Supplementary Figure Legends

Supplementary Figure S1: Representative MS spectra of SILAC telomere-IP
MS spectra of representative peptides validated by MS/MS. In the 'forward' pull-down (upper panel) the heavy peptide partners are detected (red dots) while the light partner is barely observable (blue dots). In the 'reverse' pull-down (lower panel) in contrast, the corresponding light peptides are strongly enriched.

Supplementary Figure S2: Binding of the HOT1 homeobox domain to telomeric DNA
(a) A schematic of the six fragments of HOT1 tested in preparation of the crystallization. (b) Sequence specific pull-down of recombinant HOT1 fragments. Proteins were incubated with double-stranded DNA of telomeric repeats (TTAGGG) or the control sequence (GTGAGT). All DNA substrates were concatemerized from 60bp oligonucleotides to larger DNA fragments (on average at least 1kb). (c) Quantification of recovery of recombinant His-HOT1 fragments and full length His-MBP-HOT1. Average recoveries of three independent telomere pull-downs with error bars representing standard deviation are shown.

Supplementary Figure S3: Overall structure of the two copies of the HOT1 homeobox domain bound to double stranded telomeric DNA as found in the asymmetric unit of the crystal structure
Both copies of the protein are shown as brown (copy A) and orange (copy B) cartoon representations. DNA is shown as a grey stick model with the G-C base pair triplets highlighted in blue. All secondary structure elements constituting the DBD are marked.

Supplementary Figure S4: Validation of the specificity of HOT1 immunofluorescence stainings and homeobox-dependent HOT1 localization to telomeres
(a) Immunofluorescence stainings of HOT1 using mouse anti-HOT1 with DAPI serving as a nuclear counterstain. HOT1 knock-down with two independent esiRNAs in HeLa cells results in depletion of the HOT1 signal in comparison to the RLuc (Renilla Luciferase) control, confirming the specificity of the HOT1 antibody. The number of HOT1-positive cells was counted and HOT1 was depleted with both esiRNAs from more than 95% of all cells. Scale bars represent 5 μm. (b) Co-localization analysis of TRF1 and overexpressed FLAG-HOT1 in mouse embryonic stem cells by IF staining. To visualize TRF1 a LAP cell line (Poser et al., 2008) was used, expressing GFP-tagged TRF1 under endogenous expression levels. A representative image illustrating the co-localization between several FLAG-HOT1 wt foci (green) and TRF1 (red) is shown. Both FLAG-HOT1Δhomeodomain and FLAG-HOT1 R339A fail to localize to telomeres. DAPI (blue) is used as nuclear counterstain. Co-localization events are indicated by arrows. Scale bars represent 5 μm.

Supplementary Figure S5: HOT1 localization in mouse testis
Immunofluorescence stainings of HOT1 on sections of mouse seminiferous tubules. All images shown in this panel are deconvolved projections of acquired z-stacks. (a) An overview of an entire tubule is given with stainings for HOT1 (green) and the synaptonemal complex/chromosome axis marker SYCP3 (red). The DNA is counterstained by DAPI (blue). Scale bars represent 100 μm. Individual images were acquired with a 100x objective and stitched together to cover an entire tubule. In the merged image the field of view for panel b

is marked. (b) Representative image of pachytene spermatocytes with frequent localization of HOT1 foci at chromosome ends. In the merged image one example is highlighted in which the paired homologous chromosomes can be identified individually at the level of the chromosome ends.

Supplementary Figure S6: Summary of SILAC-based protein-protein interactions from HOT1-IPs

