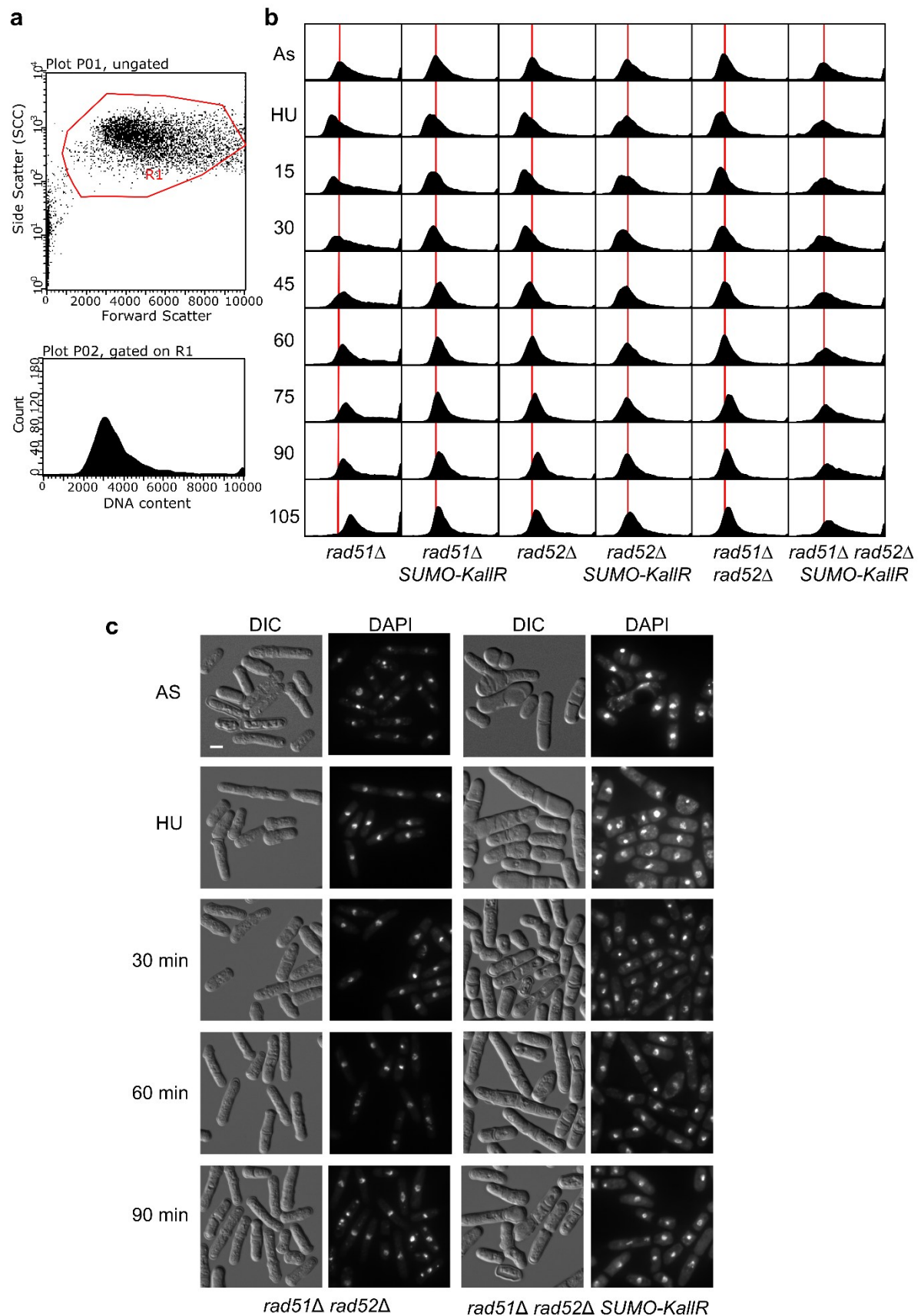
