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Supplementary Materials for

SETDB1-dependent heterochromatin stimulates alternative lengthening of telomeres

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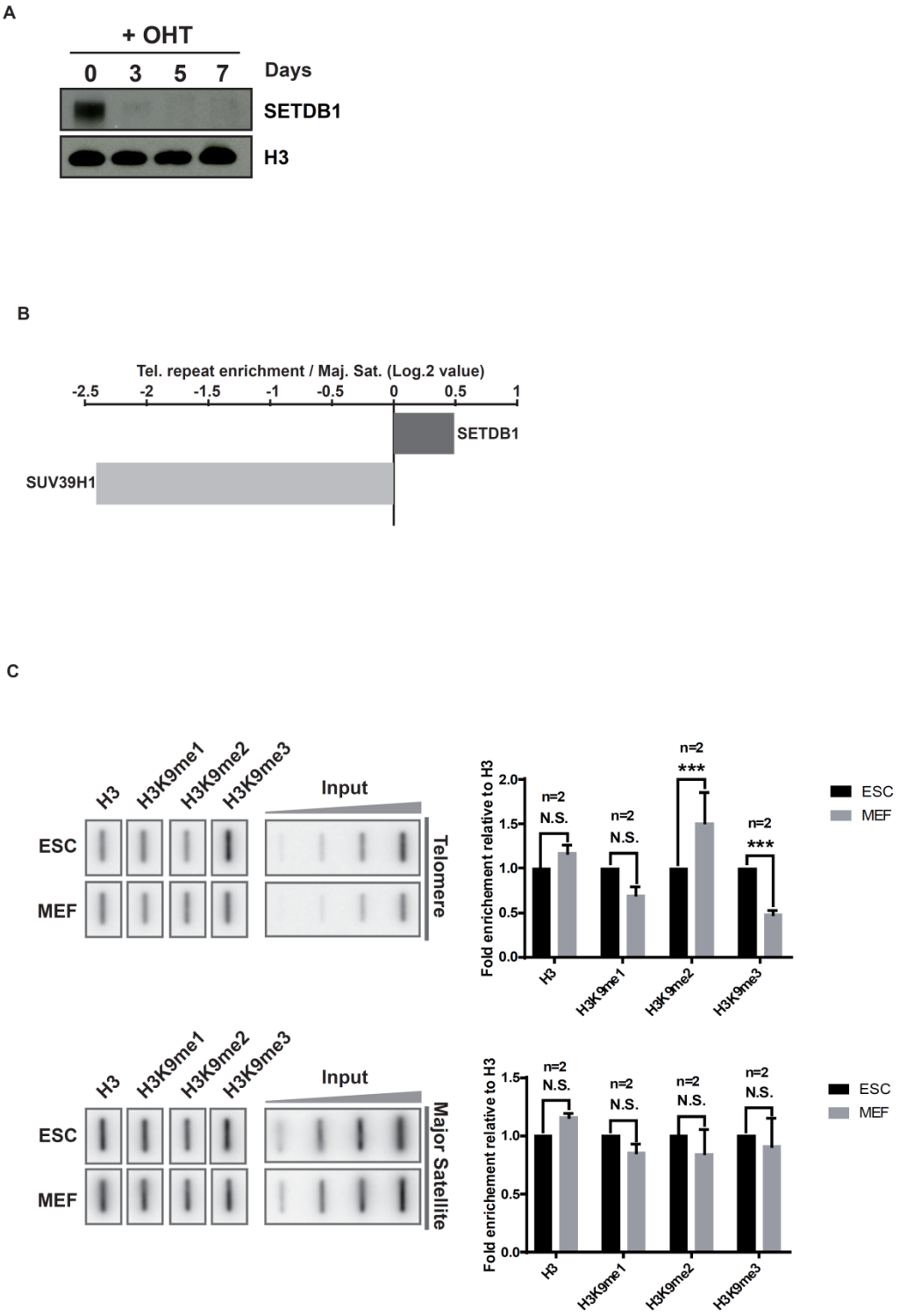

