Supplementary Figure 1

(a) HEK293T cells were transfected for 48 h with FLAG-tagged wild-type SENP3, $SENP3^{C532S}$, wild-type SENP5, $SENP5^{C713S}$ or empty vector control. After cell lysis immunoblotting against SENP3 or SENP5 was performed. (b) Transfection of HEK293T cells for 48 h with FLAG-tagged $SENP3^{C532S}$ or $SENP5^{C713S}$. Cells were fixed and analysed by immunofluorescence using FLAG antibody. (c) Whole cell lysates of U2OS WT or U2OS SENP3 KO cells were immunoblotted against SENP3, SUMO2/3 or β-Tubulin. (d) HeLa cells were transfected with two different siRNAs against SENP3, SENP5 or siControl for 72 h. Additionally cells were treated with TAK-981 (200 nM) or DMSO for 4 h directly before cell lysis. Afterwards lysates were immunoblotted against UTP14A or Vinculin. (e) HeLa cells were transfected with siRNAs against SENP3, SENP5, SENP3/5 or siControl. 72 h post transfection, endogenous SUMO2/3 or IgG control IP were performed and samples were analysed by immunoblotting using antibodies against UTP18, BMS1 or SUMO2/3. Input samples are shown in the left panel. SUMOylated proteins are indicated. Source data for a, c, d, e are provided as a Source Data file.

Supplementary Figure 2

(a) Quantification of the UTP14A protein signal in sucrose gradient density centrifugation. For quantification, the signal for UTP14A in each fraction of the gradient was determined and normalized to the input sample (representing 10% of the cleared cell extract that was loaded onto the gradient). After normalisation to the input, a scaling was performed enabling comparison of the different experiments with different overall signal intensities. The blue line represents the data of samples from control cells (siControl), while the red line represents SENP3/5 depleted cells. Two independent replicates were used for quantification. (b) & (c) UTP14A localization upon siRNA mediated depletion of SENP3 or SENP5 in HeLa cells. (b) Representative confocal images. Scale bar = 10 µm. Dashed lines represent the nucleus. (c) Quantification of the nucleolar vs nucleoplasmic ratio of the UTP14A intensity normalized to the mean nuclear intensity. 2-sided t tests were performed. Data are shown as mean ± standard deviation. (d) HEK293T cells were depleted for SENP3 and SENP5 by siRNA transfection for 72h. After 24 h cells were transfected with FLAG-tagged wild-type UTP14A or $UTP14^{K733R}$ encoding plasmids either in combination with an empty vector control or with a His-tagged SUMO2 construct for 48 h. Ni-NTA beads were used to enrich for SUMO2-UTP14A conjugates. Immunoblotting was performed against FLAG-tag, His or β-Tubulin. Input samples are shown in the left panel. The SUMOylated form of UTP14A is indicated by arrows. (e) UTP14A (red) and its main SUMOylation site K733 (yellow) were highlighted in the available SSU processome structure (PDB:7MQ8, https://doi.org/10.2210/pdb7MQ8/pdb). Source data for a, c, d are provided as a Source Data file.

Supplementary Figure 3

(a) Scheme of the pre-rRNA species detected by the 5'ITS1 (blue) or the ITS2 probe (orange), used in Figure 4b. (b) Validation of endogenous Halo-tagging by immunoblotting. Lysates of single clones of RPS3-Halo or RPL28-Halo cells were stained against RPS3 or RPL28. The clones marked with asterisks were chosen for further experiments. (c) Ribo-Halo fusion proteins were modelled into the available 40S and 60S ribosomal subunit structures (PDB:4V6X, https://doi.org/10.2210/pdb4V6X/pdb). (d) Whole-cell lysates from endogenously tagged HeLa cells (RPS3-Halo or RPL28-Halo) were separated by using sucrose density centrifugation. Peaks corresponding to (pre-)ribosomal complexes are marked on an absorbance profile at 260 nm (top). The presence of the indicated proteins in each fraction of the gradient was determined by western blotting using antibodies against Halo, RPL3, RPS9 or RPS3A (bottom). Experiments were performed with two replicates. (e) HeLa RPS3-Halo cells were depleted for SENP3 and SENP5 and were treated with both Halo-ligands.

In subsequent immunofluorescence imaging ‘old’ 40S ribosomal subunits are shown in red and ‘new’ 40S ribosomal subunits in green. Representative images are shown (upper panel). Lower panel: Quantification of the intensity of newly synthesized vs old ribosomes in the cytoplasm. 2-sided t testing was performed for statistical analysis. The data are shown as mean ± standard deviation. Source data for b, d, e are provided as a Source Data file.

Supplementary Figure 4

(a) Validation of SENP3 knock-down efficiency of samples used for whole cell proteome analysis (Figure 5a). All replicates used for MS were analysed by immunoblotting against SENP3 and Vinculin. (b), (c) Volcano plots of whole cell proteome analysis in U2OS cells (Figure 5a) transfected with siSENP3-1 (b) or siSENP3-2 (c). Highlighted are hits, that shared the same regulation with both siRNAs (log2 ratio ≥1; -log10 p-value ≥1.3), which were identified based on 2-sided Student´s t test analysis. The cell cycle regulator p21/CDKN1A is additionally highlighted. (d) Example of gating strategy used for PI staining analysis by flow cytometry. Cells were plotted with the FSC-A against the SSC-A channel. The living population was gated and then plotted for the PI-A signal as a histogram. Only PI positive cells were gated. G1, S and G2 phases were determined based on the PI signal. The same gates were transferred to all samples in the same experiment. Source data for a are provided as a Source Data file.

