Supplementary information

Supplementary Methods

Plasmids, probes, and antibodies

pcDNA4/TIP5 and antibodies against TIP5 have been published (Strohner *et al,,* 2001). Vectors encoding myc-Suv3-9h1, GFP-Suv4-20h2, GFP-TRF2, GFP-CENP-A and GFP-SIRT6 were provided by T. Jenuwein, G. Schotta, K. Rippe, S. Diekmann and L. Guarente. Cy3- and FITC-labeled telomere specific PNA probes were used for Q-FISH, Immuno FISH and CO-FISH (Panagene). ^{32}P-labeled telomeric riboprobes were used for detection of telomeric DNA ($(UUAGGG)_5$) and TERRA ($(CCCUAA)_5$) and ^{32}P-labeled riboprobe for major satellite repeat for detection of pericentromeres. Antibodies against the Flag epitope were from Sigma, α-H4K20me3, α-Myc and α-GFP from Abcam, α-TRF2 and α-PML from Santa Cruz, α-H3K9me3 from Diagenode, and GFP-Trap® from ChromoTek.

Cells and transfections

U2OS, NIH3T3 and HeLa/Kyoto cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS. To overexpress proteins, $1.2x10^6$ HEK293T cells were transfected with 2-10 μg of expression vectors in the presence of 6.25 mM $CaCl_2$. The medium was changed after 8 h and cells were harvested 24-30 h after transfection. NIH3T3 cells that conditionally express ER-tagged Suv4-20h2 were used to examine the association of Suv4-20h2 with subtelomeres.

Knockdown of TIP5

Cells ($0.8x10^6$) were transfected with 50 nM of siRNAs using Lipofectamine 2000 (Invitrogen) and harvested after 60h. For knockdown of TIP5, siGENOME SMART pools (Dharmacon, M-058835-01-005, M-020470-01-0005) were used.

Chromatin immunoprecipitation (ChIP)

Cells were fixed for 10 min with 1% formaldehyde, suspended in 200 μl lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1]) and sonicated to yield 300-500 bp DNA fragments. Chromatin was diluted 10-fold with IP-buffer (16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100), precleared for 1 h at 4°C on protein A/G-agarose in the presence of 20 mg/ml sonicated salmon

sperm DNA, and incubated overnight with the respective antibodies. Protein-DNA complexes were captured on protein A/G agarose followed by two washes in low salt buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 1% Triton X-100), high salt buffer containing 500 mM NaCl, LiCl buffer (250 mM LiCl, 10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% Na-deoxycholate, 0.5% Triton X-100) and TE buffer. After elution and reversal of the crosslink, DNA was extracted and amplified by qPCR. Alternatively, DNA was hybridized with ^{32}P-labeled telomeric or centromeric riboprobes in 50 mM sodium phosphate (pH 6.5), 50% formamide, 5xSSC, 10xDenhardts, 1 mg/ml yeast tRNA, 1% SDS (16h, 50°C), washed with 2xSSC/0.1% SDS and 0.2xSSC/0.1%SDS, and quantified by PhosphorImaging. The ratio of DNA in the immunoprecipitates *versus* DNA in the input chromatin was normalized to control reactions from mock-transfected cells. Primers used in qPCR are listed in Table S1.

Co-immunoprecipitation assays

Cells were lysed in 200 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 5 mM EDTA, 10 mM EGTA and protease inhibitors (Roche). Lysates were sonicated, treated with benzonase (100 units, 15 min, 22°C), incubated with bead-bound antibodies for 3 h at 4°C, and captured proteins were analyzed on Western blots.

Identification of protein-associated RNA

Cells were lysed in 200 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 0.5% DOC, 1% NP40, 1 mM EDTA, 1 mM EGTA and protease inhibitors (Roche). Lysates were sonicated, cleared by centrifugation, precleared on Sepharose A/G and incubated with bead-bound antibodies for 3h at 4°C in lysis buffer containing 0.02% SDS, 0.1% DOC and 0.2% NP40. After washing with IP buffer and proteinase K digestion, RNA was extracted with TRIazol (Sigma) and digested with DNase I. For quantification, RNA was either subjected to RT-qPCR or dotted on nitrocellulose filters and hybridized with ^{32}P-labeled riboprobe in 50 mM sodium phosphate (pH 6.5), 50% formamide, 5xSSC, 10xDenhardts, 1 mg/ml yeast tRNA, 1% SDS (16h, 50°C). After washing with 2xSSC/0.1% SDS and 0.2xSSC/0.1%SDS, the hybridization signal was quantified by PhosphorImaging. The ratio of RNA in the immunoprecipitates *versus* RNA in the input was normalized to control reactions from mock-transfected cells. Primers used for qPCR are listed in Table S1.