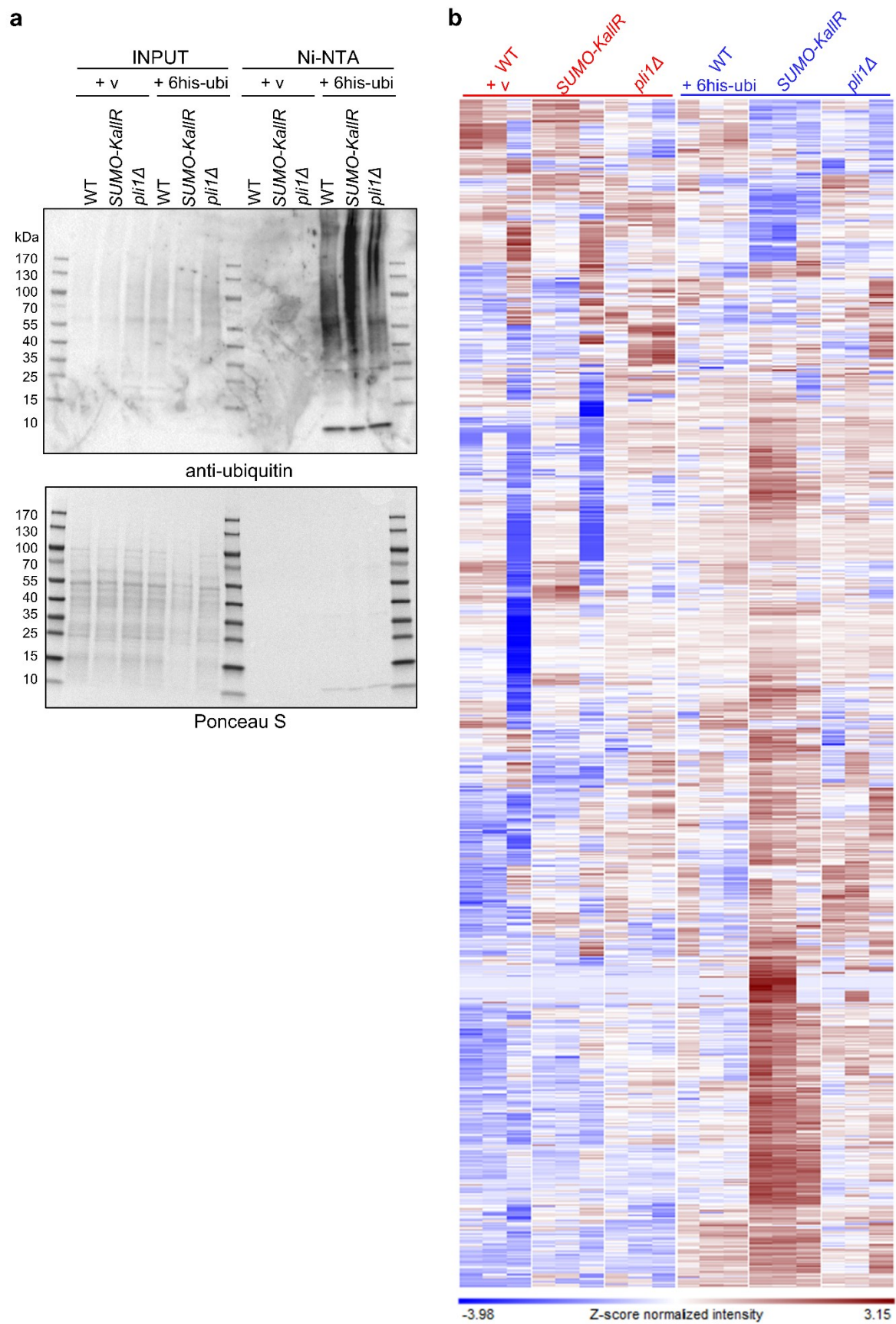




