h. These experiments have been repeated for three times with similar results (c-h). Data were presented as mean ± SD (d).

**Supplementary Figure S7**



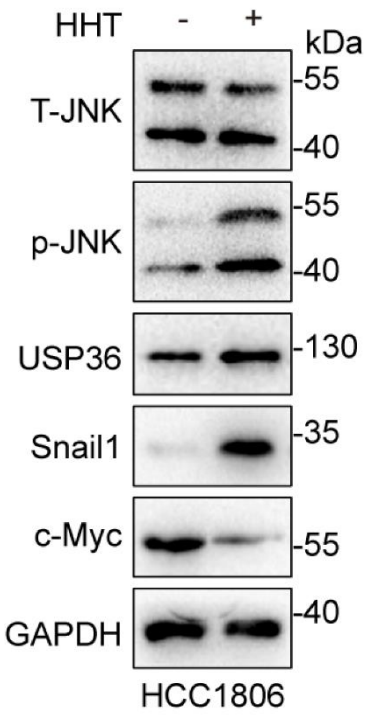
**Supplementary Figure S7. Activation of the nucleolar USP36-Snail1 axis promotes cancer cell survival in response to ribotoxic stress. (a)** HCC1806 cells stably expressing shGFP or shSnail1 (#1 or #2) were subjected to western blot analyses. **(b)** HCC1806 cells stably expressing shSnail1 were treated with HHT (20 ng/mL and hereafter) for 24h. Cells were subjected to western blot analyses. **(c)** HCC1806 cells were treated with or without HHT in the presence or absence of CX-5461 (200 nM) for 48 h, followed by FACS analyses. **(d)** HCC1806 cells stably expressing shSnail1 were infected with a recombinant lentivirus encoding HA-Snail1$^{WT}$ or HA-Snail1$^{K157R}$. Cells were treated with or without HHT for 48 h, followed by FACS analyses. **(e)** HCC1806 cells stably expressing shSnail1 were infected with a recombinant lentivirus encoding HA-Snail1$^{WT}$ or HA-Snail1$^{K157R}$. Cells were treated with or without HHT followed by MTS analyses (n=3 biologically independent samples). **(f)** HCC1806 cells stably expressing HA-Snail1 or HA-Snail1$^{K157R}$ were infected with lentivirus encoding shUSP36. Cells were treated with or without HHT for 48 h. Cells were subjected to FACS analyses. **(g)** HCC1806 cells stably expressing HA-Snail1$^{WT}$ or HA-Snail1$^{K157R}$ were infected with a recombinant lentivirus encoding shUSP36. Cells were treated with or without HHT, followed by MTS (n=3 biologically independent samples). **(h)** HCC1806 cells stably expressing Flag-USP36 were treated with or without HHT in the presence or absence of SP600125 (20 μM) for 48 h. Cells were subjected to FACS analyses. These experiments have been repeated for three times with similar results (a-c, d, f, and h). Data were presented as mean ± SD and comparisons were performed with one-way ANOVA with Tukey's test (e and g). The percentage of apoptotic cells was calculated by Annexin V+/PI+ cells (Q2) (c-d, f, and h).

**Supplementary Figure S8**



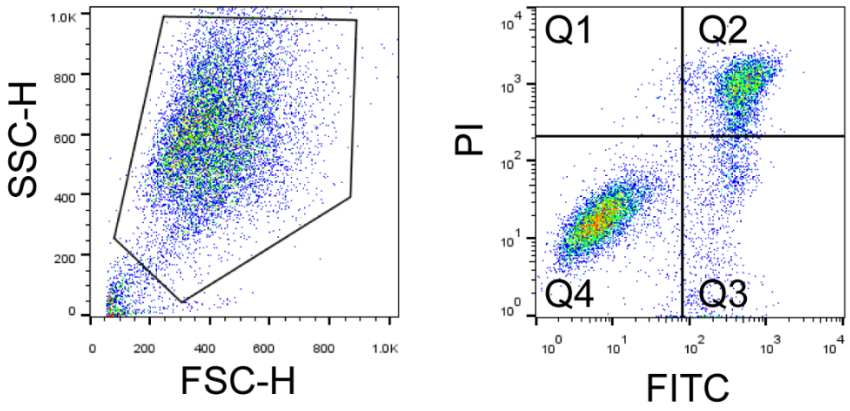
**Supplementary Figure S8. Inhibition of the JNK-USP36-Snail1 signaling sensitizes solid tumor cells to HHT. (a-b)** Triple-negative breast cancer (SUM159, Hs 578T, and HCC1806) and non-lymphocytic leukemia (K-562, OCI-AML2, and MOLM-13) cells were treated with or without HHT (20 ng/mL and hereafter). Cells were subjected to western blot analyses (a) or FACS analyses (b). **(c-d)** SUM159, Hs 578T, or HCC1806 cells were treated with or without SP600125 (20 μM and hereafter) in the presence or absence of HHT. Cells were subjected to western blot analyses (c) or FACS analyses (d). **(e-f)** HCC1806 cells were treated with HHT, anisomycin (Aniso, 50 ng/mL), or blasticidin (Blasti, 2 μg/mL) in the presence or absence of SP600125. Cells were subjected to western blot analyses (e) or FACS analyses (f). **(g)** Tumor sections derived from Figure 7J were subjected to immunofluorescence staining analyses for USP36 expression. These experiments have been repeated for three times with similar results (a-f). The percentage of apoptotic cells was calculated by Annexin V+/PI+ cells (Q2) (b, d, and f).