Supplementary Figure 5

(a) Validation of SENP3 knock-down efficiency of samples used for whole cell proteome analysis (Figure 6a). All replicates used for MS were analysed by immunoblotting against SENP3 and Vinculin. (b) & (c) Volcano plots of whole cell proteome analysis in Saos- 2 cells (Figure 6a) transfected with siSENP3-1 (b) or siSENP3-2 (c). Highlighted are hits, that shared the same regulation with both siRNAs (log2 ratio ≥1; -log10 p-value ≥1.3), which were identified based on 2-sided Student´s t test analysis. The cyclin-dependent kinase, CDK6, is additionally highlighted. (d) RNA-Seq analysis of U2OS cells transfected with siSENP3-1 (Y-axis) or siSENP3-2 (X-axis) compared to control siRNA transfection. Down-regulated RNAs are visualized in a XY diagram, comparing either of the two SENP3 siRNAs against the control. Only significant hits (log2 ratio ≥ 1; p-value < 0.05) are displayed. CDK6 is highlighted in red. Experiments were performed with four replicates. (e) U2OS cells were transfected with siControl or two different siRNAs against SENP3 for 72 h. MG132 (10 μM) or DMSO was added for 15 h before lysing the cells. Lysates were immunoblotted against CDK6 or Vinculin. (f) Whole cell lysate samples from Fig. 6f were immunoblotted against SENP5, SENP3, Vinculin or β-Tubulin. Source data for a, e, f are provided as a Source Data file. * unspecific signal.

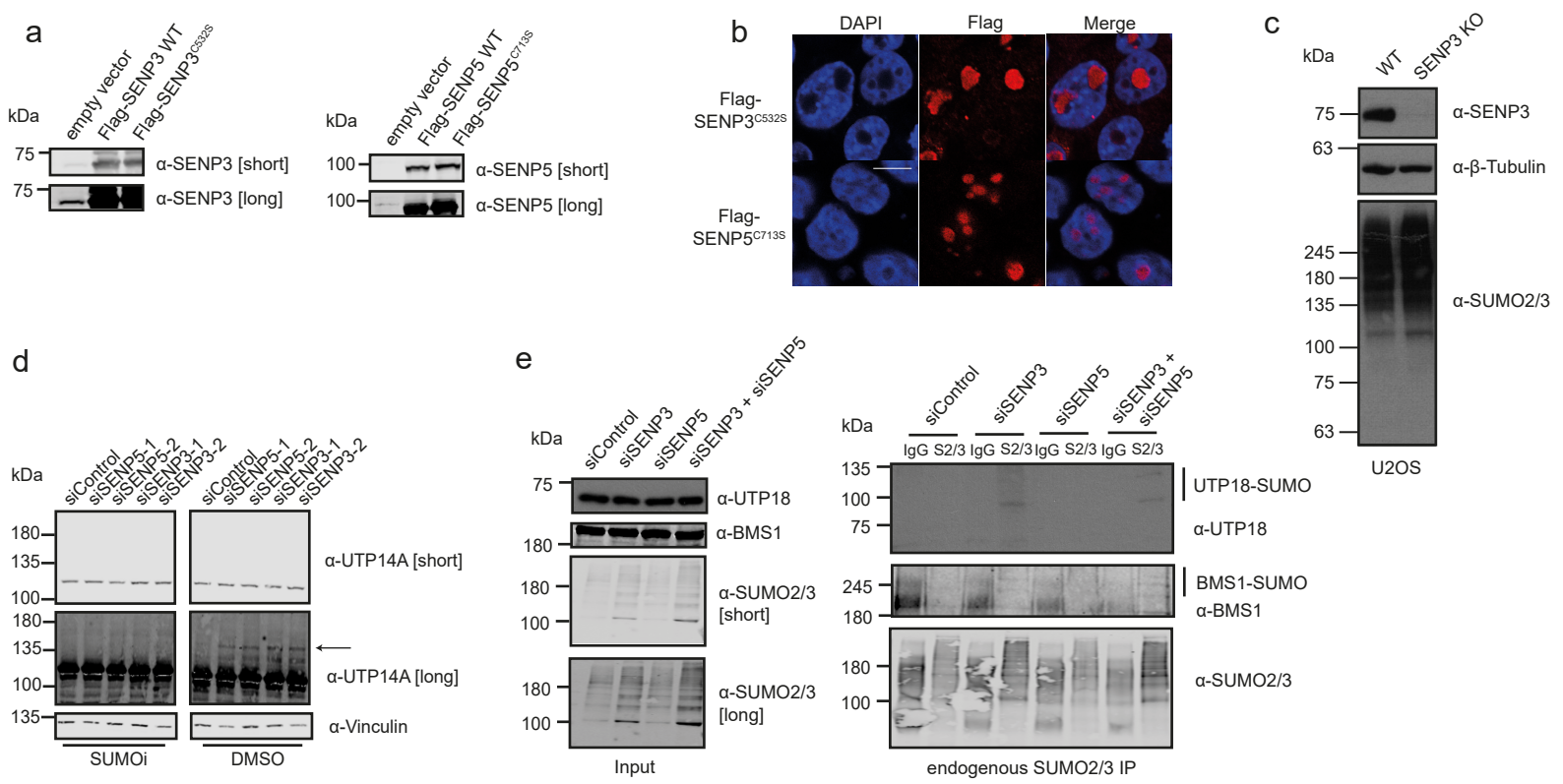
Supplementary Figure 6

(a) PI staining performed as shown before in Figure 7a in parental RPE1 cells (left) or RPE1$^{\Delta p53}$ cells (right) transfected with siCDK6 (green) or siControl (blue) for 72 h. Significance level was calculated by 2-sided t testing. Experiments were performed with four replicates and are shown as mean ± standard deviation. Mean values of percentages are added above the bars. (b) Proliferation assay of parental RPE1 cells (left) or RPE1$^{\Delta p53}$ cells (right) transfected with siSENP3 or siControl. The cell index was measured by an XCELLigence RTCA SP instrument for 100 h. Experiments were performed as duplicates and the mean value of both replicates is shown here. (c) Cell cycle analysis using PI staining of U2OS cells transfected for 72 h with siRNAs as indicated. Percentages of cells in the S phase were determined and significance level was calculated using 2-sided t testing. Experiments were performed with four replicates and are shown as mean ± standard deviation. (d) Whole cell lysates related to Figure 7c were immunoblotted against phospho-RB (S807/811). (e) U2OS cells stably expressing FLAG-CDK6 under a doxycycline-inducible promoter were transfected with control siRNA or siRNA against SENP3 (siSENP3-2) for 72 h. FLAG-CDK6 expression was induced by supplementing

the media with doxycycline (0.05 µg/ml) 15 h before cell lysis. Immunoblots were performed as indicated. Source data for a-e are provided as a Source Data file.