Supplementary Figure 1: SUMO chains enable completion of disturbed replication in HR-deficient cells.
a Gating strategy for conducted flow cytometry analysis; upper panel: ungated SSC-FSC plot; lower panel:

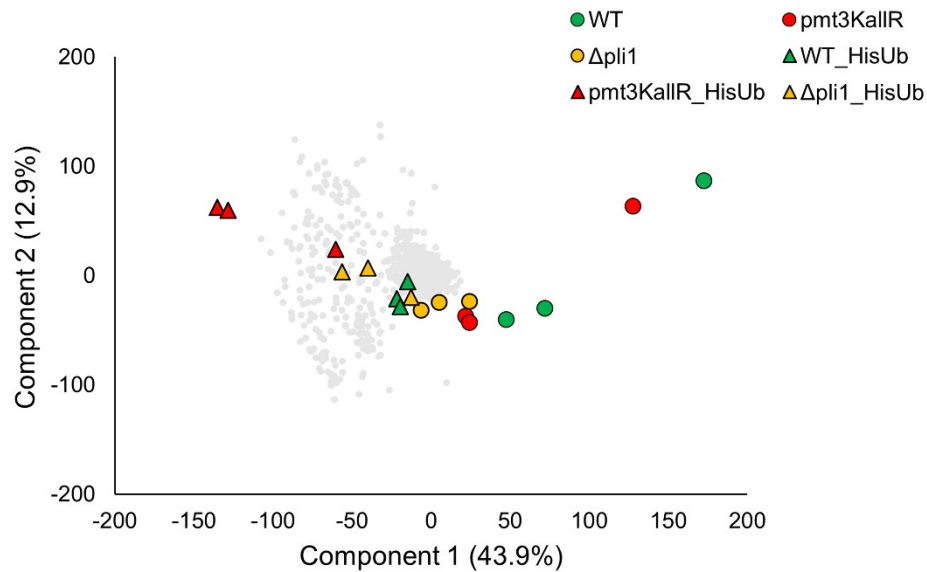
gated histogram based on SSC-FSC plot. **b** Flow cytometry analysis of DNA content in indicated strains. Logarithmically growing cells (As, asynchronous cells) were exposed to 20 mM HU for 4 h (HU time point) and then released into fresh, HU-free, rich YES medium at 30°C to monitor S-phase progression in the 15 min intervals after release. Red line marks G2 phase in asynchronous cells. **c** Representative fluorescence microscopy images of the indicated strains. Cells were treated as in (a), then collected at 30 min intervals after release, fixed with ethanol, and stained with DAPI to visualize nuclei. Differential interference contrast (DIC) images show cell morphology. Scale bar = 5 μ m



Supplementary Figure 2: Changes in protein ubiquitination upon SUMO chain depletion and *pli1+* deletion (related to Figure 4). **a** Ubiquitination pattern from indicated strains transformed with empty

vector (marked as '+v') or pREP1-6His-ubiquitin (marked as '+6his-ubi'). Cells were disrupted in denaturing conditions and subjected to Ni-NTA pulldown. Ubiquitination status of precipitated proteins was examined with anti-ubiquitin antibody. Ponceau S was included for both Western blots to show the amount of protein loaded onto the gels. Representative blots from n=3 biological replicates used for mass spectrometry analysis. **b** Heat map presenting the effects of SUMO chains depletion and Pli1 deletion on ubiquitination of proteins. Ubiquitination pattern from indicated strains transformed with empty vector (marked as '+v', in red font) or pREP1-6His-ubiquitin (marked as '+6his-ubi', in blue font). Only statistically significant changes are presented.