**Supplementary Figure S9**




**Supplementary Figure S9. HHT inhibits c-Myc expression.** HCC1806 cells were treated with or without HHT (20 ng/mL) for 24h. Cells were subjected to western blot analyses. This experiment has been repeated for three times with similar results.

**Supplementary Figure S10**




**Supplementary Figure S10.** The representative FACS gating strategies for analyzing Annexin V-FITC/ PI staining were shown.

**Supplementary Table 1.** The nucleolar Snail1 positive cells derived from Figure 1B and 1F out of approximately 200 cells were counted. The percentage of positive cells (%) was presented.

| Group | Total HCC1806 cells | Nucleolar Snail1 positive HCC1806 cells | Percentage of Nucleolar Snail1 positive HCC1806 cells (%) |
|---|---|---|---|
| Ctrl | 197 | 8 | 4.06 |
| HHT | 197 | 194 | 98.48 |
| Anisomycin | 201 | 197 | 98.01 |
| Puromycin | 208 | 202 | 97.12 |
| G418 | 216 | 211 | 97.69 |
| Blasticidin | 217 | 213 | 98.16 |

**Supplementary Table 2. A substantial amount of Snail1 proteins were attributable to the nucleolus.**

| Cell number | Fluorescence intensity in nucleoli | Fluorescence intensity in nucleus | Nucleolar/Nuclear fluorescence intensity (%) | Mean (%) |
|---|---|---|---|---|
| #1 | 21.906 | 115.648 | 18.94 | 18.24 |
| #2 | 24.92 | 120.161 | 20.74 | |
| #3 | 20.579 | 116.95 | 17.60 | |
| #4 | 15.286 | 97.532 | 15.67 | |

Nucleolar Snail1 positive HCC1806 cells (n=4) derived from Figure 1A were subjected to quantify the fluorescence intensity of nucleolar Snail1 and fluorescence intensity of nuclear Snail1 by Image J. The percentage (%) of fluorescence intensity of nucleolar Snail1/fluorescence intensity of nuclear Snail1 was presented in each of the 4 cells.

**Supplementary Table 3.** Putative nucleoli DUBs detected by immunofluorescence staining from www.proteinatlas.org/humanproteome/cell/nucleoli.

| Gene | Nucleolar localization verified | Nucleolar substrates |
|---|---|---|
| USP35 | No | Unkown |
| USP36 | Yes[1, 2, 3] | B23, c-Myc, Fibrillarim |
| USP37 | No | Unkown |
| USP44 | No | Unkown |
| USP46 | No | Unkown |
| OTUD5 | No | Unkown |
| OTUD7A | No | Unkown |
| MPND | No | Unkown |
| ATXN3 | No | Unkown |

**Supplementary Table 4.** Primers used in this study.

| Targets | Sequence | Application |
|---|---|---|
| GFP | 5′-GAAGCAGCACGACTTCTTC-3′ | shRNA |
| Human Snail1 | #1: 5′-CCACTCAGATGTCAAGAAGTA-3′<br>#2: 5′-CCAGGCTCGAAAGGCCTTCAA-3′ | shRNA |
| Human USP36 | #1: 5′-GCGGTCAGTCAGGATGCTATT-3′<br>#2: 5′-ACAGAACATCCAACGTCTTAA-3′ | shRNA |
| Human GAPDH | F: 5′-TGGACTCCACGACGTACTCA-3′<br>R: 5′-GAGGGGCCATCCACAGTCTTCT-3′ | qPCR |
| Human USP36 | F: 5′-AGCACTTTTCCCCCAGAACTG-3′<br>R: 5′-GGCTCCCAGATCTGCTGCTA-3′ | qPCR |
| Human Snail1 | F: 5′-GGCCCTGGCTGCTACAAGGC-3′<br>R: 5′-CTCGAGGGTCAGCGGGGACA-3′ | qPCR |
| Human 47S pre-rRNA | F: 5′-TGTCAGGCGTTCTCGTCTC-3′<br>R: 5′-AGCACGACGTCACCACATC-3′ | qPCR |
| Human E-cadherin | F: 5′-CGAGAGCTACACGTTCACGG-3′<br>R: 5′-GGGTGTCGAGGGAAAAATAGG-3′ | qPCR |
| Human USP36 NC | F: 5′-GTCTGCACATCCCAGGTGAGTCTG-3′<br>R: 5′-GCCTTTTCTCATGAGAATTATCAAAGACTTCTCACC-3′ | ChIP |
| Human USP36 P1 | F: 5′-AAGATGGGGTCTCACGATGTTGC-3′<br>R: 5′-GTCGTCCCAGATGCTTGGGAG-3′ | ChIP |
| Human USP36 P2 | F: 5′-CCTGTTTTGGAGCAGACATTTGGACC-3′<br>R: 5′-CCCTGCCATGTGCATCTCTTCATC-3′ | ChIP |
| Human 47S pre-rRNA | 5’-CGGAGGCCCAACCTCTCCGACGACA GGTCGCCAGAGGACAGCGTGTCAGC-3’ | Northern blotting |

**Supplementary References**


1. Endo A, Kitamura N, Komada M. Nucleophosmin/B23 regulates ubiquitin dynamics in nucleoli by recruiting deubiquitylating enzyme USP36. *The Journal of biological chemistry* **284**, 27918-27923 (2009).

2. Sun XX, He X, Yin L, Komada M, Sears RC, Dai MS. The nucleolar ubiquitin-specific protease USP36 deubiquitinates and stabilizes c-Myc. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 3734-3739 (2015).

3. Endo A*, et al.* Nucleolar structure and function are regulated by the deubiquitylating enzyme USP36. *Journal of cell science* **122**, 678-686 (2009).