Supplementary Figure 7

(a) U2OS cells or (b) Saos-2 cells were transfected with the indicated siRNAs. 72 h after transfection cells were lysed and lysates were immunoblotted against CDK6 or Vinculin. (c) Lysates related to Figure 9 b, c were immunoblotted as indicated to validate proper efficiency of protein depletion. (d) Parental RPE1 cells were transfected for 72h with the indicated siRNAs. Cell lysates were analysed by immunoblotting as indicated. Source data for a-d are provided as a Source Data file.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Flag-SENP3C532S
75
75
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kDa
empty vector
Flag-SENP5 WT
Flag-SENP5C713S
100
100
α-SENP5 [short]
α-SENP5 [long]
b
DAPI
Flag
Merge
Flag-SENP3C532S
Flag-SENP5C713S
c
kDa
WT
SENP3 KO
75
63
α-SENP3
α-β-Tubulin
245
180
135
100
75
63
α-SUMO2/3
U2OS
d
kDa
siControl
siSENP5-1
siSENP5-2
siSENP3-1
siSENP3-2
180
135
100
α-UTP14A [short]
α-UTP14A [long]
135
α-Vinculin
SUMOi
DMSO
e
kDa
siControl
siSENP3
siSENP5
siSENP3 + siSENP5
75
α-UTP18
180
α-BMS1
180
100
α-SUMO2/3 [short]