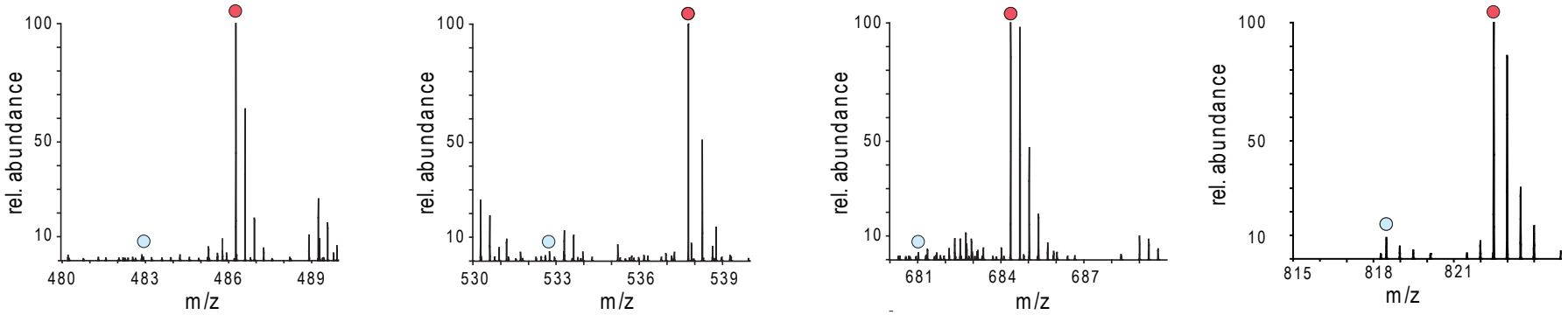
Identification and normalized SILAC ratios are indicated for HOT1 (bait) and the identified interaction partner relevant for telomere biology. (a) Data from immunoprecipitations using both a rabbit and a mouse anti-HOT1 and nuclear protein extracts from HeLa cells. (H)* indicates that only heavy peptides were identified so that no SILAC ratio could be assigned. (b) Data from an immunoprecipitation using a mouse anti-HOT1 antibdy and nuclear protein extracts from mouse embryonic stem cells. (L)* indicates that only light peptides were identified in at least two quantification events so that no SILAC ratio could be assigned. (c) Two-dimensional interaction plot of a POT1 immunoprecipitation from a POT1-LAP cell line (Poser et al., 2008): All six shelterin components cluster together (light blue background).

Supplementary Figure S7: HOT1 acts as a positive regulator of telomere length

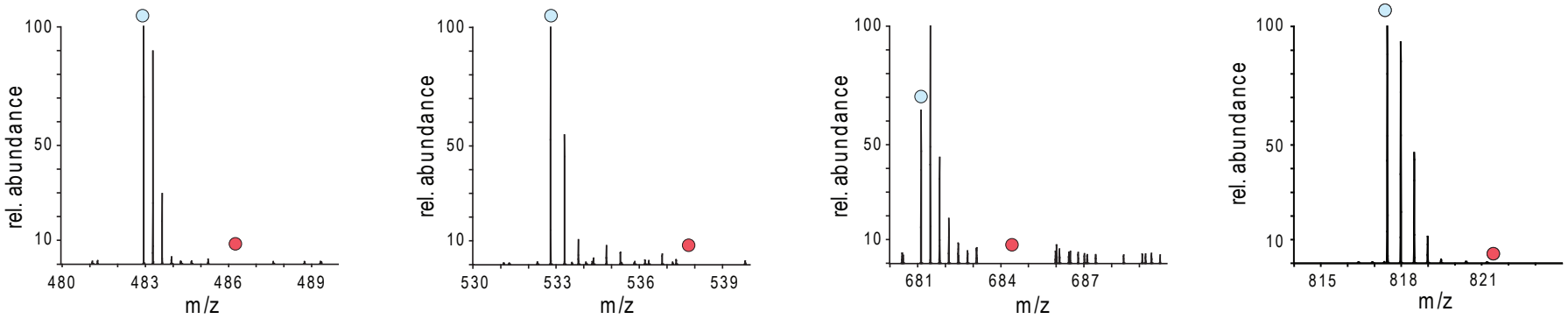
(a) Verification of HOT1 esiRNA knock-down efficiency using a HOT1-LAP cell line (Poser et al., 2008) as a reporter for HOT1 expression by Western blot 48h post transfection. (b) Quantification of telomere length by quantitative telomeric FISH after transient knockdown of HOT1. The distributions of fluorescence intensities, in arbitrary units of fluorescence (a.u.f.), of individual telomeres from a total of 30 metaphases per treatment are displayed; the average intensity is indicated in red. For the gene-specific knockdowns, changes of average telomere signal intensity relative to the RLuc (Renilla Luciferase) control are shown (left). Representative FISH images are shown for each treatment and signal free ends are indicated by arrows (right). Examples of individual chromosomes are magnified and the respective chromosomes are marked by rectangles (right). Scale bars represent 5 μm. (c) Summary of the quantification of signal free ends per metaphase after gene-specific knockdown.