A
+ OHT
0 3 5 7
Days
SETDB1
H3
B
Tel. repeat enrichment / Maj. Sat. (Log.2 value)
-2.5 -2 -1.5 -1 -0.5 0 0.5 1
SETDB1
SUV39H1
C
H3 H3K9me1 H3K9me2 H3K9me3
Input
ESC
MEF
Telomere
Major Satellite
Fold enrichement relative to H3
n=2
N.S.
***
ESC
MEF

Figure S1

Fig. S1. SETDB1 telomeric binding is developmentally regulated. (**A**) SETDB1 immuno-blots against SETDB1) in non-induced wild-type mESCs and *Setdb1* KO cells after different days of tamoxifen (OHT) treatment. (**B**) ChIP sequencing data from SETDB1 and SUV39H1 libraries plotting the amounts of telomeric reads obtained in both libraries, normalized to the amounts of pericentromeric reads (*26, 27*). (**C**) (Left) ChIP experiments using antibodies raised against H3 and mono-, di- and tri-methylated H3K9 in mESCs or MEF. 20% of the immunoprecipitated DNA was blotted and probed with a telomere specific or a major satellite specific probe. 0.01%, 0.05%, 0.25% and 1.25% of input were loaded. (Right) Quantifications representing the enrichment of H3K9 methylation normalized to the total H3 signal and the input relative to ESC. Triple asterisks represent p values below 0.005 (from a Student's t-test).