Supplementary Figure Legends

Figure S1. TIP5 affects cell proliferation.

A. Knockdown of TIP5 impairs cell proliferation.

NIH3T3, U2OS and HeLa/Kyoto cells were transfected with control (ctrl) or TIP5-specific (siTIP5) siRNA and cell numbers were counted. The data representing average cell numbers at the indicated time after siRNA transfection were compared for each time point between control and siTIP5-treated cells by unpaired t-test and the following significances were obtained:

	48h	**72h**	**96h**
NIH 3T3 (n=6)	$p<0,005$	$p<0,005$	$p<0,001$
U2OS (n=10)	$p<0,005$	$p<0,0001$	$p<0,00005$
HeLa/Kyoto (n=3)	$p=0,06$	$p=0,09$	$p<0,05$

Error bars indicate standard deviations.

B. NIH3T3, U2OS and HeLa/Kyoto cells were transfected with siRNAs targeting mouse or human TIP5, and TIP5 mRNA was monitored by RT-PCR after 72h. Panels depict normalized level of TIP5 mRNA. Error bars represent standard deviation (n=3).

C. Western blot showing knockdown of TIP5 in NIH3T3, U2OS and HeLa/Kyoto cells 72h after transfection.

D. Immunostaining of TIP5 in U2OS cells transfected with siRNA against TIP5 (red). DNA was counterstained with DAPI. Scale bar 5μm.

Figure S2. Specificity of TIP5 ChIPs.

Crosslinked chromatin from NIH3T3 or U2OS cells was precipitated with α-TIP5 antibody and recovered DNA was analyzed by qPCR using primers specific for the indicated regions. Primer sequences to amplify X chromosome- (C21) and SINE-specific sequences have been published [25,26]. Data are normalized to input levels. Error bars represent standard error of the mean (n=3).

Figure S3. TIP5 interacts with CENP-A and HP1α.

Co-immunoprecipitation experiments showing the interaction of Flag-tagged TIP5 with GFP-tagged CENP-A (**A**), GFP-tagged HP1α (**B**), myc-tagged Suv3-9h1 (**C**), but not with GFP-tagged TIF-IA (**D**). Non-adjacent lanes in the original blots are indicated by dotted lines.

Figure S4. NoRC impacts the level of telomeric and centromeric RNA.

A. Overexpression of TIP5 increases the level of TERRA and major satellite RNA. Left panel: RNA (20, 100 and 500 ng) from mock-transfected cells (ctrl) or cells overexpressing TIP5 (TIP5) were analyzed by dot hybridization using a TERRA-specific riboprobe. The bars represent quantification of radioactive signals normalized to β-actin mRNA (unpaired t-test, $p=0{,}06$ for TERRA and $p<0{,}05$ for satellite RNA). Right panel: Major satellite RNA and GAPDH mRNA were monitored by semi-quantitative RT-PCR using 2 μg of total RNA. Satellite RNA in control samples was used as a reference (=1). Error bars represent standard deviation (n=3).

B. Depletion of TIP5 decreases the level of TERRA and major satellite RNA. RNA from cells transfected with control (ctrl) or TIP5-specific siRNA (siTIP5) was analyzed by hybridization or semi-quantitative RT-PCR (unpaired t-test, $p<0{,}05$ for TERRA and $p<0{,}05$ for satellite RNA). Error bars represent standard deviation (n=3).

Figure S5. TIP5 is involved in telomere length maintenance.

A. Knockdown of TIP5 leads to telomere elongation. Telomeric DNA was assayed in control (ctrl) and TIP5-deficient (siTIP5) U2OS cells (left panels) and NIH3T3 cells (right panels) by dot blot hybridization of genomic DNA using a telomere-specific riboprobe. Representative blots are shown. Data represent the average of telomeric signals intensity normalized to β-actin, error bars represent standard error of the mean (n=3). Values for control cells were used as a reference (ctrl=1).

B. Knockdown of TIP5 does not induce DNA damage response at telomeres. Control and siTIP5-treated cells were stained with α−p53BP (upper panels,

U2OS cells) or γH2A.X antibody (bottom panels, NIH3T3 cells) followed by Q-FISH with a telomere-specific PNA probe. Scale bars 5μm.