a

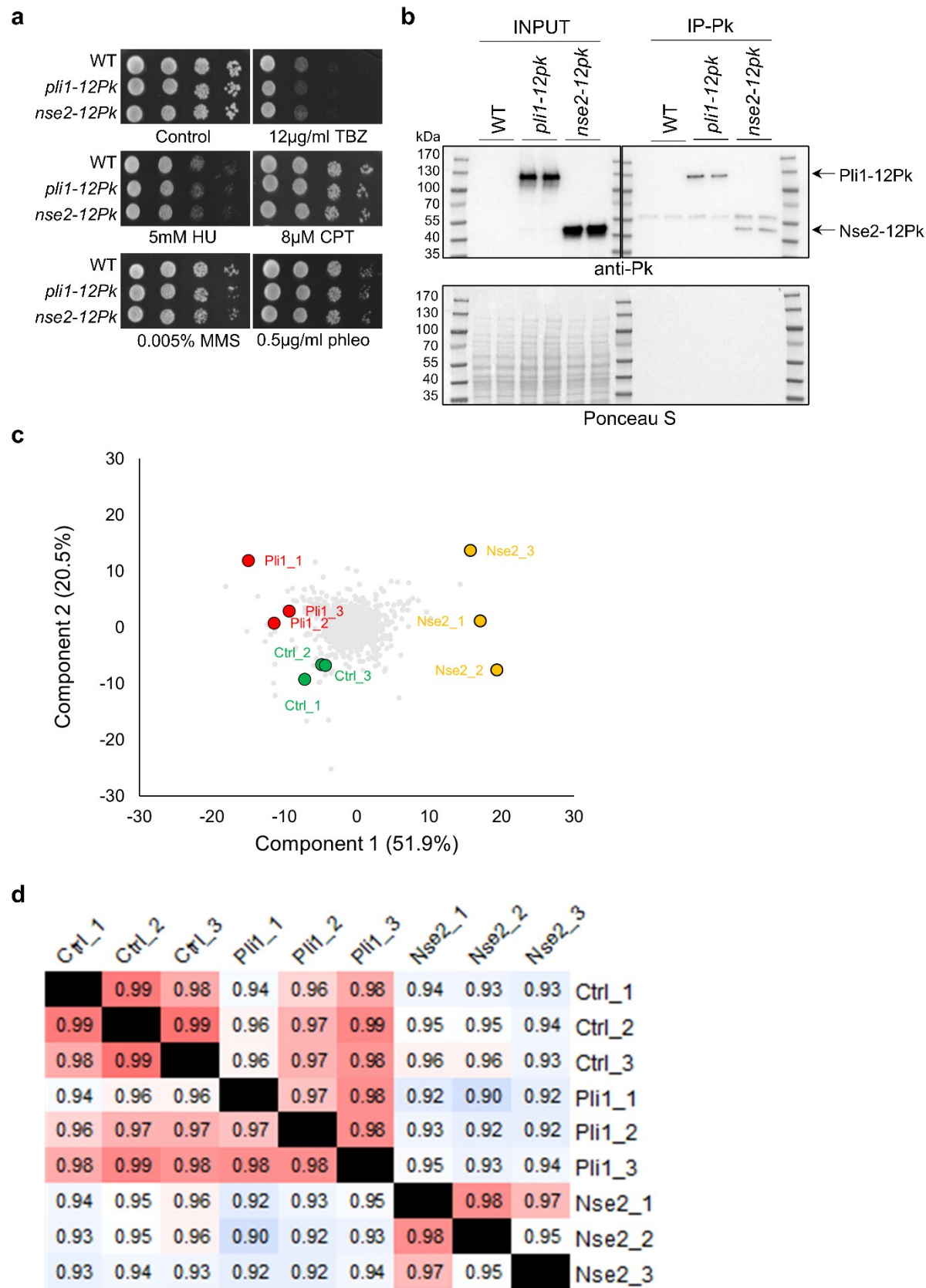


b

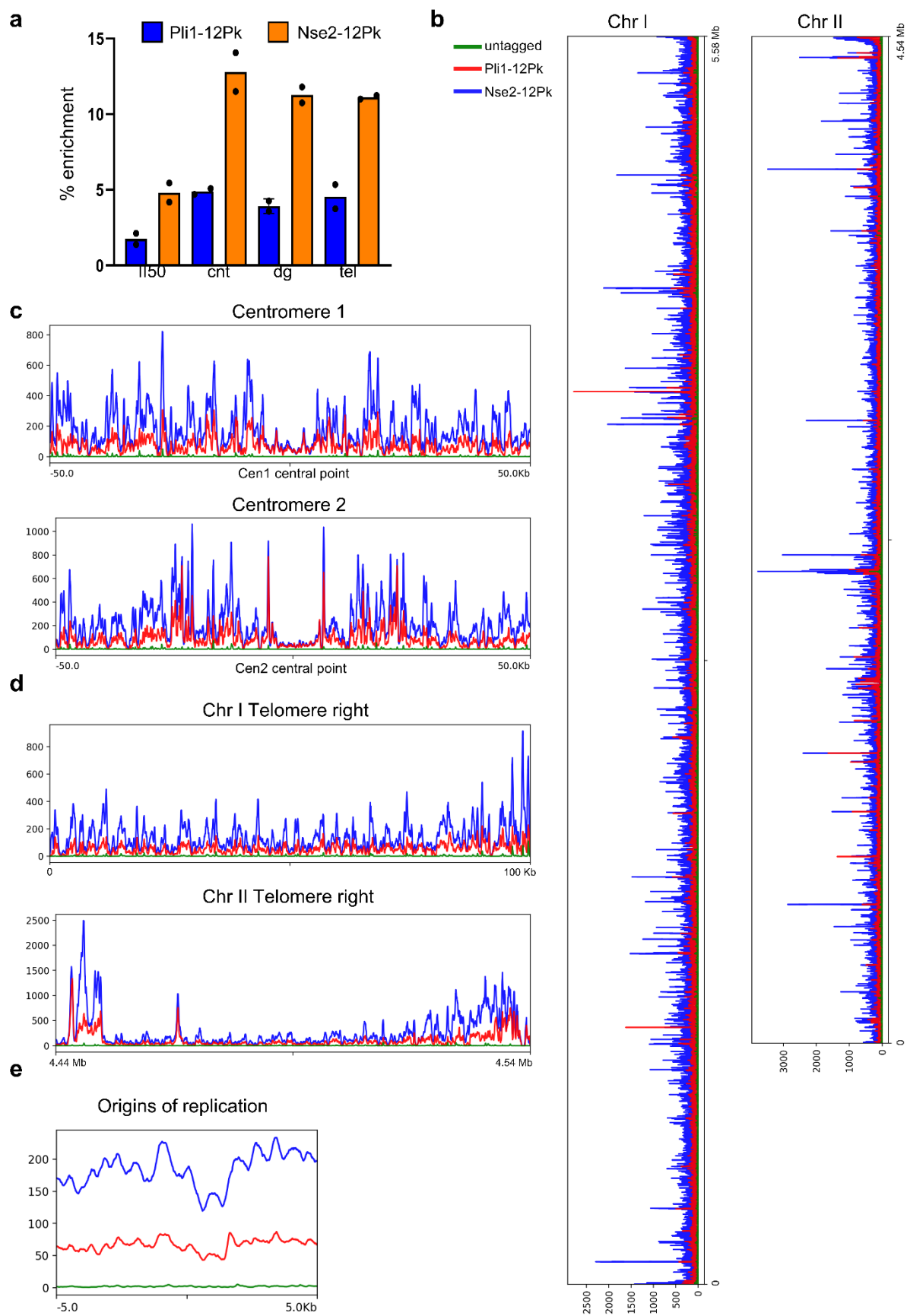
WT_1	WT_2	WT_3	pmt3KallR_1	pmt3KallR_2	pmt3KallR_3	Δpli1_1	Δpli1_2	Δpli1_3	WT_HisUb_1	WT_HisUb_2	WT_HisUb_3	pmt3KallR_HisUb_1	pmt3KallR_HisUb_2	pmt3KallR_HisUb_3	Δpli1_HisUb_1	Δpli1_HisUb_2	Δpli1_HisUb_3	
	0.89	0.77	0.85	0.86	0.79	0.8	0.82	0.82	0.8	0.8	0.83	0.58	0.61	0.69	0.75	0.74	0.73	WT_1
0.89		0.76	0.84	0.87	0.75	0.84	0.83	0.84	0.78	0.85	0.86	0.59	0.61	0.7	0.79	0.78	0.74	WT_2
0.77	0.76		0.72	0.72	0.82	0.71	0.73	0.75	0.72	0.7	0.69	0.62	0.62	0.66	0.69	0.68	0.67	WT_3
0.85	0.84	0.72		0.91	0.76	0.87	0.81	0.8	0.83	0.8	0.85	0.58	0.61	0.71	0.86	0.78	0.8	pmt3KallR_1
0.86	0.87	0.72	0.91		0.76	0.89	0.84	0.81	0.83	0.8	0.84	0.59	0.61	0.71	0.86	0.8	0.77	pmt3KallR_2
0.79	0.75	0.82	0.76	0.76		0.73	0.72	0.72	0.75	0.68	0.69	0.59	0.61	0.65	0.74	0.67	0.72	pmt3KallR_3
0.8	0.84	0.71	0.87	0.89	0.73		0.87	0.84	0.86	0.82	0.85	0.6	0.62	0.75	0.92	0.84	0.82	Δpli1_1
0.82	0.83	0.73	0.81	0.84	0.72	0.87		0.91	0.82	0.83	0.82	0.63	0.63	0.77	0.82	0.84	0.77	Δpli1_2