α-SUMO2/3 [long]
Input
kDa
siControl
siSENP3
siSENP5
siSENP3 + siSENP5
IgG S2/3 IgG S2/3 IgG S2/3 IgG S2/3
135
100
75
UTP18-SUMO
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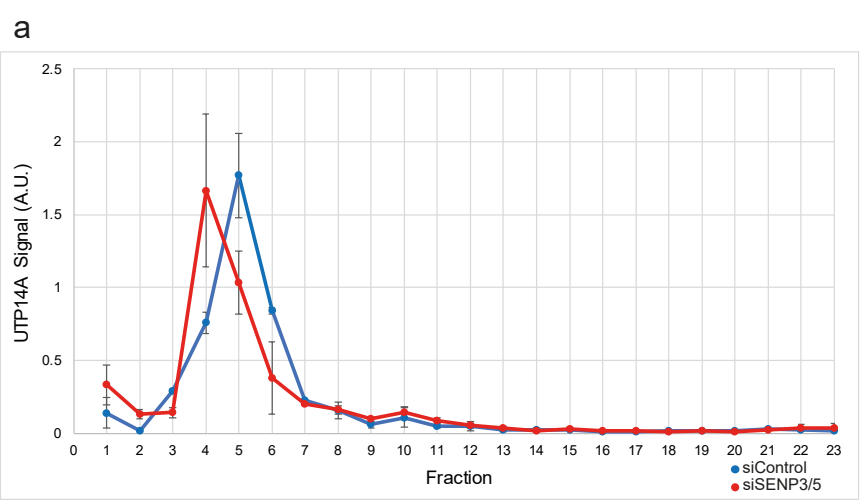
endogenous SUMO2/3 IP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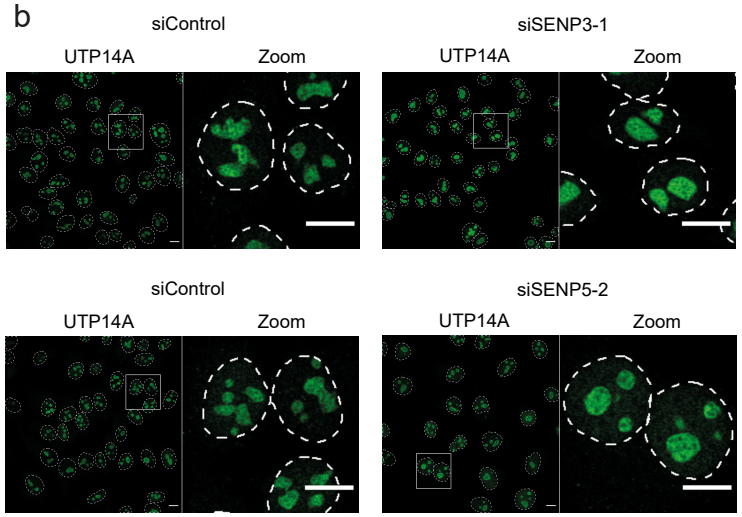
UTP14A Signal (A.U.)
Fraction
siControl
siSENP3/5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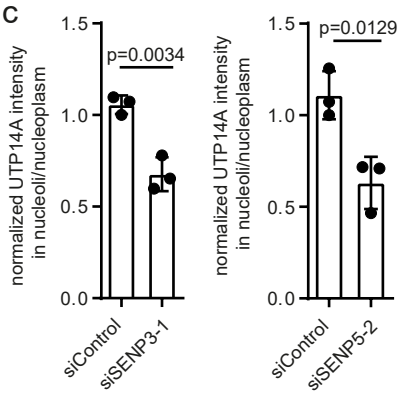
siControl
siSENP3-1
siControl
siSENP5-2
UTP14A
Zoom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p=0.0129
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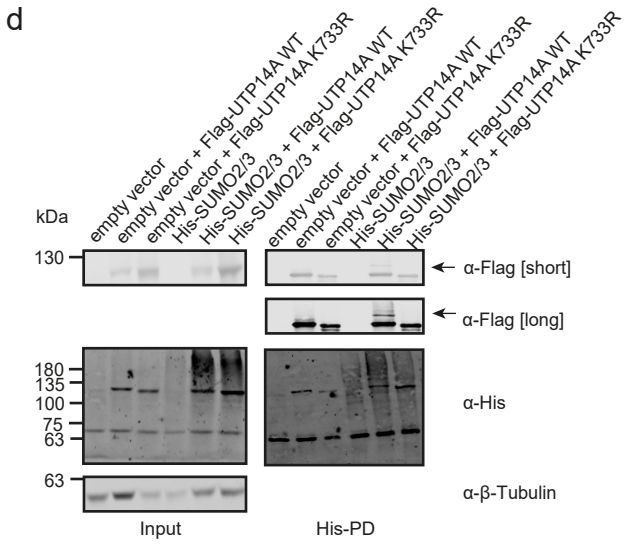
siSENP3-1
siSENP5-2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empty vector + Flag-UTP14A WT
empty vector + Flag-UTP14A K733R
His-SUMO2/3
His-SUMO2/3 + Flag-UTP14A WT
His-SUMO2/3 + Flag-UTP14A K733R
kDa
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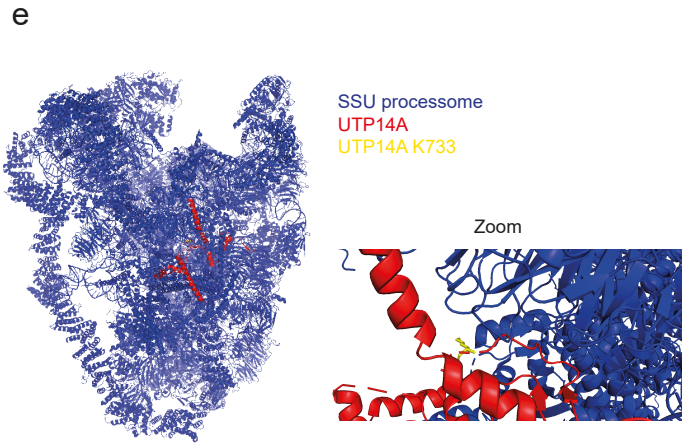
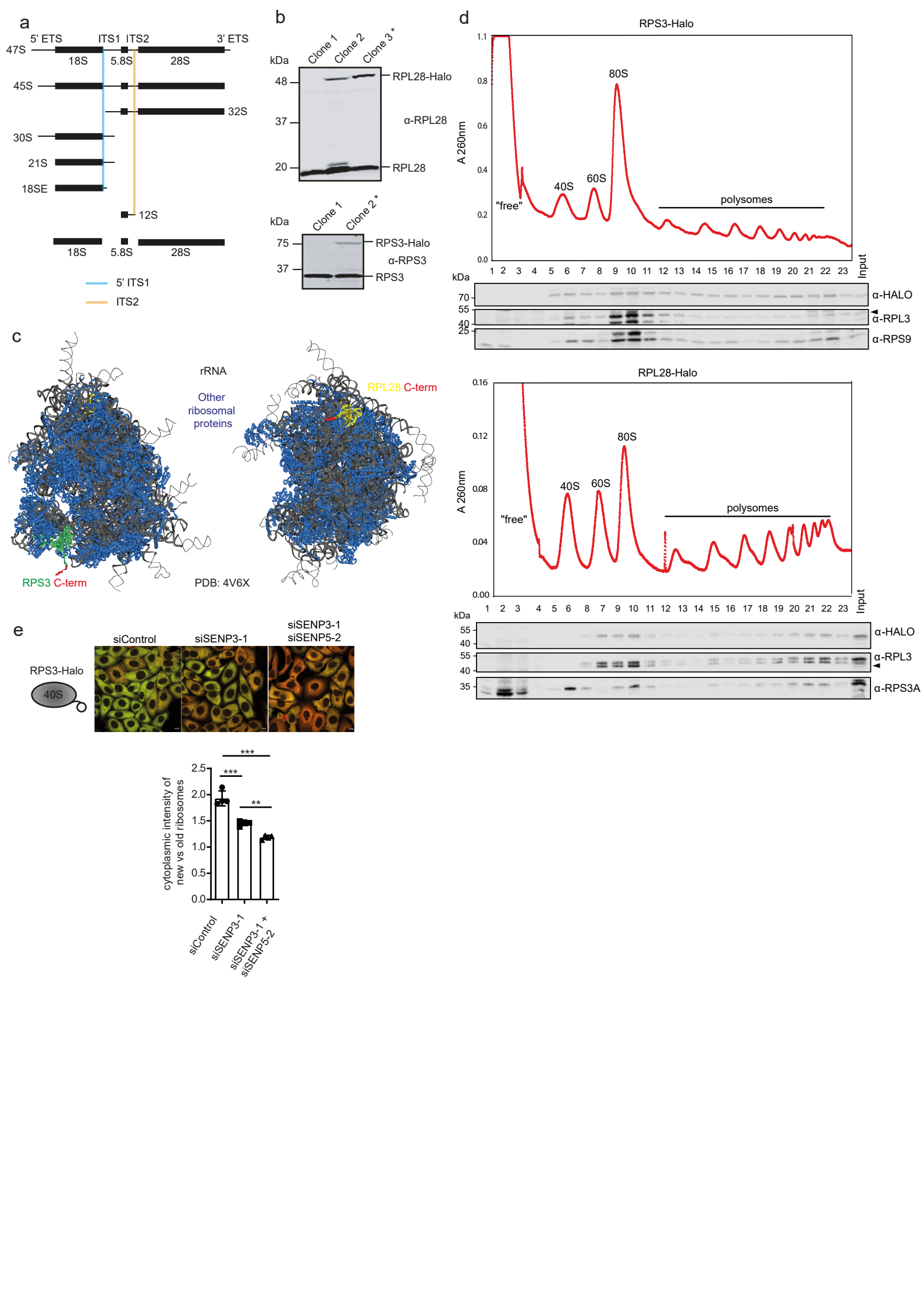
α-Flag [long]
α-His
α-β-Tubulin
Input
His-PD