Supplementary Figure S1

telomere forward experiment (telomere heavy: control light)



telomere reverse experiment (telomere light: control heavy)



HOT1
HEILHALETLDR^{3+}

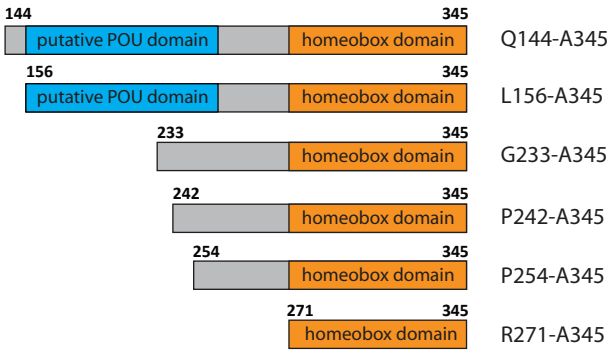
RAP1
ADGYPIWSR^{2+}

TRF1
NSAESIIHGLSSLTACQLR^{3+}

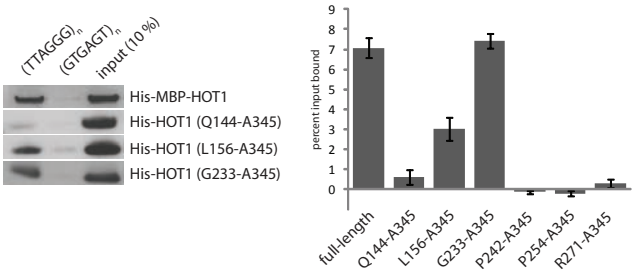
TRF2
DLVLPTQALPASPALK^{2+}

Supplementary Figure S2

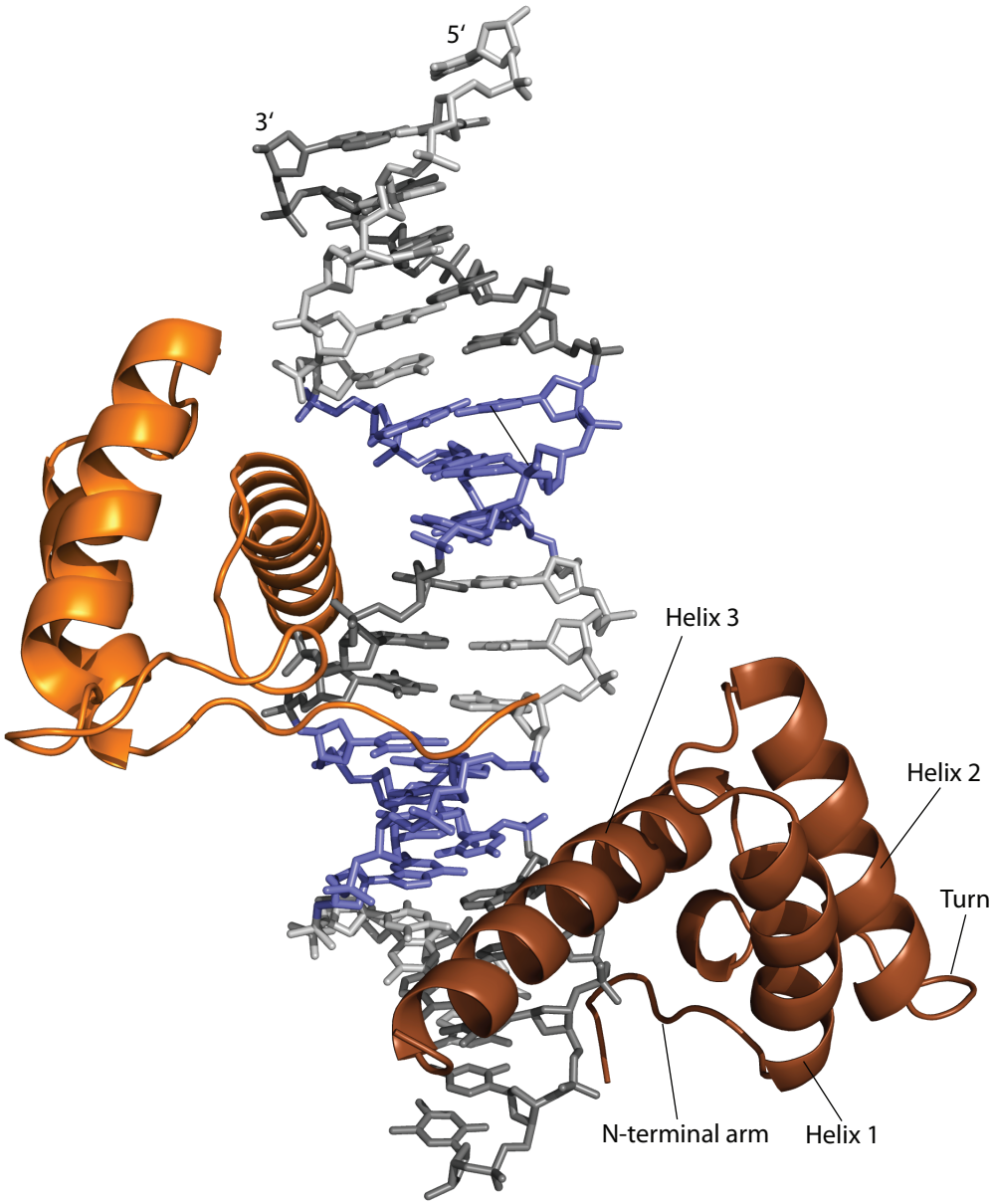
a



b