0.82	0.84	0.75	0.8	0.81	0.72	0.84	0.91		0.75	0.83	0.8	0.61	0.59	0.73	0.76	0.8	0.75	Δpli1_3
0.8	0.78	0.72	0.83	0.83	0.75	0.86	0.82	0.75		0.81	0.84	0.6	0.66	0.76	0.88	0.8	0.85	WT_HisUb_1
0.8	0.85	0.7	0.8	0.8	0.68	0.82	0.83	0.83	0.81		0.9	0.63	0.63	0.76	0.78	0.84	0.77	WT_HisUb_2
0.83	0.86	0.69	0.85	0.84	0.69	0.85	0.82	0.8	0.84	0.9		0.61	0.64	0.78	0.81	0.79	0.79	WT_HisUb_3
0.58	0.59	0.62	0.58	0.59	0.59	0.6	0.63	0.61	0.6	0.63	0.61		0.95	0.7	0.6	0.66	0.62	pmt3KallR_HisUb_1
0.61	0.61	0.62	0.61	0.61	0.61	0.62	0.63	0.59	0.66	0.63	0.64	0.95		0.72	0.63	0.63	0.66	pmt3KallR_HisUb_2
0.69	0.7	0.66	0.71	0.71	0.65	0.75	0.77	0.73	0.76	0.76	0.78	0.7	0.72		0.73	0.74	0.76	pmt3KallR_HisUb_3
0.75	0.79	0.69	0.86	0.86	0.74	0.92	0.82	0.76	0.88	0.78	0.81	0.6	0.63	0.73		0.8	0.84	Δpli1_HisUb_1
0.74	0.78	0.68	0.78	0.8	0.67	0.84	0.84	0.8	0.8	0.84	0.79	0.66	0.63	0.74	0.8		0.76	Δpli1_HisUb_2
0.73	0.74	0.67	0.8	0.77	0.72	0.82	0.77	0.75	0.85	0.77	0.79	0.62	0.66	0.76	0.84	0.76		Δpli1_HisUb_3