e
SSU processome
UTP14A
UTP14A K733
Zoom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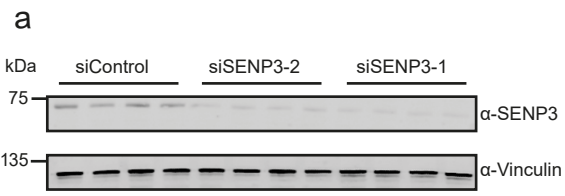
5' ETS
ITS1
ITS2
3' ETS
47S
18S
5.8S
28S
45S
32S
30S
21S
18SE
12S
18S
5.8S
28S
5' ITS1
ITS2
b
kDa
Clone 1
Clone 2
Clone 3*
48
37
20
RPL28-Halo
α-RPL28
RPL28
kDa
Clone 1
Clone 2*
75
37
RPS3-Halo
α-RPS3
RPS3
c
rRNA
Other ribosomal proteins
RPL28 C-term
RPS3 C-term
PDB: 4V6X
d
RPS3-Halo
A 260nm
"free"
40S
60S
80S
polysomes
Input
kDa
70
55
40
25
α-HALO
α-RPL3
α-RPS9
RPL28-Halo
A 260nm
"free"
40S
60S
80S
polysomes
Input
kDa
55
40
55
40
35
α-HALO
α-RPL3
α-RPS3A
e
RPS3-Halo
40S
siControl
siSENP3-1
siSENP3-1
siSENP5-2
cytoplasmic intensity of new vs old ribosomes
***
***
**
siControl
siSENP3-1
siSENP3-1 + siSENP5-2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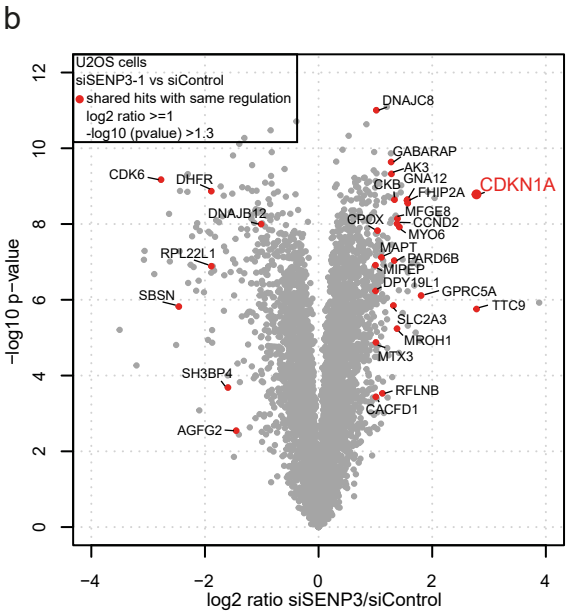
kDa
siControl
siSENP3-2
siSENP3-1
75
α-SENP3
135
α-Vinculin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U2OS cells
siSENP3-1 vs siControl
shared hits with same regulation
log2 ratio >=1
-log10 (pvalue) >1.3
−log10 p−value
log2 ratio siSENP3/siControl
CDKN1A
DNAJC8
GABARAP
AK3
GNA12
CKB
FHIP2A
CDK6
DHFR
DNAJB12
MFGE8
CPOX
CCND2
MYO6
MAPT
RPL22L1
PARD6B
MIPEP
DPY19L1
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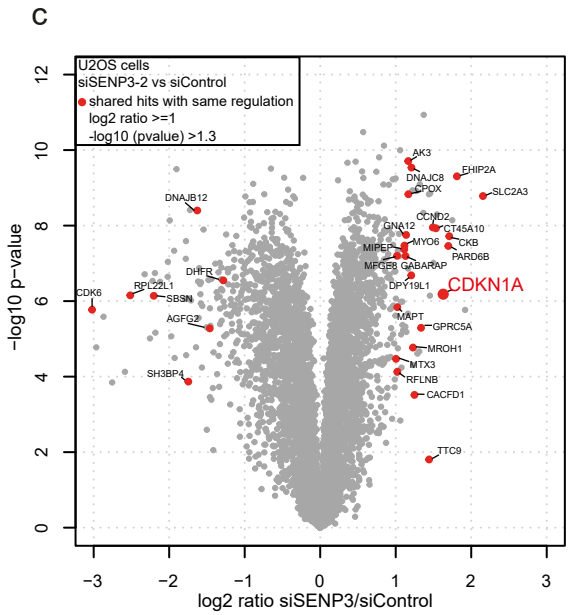
c
U2OS cells
siSENP3-2 vs siControl
shared hits with same regulation
log2 ratio >=1
-log10 (pvalue) >1.3