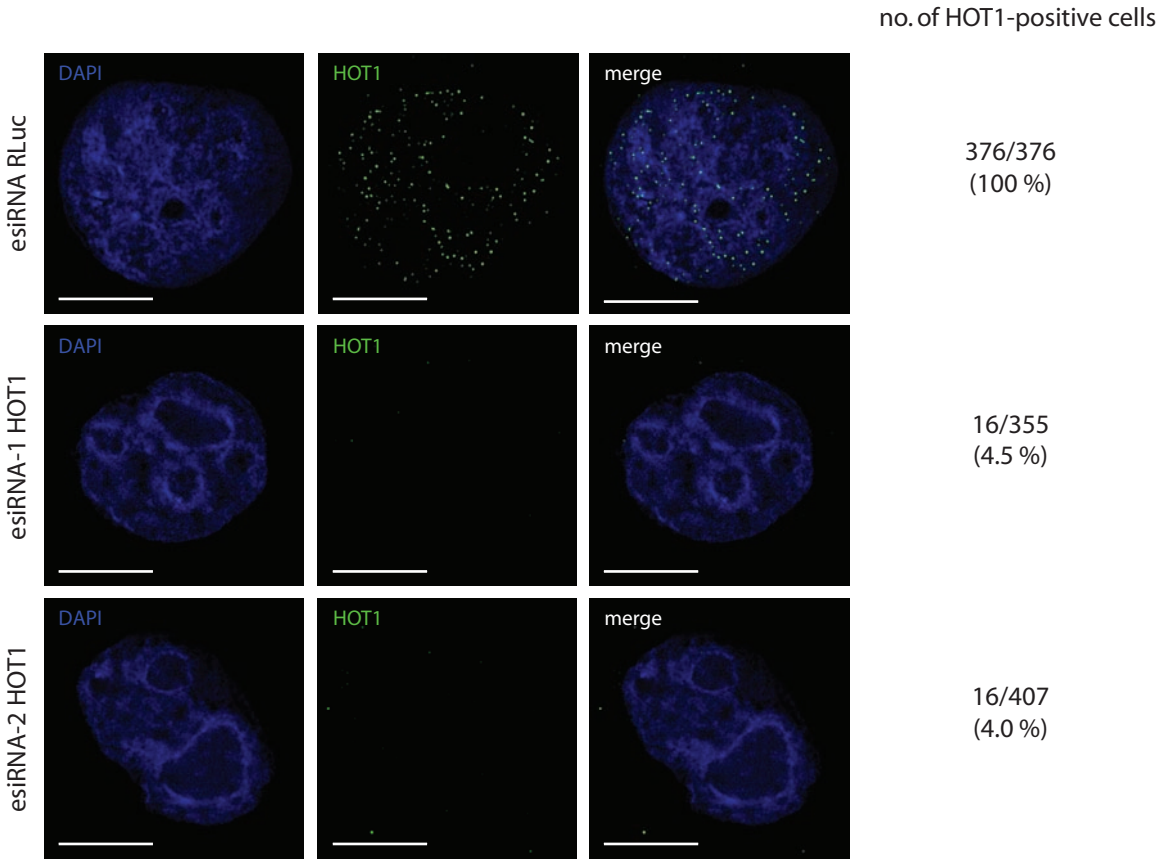


Supplementary Figure S3



Supplementary Figure S4

a



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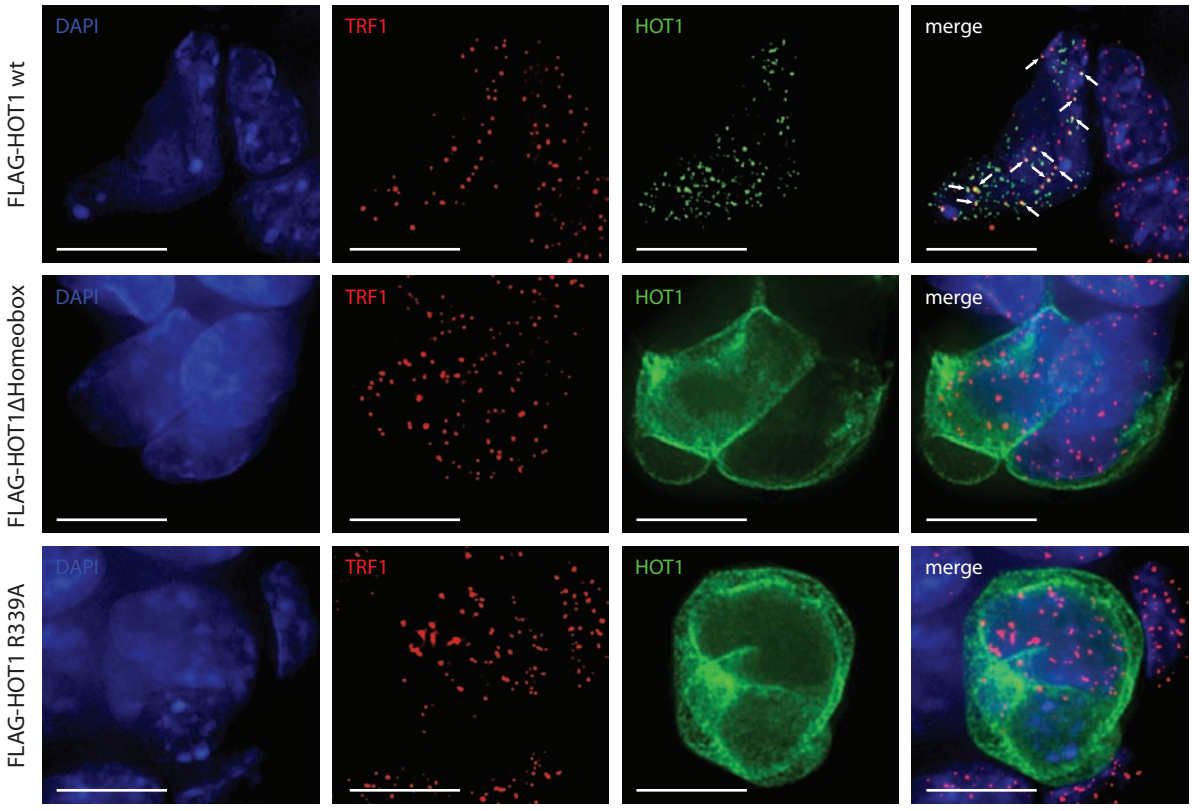
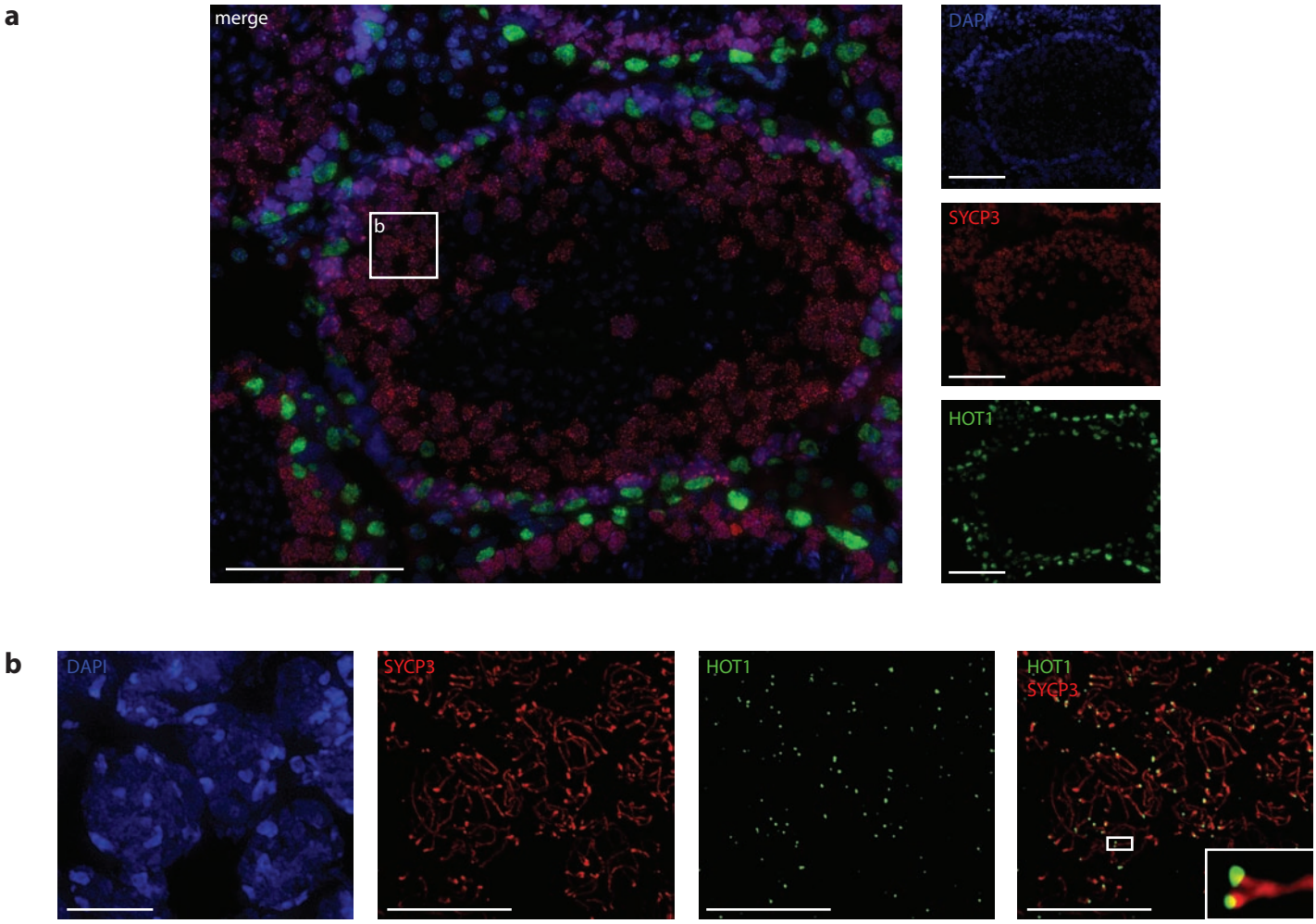