C. Knockdown of TIP5 does not induce telomere aberrations, such as telomeric duplications and telomere losses. Control and siTIP5-treated U2OS were treated with colcemid for 3h, spread and subjected to telomeric FISH. Scale bar 5μm.

Supplemental movie 1. The frequency of apoptosis is increased in TIP5-deficient cells. Live cell microscopy of HeLa/Kyoto cells transfected with control siRNA (ctrl) or siRNA against TIP5 (siTIP5). Images of representative TIP5-deficient cells undergoing apoptosis are shown.

Supplemental movie 2. Chromosome alignment in the metaphase plate is delayed in TIP5-deficient cells. Live cell microscopy of HeLa/Kyoto cells transfected with control siRNA (ctrl) or siRNA against TIP5 (siTIP5) monitored between 32 and 92 h after transfection using a motorized Olympus IX 81-ZDC microscope equipped with an environmental chamber (37°C, 5% CO_2, 40% humidity) and a Hamatsu Digital Camera C10600. Progression of through mitosis of representative control and TIP5-depleted cells is shown 72 h after siRNA transfection.

Supplementary References

25. Rougeulle C, Chaumeil J, Sarma K, Allis DC, Reinberg D, Avner P, Heard E (2004) Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome. *Mol Cell Biol* **24:** 5475-5484

26. Martens JHA, O'Sullivan RJ, Braunschweig U, Opravil S. Radolf M, Steinlein P, Jenuwein T (2005) The profile of repeat-associated histone lysine methylation states in the mouse epigenome. *EMBO* **24:** 800-812.

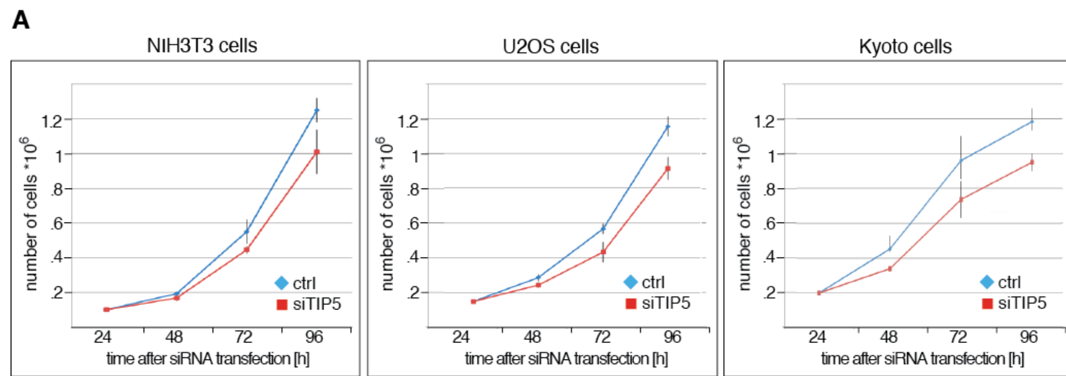
Supplemental Table 1. Primers used in PCR.

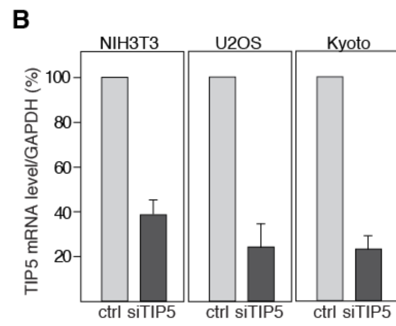
Primer name	**Sequence**
mrDNA -105/-87 for	5'- CCCAGGTATGACTTCCAG-3'
mrDNA -21/-1 rev	5'- ACCTATCTCCAGGTCCAATAG-3'
m/hrDNA 28S rRNA for	5'- GCGACCTCAGATCAGACGTGG-3'
m/hrDNA 28S rRNA rev	5'- CTGTTCACTCGCCGTTACTGAG-3'
mSubtelomere 2 for	5'- CACCTTTAACACCCCTTCCA-3'
mSubtelomere 2 rev	5'- CAGAGCACATGGAGTGGAGA-3'
mβ-actin for	5'- CCATCACCATCTTCCAGGAGC-3'
mβ-actin rev	5'- CAAGAAGGTGGTGAAGCAGG-3'
mTIP5 for	5'- CACACTACTACTTCAGGG-3'
mTIP5 rev	5'- GGGTACTGTGAGTAGTTC-3'
hTIP5 for	5'- ACCGGCGCATCATCAAAAATCCTA-3'
hTIP5 rev	5'- ACTCCCCACCTCCCTTGCCTCAC-3'
mGAPDH for	5'- CCATCACCATCTTCCAGGAGC-3'
mGAPDH rev	5'- CAAGAAGGTGGTGAAGCAGG-3'
hGAPDH for	5'- CCCATCACCATCTTCCAGGAG-3'
hGAPDH rev	5'- CTTCTCCATGGTGGTGAAGACG-3'
C21 for	5'- ACAGGCTGTGAACCAGAGTACC-3'
C21 rev	5'- ACAGGAATGTAGGATTCACCAA-3'
hSubtelomere 17 for	5'- CACCCAGGCACAGATAGA-3'
hSubtelomere 17 rev	5'- AGAAATGACCAGGCTCCA-3'
Maj Sat for	5'- GACGACTTGAAAAATGACGAAATC-3'
Maj Sat rev	5'- CATATTCCAGGTCCTTCAGTGTGC-3'