−log10 p−value
log2 ratio siSENP3/siControl
CDKN1A
AK3
EHIP2A
DNAJC8
CPOX
SLC2A3
DNAJB12
CCND2
GNA12
CT45A10
MYO6
CKB
MIPEP
PARD6B
MFGE8
GABARAP
DPY19L1
DHFR
CDK6
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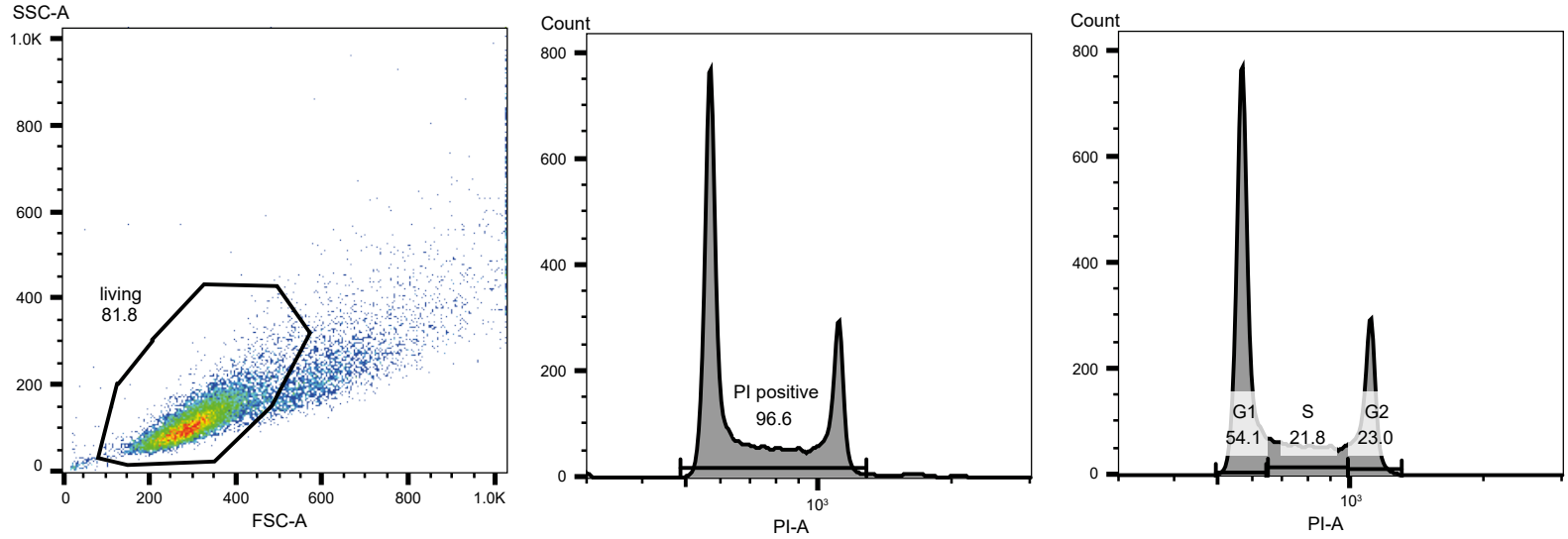
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FSC-A
living
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Count
PI positive
96.6
PI-A
Count
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S
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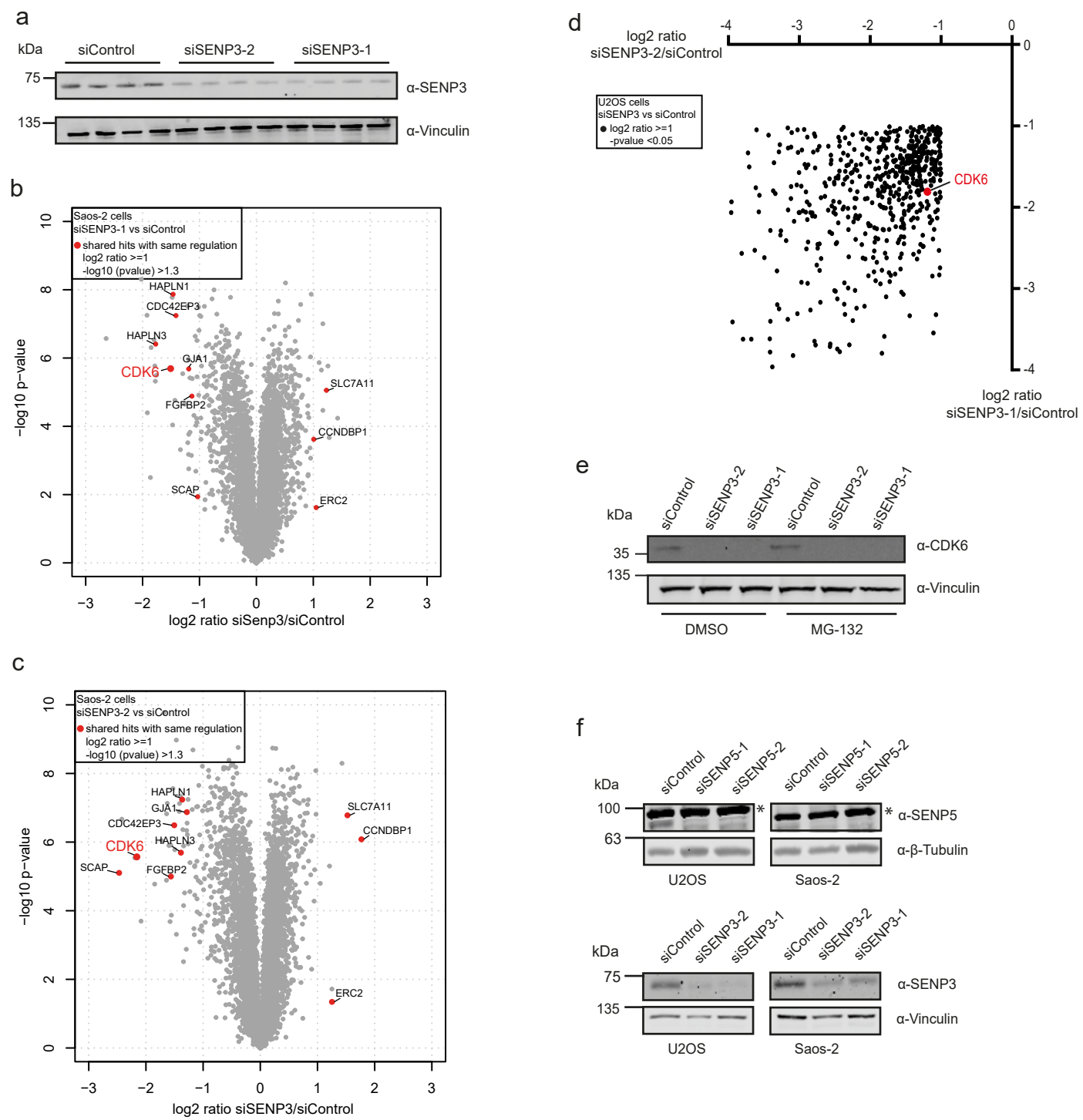
PI-A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kDa
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α-Vinculin
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siSENP3-1 vs siControl
shared hits with same regulation
log2 ratio >=1
-log10 (pvalue) >1.3
HAPLN1
CDC42EP3
HAPLN3
GJA1
CDK6
SLC7A11
FGFBP2
CCNDBP1
SCAP
ERC2
−log10 p−value
log2 ratio siSenp3/siControl
c
Saos-2 cells
siSENP3-2 vs siControl
shared hits with same regulation
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-log10 (pvalue) >1.3
HAPLN1
GJA1
SLC7A11
CDC42EP3
CCNDBP1
CDK6
HAPLN3
SCAP
FGFBP2
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log2 ratio siSENP3/siControl
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log2 ratio
siSENP3-2/siControl
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siSENP3 vs siControl