Supplementary Figure 3: Reproducibility analyses (related to Figure 4 and Supplementary Figure 2). **a** Principal component analysis (PCA) of protein log₂ intensities for independent biological replicates from proteomic analyses of the indicated strains: WT, *pli1Δ* and *SUMO-KallR* (pmt3KallR) transformed with empty vector or pREP1-6His-ubiquitin (marked as _HisUb). Small grey points indicate each protein's resultant cluster, while large colored points with black outlines indicate each sample's resultant cluster. **b** Protein log₂ intensity Pearson correlation coefficient matrix for independent biological replicates from

proteomic analyses of the indicated strains: WT, *pli1Δ* and *SUMO-KallR* (*pmt3KallR*) transformed with empty vector or pREP1-6His-ubiquitin (marked as _HisUb).



Supplementary Figure 4: Strains validation, pulldown verification and reproducibility analyses (related to Figure 5). **a** Verification of Pli1-12Pk and Nse2-12Pk functionality. Ten-fold dilutions of indicated strains were spotted on plates containing TBZ, HU, CPT, MMS or Phleomycin (phleo) at indicated concentrations. **b** Pulldown of Pli1-12Pk and Nse2-12Pk performed using anti-Pk antibodies. Protein extracts (INPUT) and immunoprecipitated samples (IP-Pk) were analyzed by Western blot using anti-Pk antibodies. Ponceau S staining of the membrane was used as a loading control. **c** Principal component analysis (PCA) of protein log₂ intensities for independent biological replicates from proteomic analyses of the indicated strains: untagged (Ctrl), Pli1-12Pk and Nse2-12Pk. Small grey points indicate each protein's resultant cluster, while large colored points with black outlines indicate each sample's resultant cluster. **d** Protein log₂ intensity Pearson correlation coefficient matrix for independent biological replicates from proteomic analyses of the indicated strains: untagged (Ctrl), Pli1-12Pk and Nse2-12Pk.



Supplementary Figure 5: Analysis of chromatin distribution of Nse2 and Pli1 (related to Figure 6). a Binding of Pli1-12Pk and Nse2-12Pk to the indicated loci analyzed by ChIP-qPCR using anti-Pk antibody.

Chromosome II intergenic locus (II-50) was used as internal specificity control; cnt – core of centromere, dg – outer repetitive sequences of centromere, tel – telomere. Bars show the mean values; dots represent individual biological experiments (n=2). **b-e** Calibrated ChIP-seq profiles of Pli1-12Pk, Nse2-12Pk and untagged control across fission yeast chromosomes. Logarithmic cultures of indicated strains were mixed with budding yeast calibrator aliquot (exponential culture of Ku70-cMyc) and extracted DNA was used to construct DNA libraries and NGS analysis. Red line corresponds to signal obtained for Pli1-12Pk, blue line – Nse2-12Pk, green line – untagged control. **(b)** Binding of SUMO ligases across chromosome I and II. **(c)** Enrichment of Pli1 and Nse2 at centromeres of chromosome I and II. **(d)** Enrichment of Pli1 and Nse2 at right telomeres of chromosome I and II. **(e)** Analysis of Nse2 and Pli1 enrichment at active replication origins (averaged).