Supplementary Figure S5



Supplementary Figure S6

a

HeLa cells																						
	rabbit HOT1 antibody (polyclonal)						mouse HOT1 antibody (monoclonal)															
	150 mM NaCl						150 mM NaCl						250 mM NaCl				400 mM NaCl					
	Forward experiment			Reverse experiment			Forward experiment			Reverse experiment			Forward experiment			Reverse experiment	Forward experiment			Reverse experiment		
Protein	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio
HOT1	**10**	**6**	**12.4**	**9**	**2**	**0.02**	**9**	**14**	**13.7**	**9**	**7**	**0.08**	**13**	**32**	**25.3**	**16**	**24**	**0.02**	**12**	**23**	**23.8**	**13**	**19**	**0.03**
GAR1	5	6	11.7	6	4	0.07	9	19	6.9	9	26	0.14	3	2	17.8	4	4	0.01	5	7	10.0	3	3	0.02
NHP2	3	6	12.9	3	7	0.04	6	13	6.4	6	18	0.13	2	0	(H)*	2	3	0.05	2	2	16.9	3	4	0.01
NOP10	3	3	27.1	4	2	0.04	2	3	5.7	3	8	0.13	4	3	20.5	4	6	0.02	4	9	22.1	4	6	0.03
DKC1	4	2	21.8	11	9	0.04	27	58	7	27	69	0.13	12	15	18.0	18	17	0.01	12	13	12.2	8	7	0.05
Ku70	28	34	15.0	34	33	0.02	18	19	4.6	22	23	0.09	9	7	13.0	7	5	0.25	---	---	---	---	---	---
Ku80	29	33	14.0	38	43	0.02	5	5	4.4	12	11	0.08	5	5	16.0	4	4	0.08	---	---	---	---	---	---
Coilin	3	1	14.5	3	1	0.03	14	31	7.2	12	23	0.26	---	---	---	---	---	---	---	---	---	---	---	---