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-pvalue <0.05
CDK6
log2 ratio
siSENP3-1/siControl
e
kDa
siControl
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siSENP3-1
siControl
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siSENP3-1
35
α-CDK6
135
α-Vinculin
DMSO
MG-132
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63
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Saos-2
kDa
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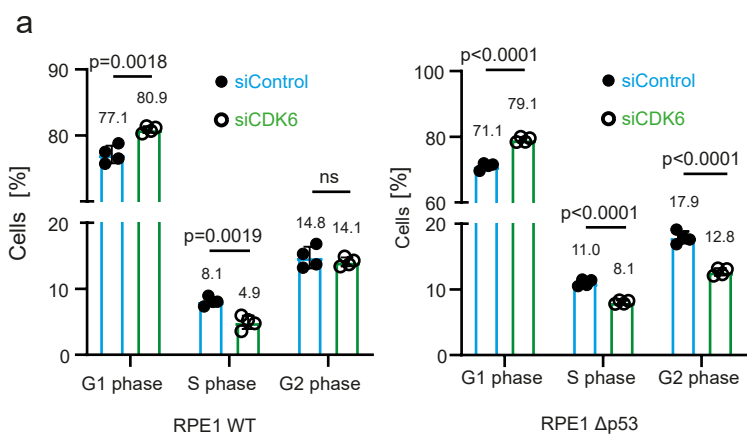
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p=0.0018
77.1
80.9
siControl
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ns
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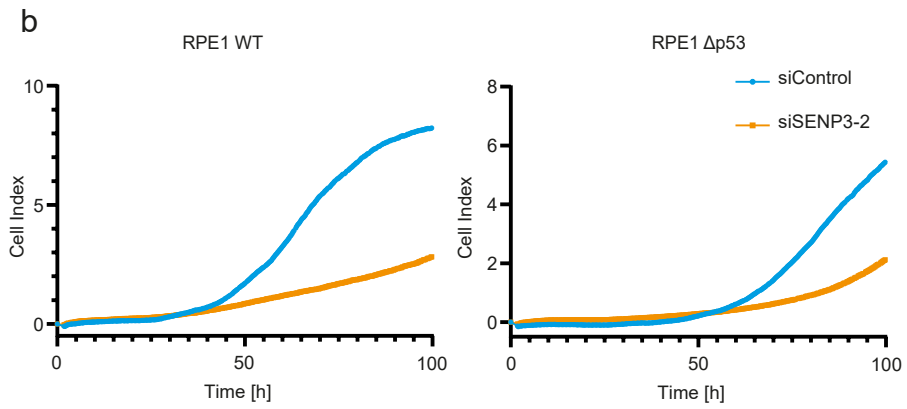
8.1
4.9
14.8
14.1
Cells [%]
G1 phase
S phase
G2 phase
RPE1 WT
p<0.0001
71.1
79.1
p<0.0001
11.0
8.1
p<0.0001
17.9
12.8
RPE1 Δp53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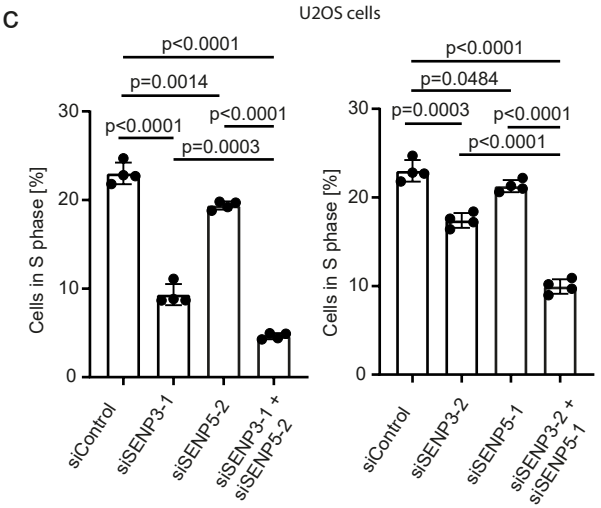
RPE1 WT
RPE1 Δp53
siControl
siSENP3-2
Cell Index
Time [h]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U2OS cells
p<0.0001
p=0.0014
p<0.0001
p<0.0001
p=0.0003
Cells in S phase [%]
siControl
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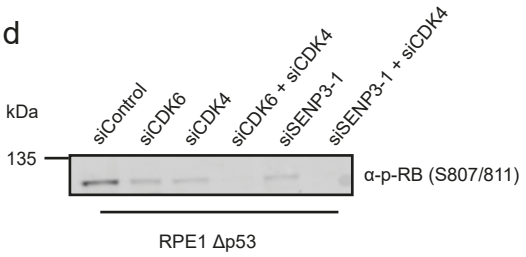
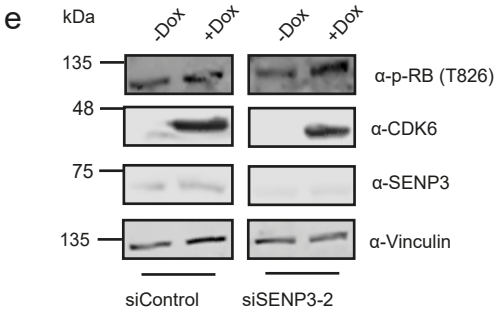
siSENP5-2
siSENP3-1 + siSENP5-2
p<0.0001
p=0.0484
p=0.0003
p<0.0001
p<0.0001
siControl
siSENP3-2
siSENP5-1
siSENP3-2 + siSENP5-1