Supplementary Table 1: Strains and plasmids used in this study

Strain number	Genotype	Source
IDN1	<i>ade6-704 leu1-32 ura4-D18, h+</i>	S. Lambert
IDN365	<i>ade6-704 leu1-32 ura4-D18 pmt3-KallR, h+</i>	Markowska et al., 2025
IDN375	<i>leu1-32 ura4-D18 gar2-mCherry:Kan rad52-YFP:Kan, h?</i>	this study
IDN378	<i>leu1-32 ura4-D18 gar2-mCherry:Kan rad52-YFP:Kan pmt3-KallR, h?</i>	this study
IDN372	<i>ade6-704 leu1-32 ura4-D18 rad52-YFP:Kan, h?</i>	S. Lambert
IDN373	<i>ade6-704 leu1-32 ura4-D18 rad52-YFP:Kan pmt3-KallR, h?</i>	Markowska et al., 2025
IDN470	<i>ade6-704 leu1-32 ura4-D18 pcn1-K164R h?</i>	S. Lambert
IDN486	<i>ade6-704 leu1-32 ura4-D18 pcn1-K164R pmt3-KallR h?</i>	Markowska et al., 2025
IDN180	<i>ade6-704 leu1-32 ura4-D18 rad51::Kan, h?</i>	S. Lambert
IDN318	<i>ade6-704 leu1-32 ura4-D18 rad51::Kan pmt3-KallR, h?</i>	this study
IDN179	<i>ade6-704 leu1-32 rad52::Kan, h?</i>	S. Lambert
IDN341	<i>ade6-704 leu1-32 ura4-D18 rad52::Kan pmt3-KallR, h?</i>	this study
IDN421	<i>ade6-704 leu1-32 ura4-D18 rad52::Kan rad51::Kan, h?</i>	this study
IDN423	<i>ade6-704 leu1-32 rad52::Kan rad51::Kan pmt3-KallR, h?</i>	this study
IDN640	<i>ade6-704 leu1-32 ura4-D18 rad52-6his-3flag:Kan, h-</i>	Markowska et al., 2025
IDN642	<i>leu1-32 ura4-D18 rad52-6his-3flag:Kan pmt3-KallR h+</i>	Markowska et al., 2025
IDN520	<i>ade6-704 leu1-32 ura4-D18 pli1::Hph, h+</i>	Markowska et al., 2025
IDN254	<i>ade6-704 leu1-32 ura4-D18 nse2-12Pk:Kan, h+</i>	this study
IDN244	<i>ade6-704 leu1-32 ura4-D18 pli1-12Pk:Kan, h+</i>	this study
AN008	<i>MATa Ku70-13cMyc ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 (S. cerevisiae)</i>	laboratory collection

IDN_P13	<i>pREP41-MSC+</i>	Addgene 52690
IDN_P107	<i>pREP1-6his-Ubi</i>	D. Dziadkowiec

Supplementary Table 2: Primers used in this study

Primer name	Sequence (5'-3')	Experiment
II50_F	CACCGCAGTTCTACGTATCCT	ChIP (Control locus on ChrII)
II50_R	CGATGTAACGGTATGCGGTA	ChIP (Control locus on ChrII)
rdna_F	GGACGGTGGCCATGGAA	Rad52-YFP
rdna_R	CATTCGGCCGGTGAG	Rad52-YFP
cnt_F	CAACCGTTGCAACTTACATCAGCA	Pli1-12Pk, Nse2-12Pk
cnt_R	CCGGTCGCCAAATAGCAATGAGAT	Pli1-12Pk, Nse2-12Pk
dg_F	TACCGTGATTAGCCTTACTCCGCA	Pli1-12Pk, Nse2-12Pk
dg_R	ACCGCAAGATAGAGTAGGATGGGT	Pli1-12Pk, Nse2-12Pk
tel_F	TCAAAGTTGGCGACGTTGCTGATG	Pli1-12Pk, Nse2-12Pk
tel_R	AAGCAATGTGTGGAGCAACAGTGG	Pli1-12Pk, Nse2-12Pk
KK146	GAATTCGGTAAGCGTTGGATTG	southern rDNA 2DGE
KK147	GAATTCTTCTTTCACATC	southern rDNA 2DGE
KK161	CCTGGGTGAGATCAGTAGTC	southern 28S rDNA
KK162	TTCACCATCTTTCGGGTCCC	southern 28S rDNA