H/ACA snoRNP complex

Ku70/Ku80 heterodimer

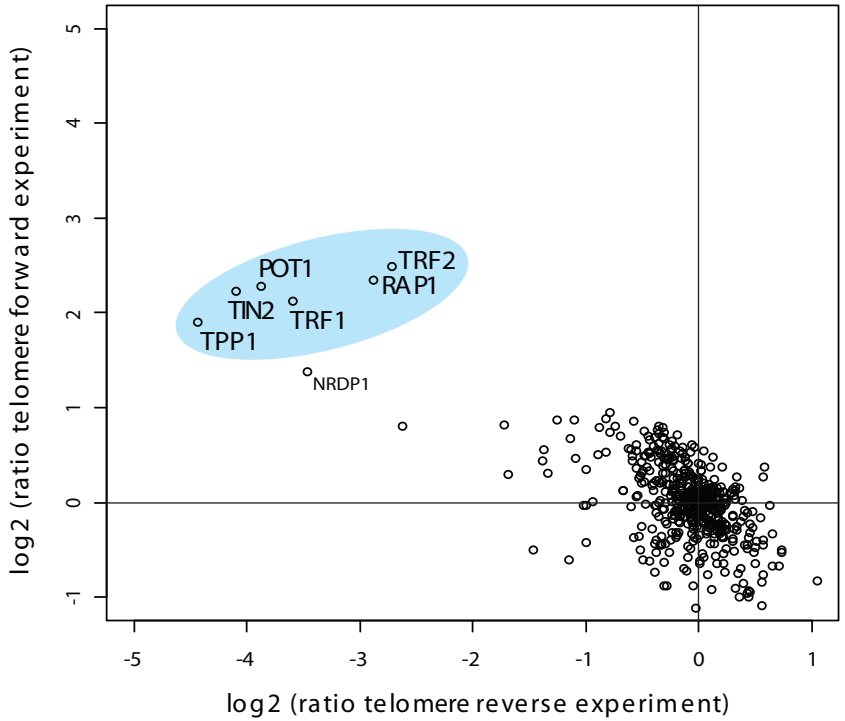
b

mouse embryonic stem cells						
	mouse HOT1 antibody (monoclonal)					
	150 mM NaCl					
	Forward experiment			Reverse experiment		
Protein	unique peptides identified	quant events	SILAC ratio	unique peptides identified	quant events	SILAC ratio
HOT1	**8**	**19**	**19.5**	**14**	**21**	**0.03**
GAR1	6	8	42.1	4	4	0.02
NHP2	5	11	24.5	2	1	(L)*
NOP10	6	7	15.7	0	0	n.d.
DKC1	19	31	34.3	22	30	0.01
Ku70	9	10	40.5	10	10	0.01
Ku80	8	8	15.2	8	10	0.06
Coilin	8	12	28.2	9	10	0.01

H/ACA snoRNP complex

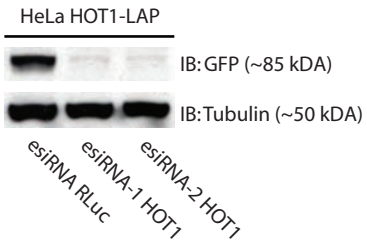
Ku70/Ku80 heterodimer

c



Supplementary Figure S7

a



c

Knock-down	no. of metaphases analyzed	Signal free ends (SFE/metaphase)	significance
RLuc esiRNA	30	1.8 ± 0.1	
HOT1 1st esiRNA	29	5.8 ± 0.2	p<0.001
HOT1 2nd esiRNA	30	6.7 ± 0.2	p<0.001

b