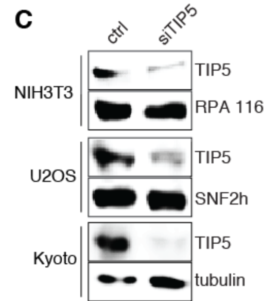
Supplemental Table 2. Chromosomal aberrations are increased upon depletion of NoRC.

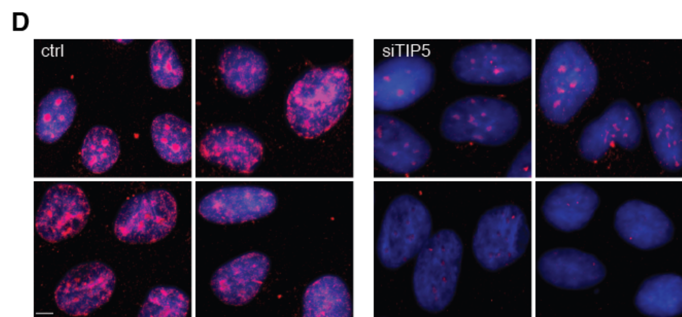
Chromosome number	Unique translocations (control siRNA)	Unique translocations (siTIP5)
1		t(1;8)
4		t(4;22;7)
		t(4;11;12)
		t(4;6)
8	2t(12;8;15;18)	t(17;8;3)
	2t(8;16)	t(8;1)
		2t(12;8;15;5;12)
13	t(13;19;17)	t(13;5)
		2t(13;19)
16	t(16;6)	t(16;10)
		2t(16;10)

Metaphase spreads were prepared 30 h after transfection of U2OS cells with control siRNA or TIP5-specific siRNA. Chromosomal translocations were monitored by M-FISH, focusing on chromosomes 1, 4, 8, 13, and 16. While control cells showed 4 new translocations, 11 non-recurrent, unique translocations were detected in TIP5-depleted cells. Similar results were obtained in 3 independent experiments.









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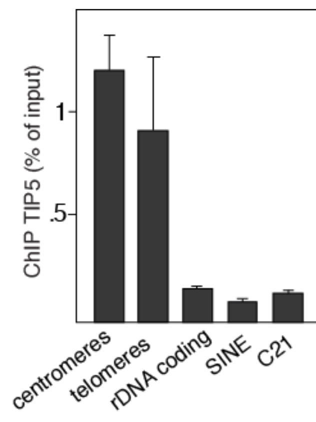
Supplemental Figure 1



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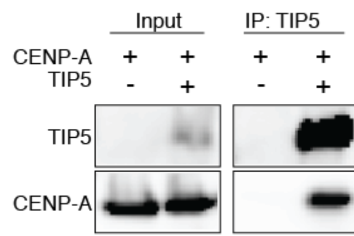
Supplemental Figure 2

A



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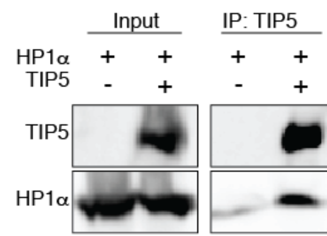

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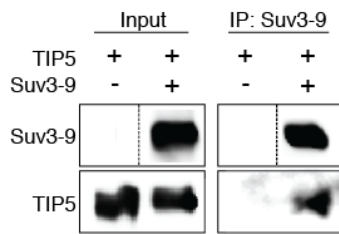

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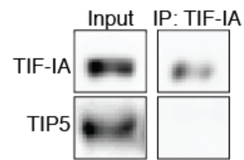