d
kDa
siControl
siCDK6
siCDK4
siCDK6 + siCDK4
siSENP3-1
siSENP3-1 + siCDK4
135
α-p-RB (S807/811)
RPE1 Δp53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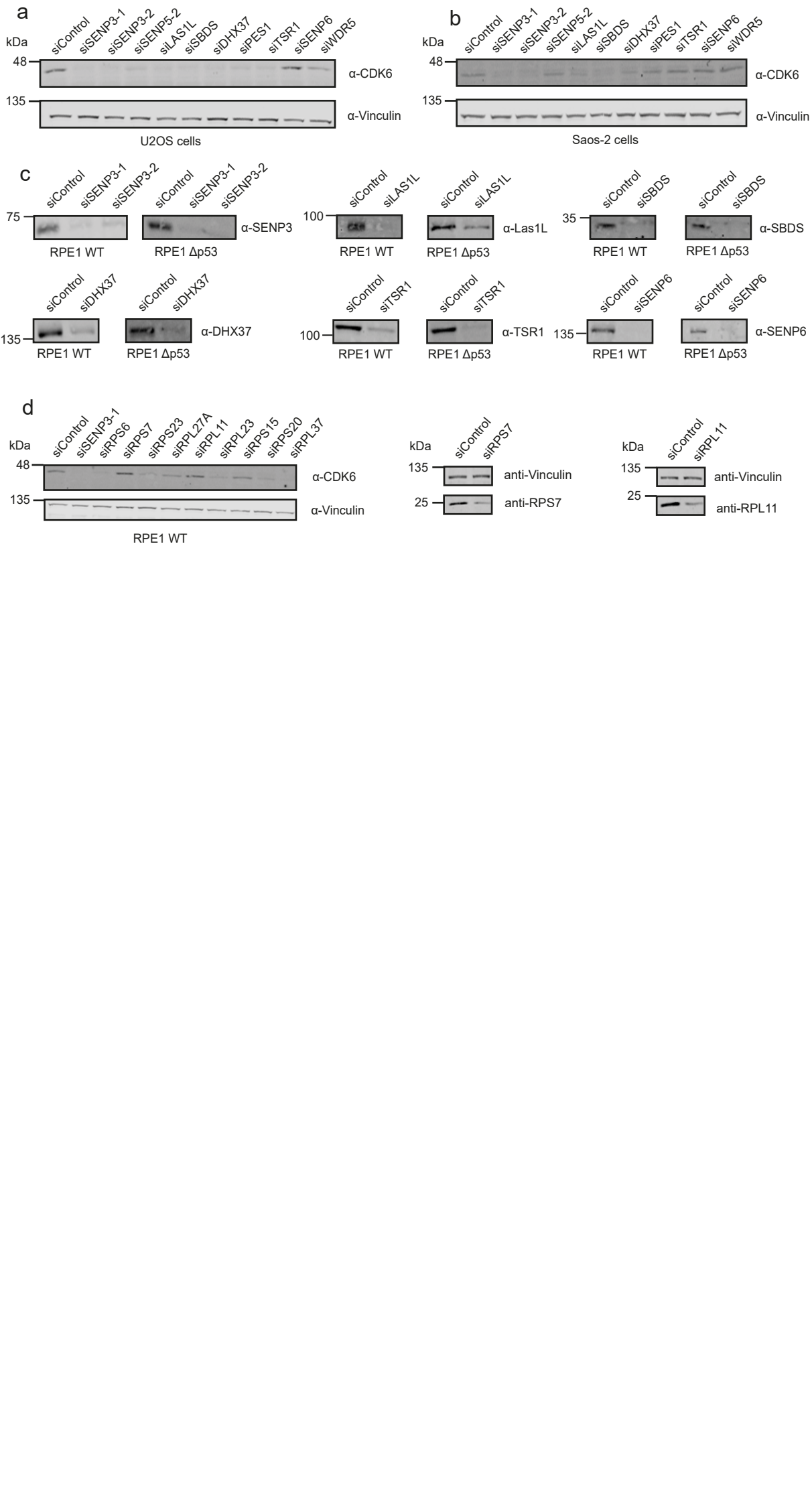
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siSENP6
siWDR5
48
α-CDK6
135
α-Vinculin
U2OS cells
b
kDa
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siSENP3-2
siSENP5-2
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siSBDS
siDHX37
siPES1
siTSR1
siSENP6
siWDR5
48
α-CDK6
135
α-Vinculin
Saos-2 cells
c
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siSENP3-2
75
α-SENP3
RPE1 WT
RPE1 Δp53
siControl
siLAS1L
100
α-Las1L
RPE1 WT
RPE1 Δp53
siControl
siSBDS
35
α-SBDS
RPE1 WT
RPE1 Δp53
siControl
siDHX37
135
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RPE1 WT
RPE1 Δp53
siControl
siSENP6
135
α-SENP6
RPE1 WT
RPE1 Δp53
d
kDa
siControl
siSENP3-1
siRPS6
siRPS7
siRPS23
siRPL27A
siRPL11
siRPL23
siRPS15
siRPS20
siRPL37
48
α-CDK6
135
α-Vinculin
RPE1 WT
kDa
siControl
siRPS7
135
anti-Vinculin
25
anti-RPS7
kDa
siControl
siRPL11
135
anti-Vinculin
25
anti-RPL11