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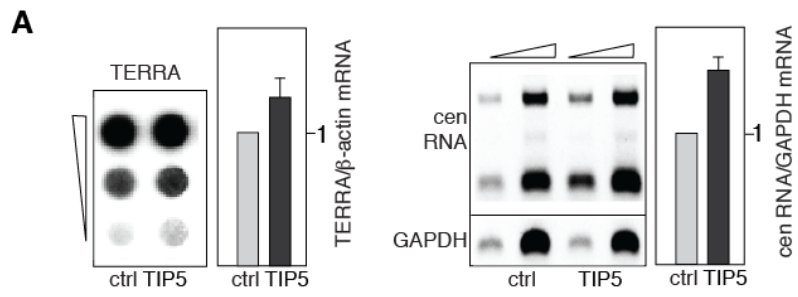
Supplemental Figure 3

A



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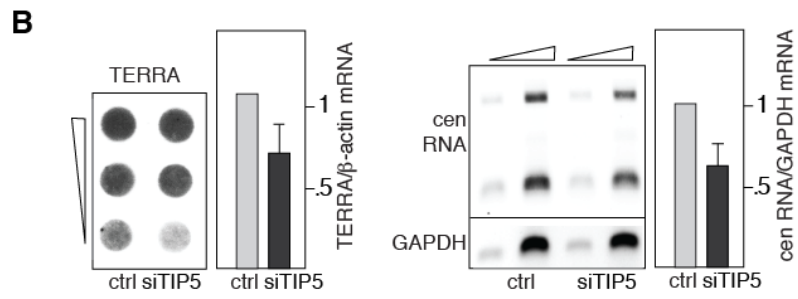

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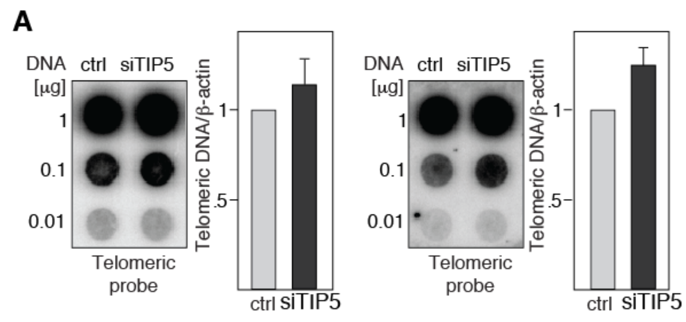
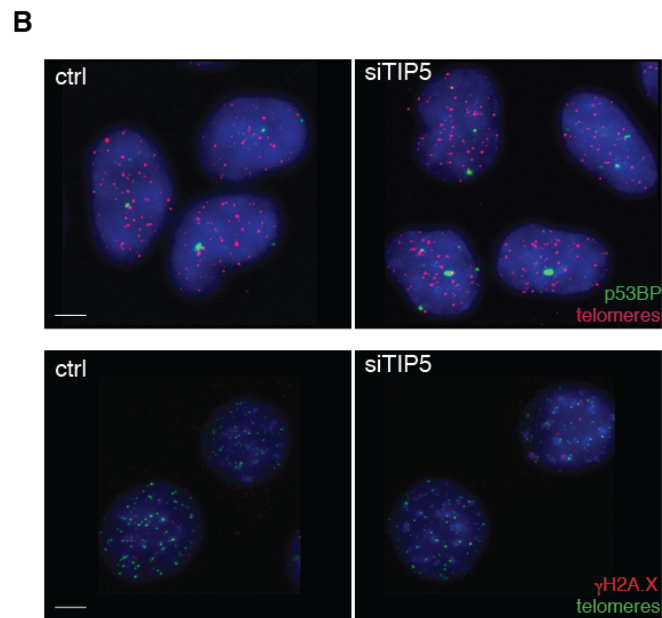
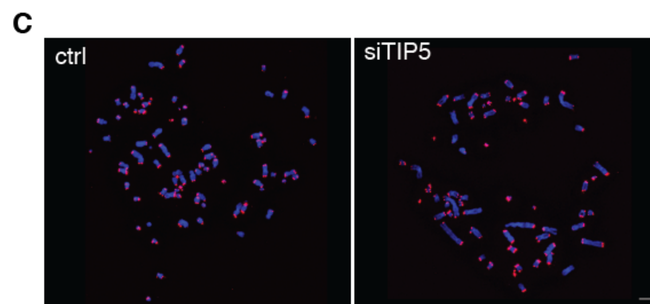
Supplemental Figure 4







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Supplemental Figure 5