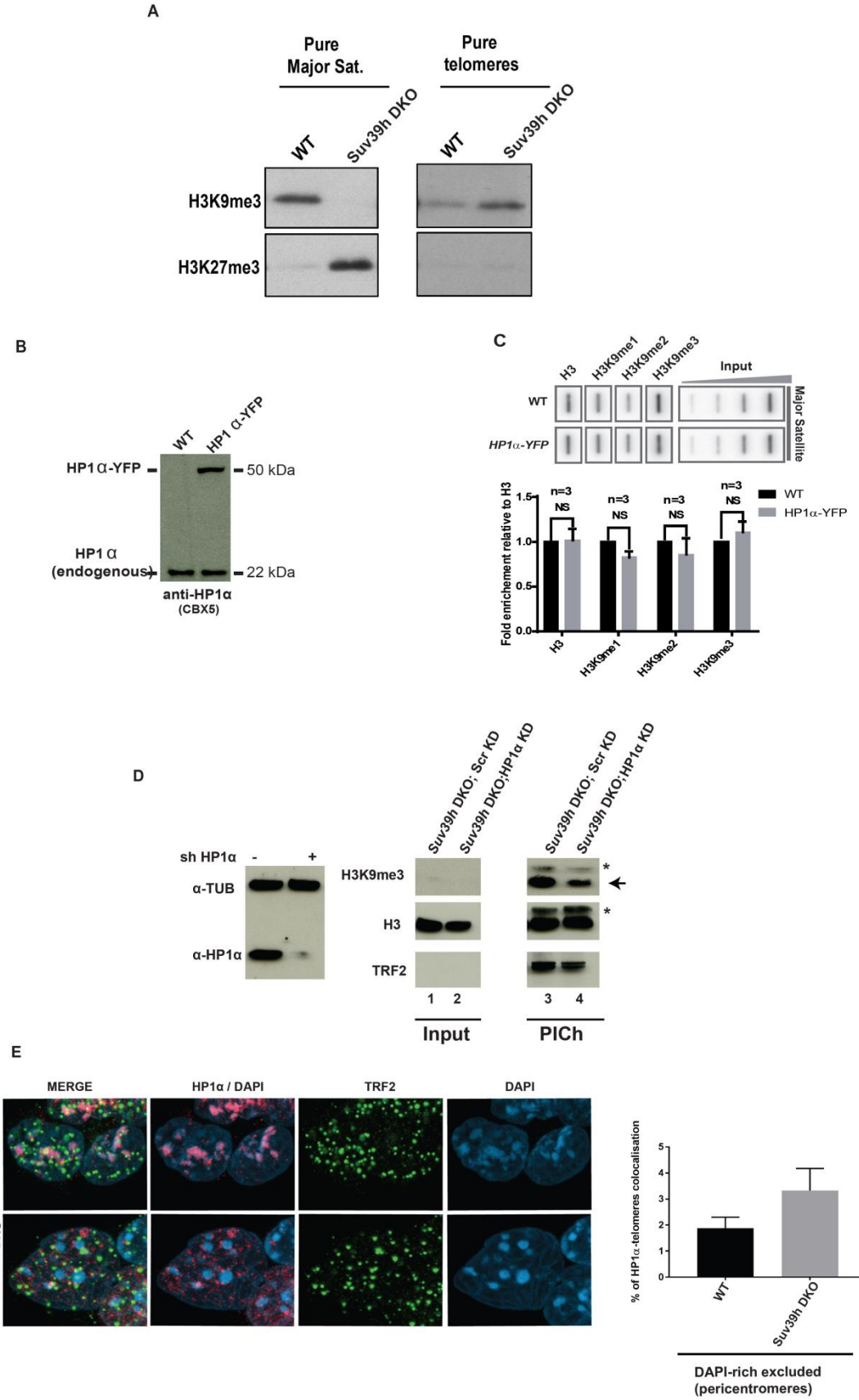

A
Pure Major Sat.
Pure telomeres
WT
Suv39h DKO
WT
Suv39h DKO
H3K9me3
H3K27me3
B
WT
HP1 α-YFP
HP1 α-YFP
50 kDa
HP1 α (endogenous)
22 kDa
anti-HP1α (CBX5)
C
H3
H3K9me1
H3K9me2
H3K9me3
Input
WT
HP1α-YFP
Major Satellite
Fold enrichement relative to H3
n=3
NS
WT
HP1α-YFP
D
sh HP1α
-
+
α-TUB
α-HP1α
Suv39h DKO; Scr KD
Suv39h DKO;HP1α KD
H3K9me3
H3
TRF2
1 2
3 4
Input
PICh
E
MERGE
HP1α / DAPI
TRF2
DAPI
WT
Suv39h DKO
% of HP1α-telomeres colocalisation
DAPI-rich excluded (pericentromeres)

Figure S2

Fig. S2. HP1α stimulates SETDB1-dependent heterochromatin formation at telomeres. (**A**) Immuno-blots performed on telomeres and pericentromeres nucleosomes obtained from specific PICh purifications in WT or *Suv39h* KO cells for the analysis of the histone marks H3K9me3 and H3K27me3. (**B**) Immunoblot of HP1α in control cells and HP1α-YFP-overexpressing cells. (**C**) (Top) ChIP experiments in HP1α-YFP overexpressing cells using antibodies raised against H3 and mono-, di- and tri-methylated H3K9. 20% of the immunoprecipitated DNA was blotted and probed with a pericentromere-specific probe. 0.01%, 0.05%, 0.25% and 1.25% of input were loaded. (Bottom) Quantifications of the fold enrichment of H3K9 methylation normalized to the total H3 signal and the inputrelative to wild-type cells. (**D**) (Left): Immunoblot of HP1α in *Suv39h* KO cells expressing a shRNA scramble (scr) and *Suv39h* KO cells expressing an shRNA for HP1α. (Right): Immunoblots of TRF2, H3 and H3K9me3 in input samples and in telomere PICh preparations from the Suv39h KO;Scr and the *Suv39h* KO cells knockdown forHP1α. The asterisk denotes a non-specific band that we obtain upon PICh material decrosslinking. (**E**) Co-immuno-staining analysis showing HP1α (red) and TRF2 (green) signals co-localization in *Suv39h* KO cells. Due to the large enrichment of HP1α in the chromocenters (pericentromeric region corresponding to DAPI rich region) in the wild-type background, only the TRF2 signals found outside the DAPI bright regions were taken into account for counting and statistical analyses (right panels).

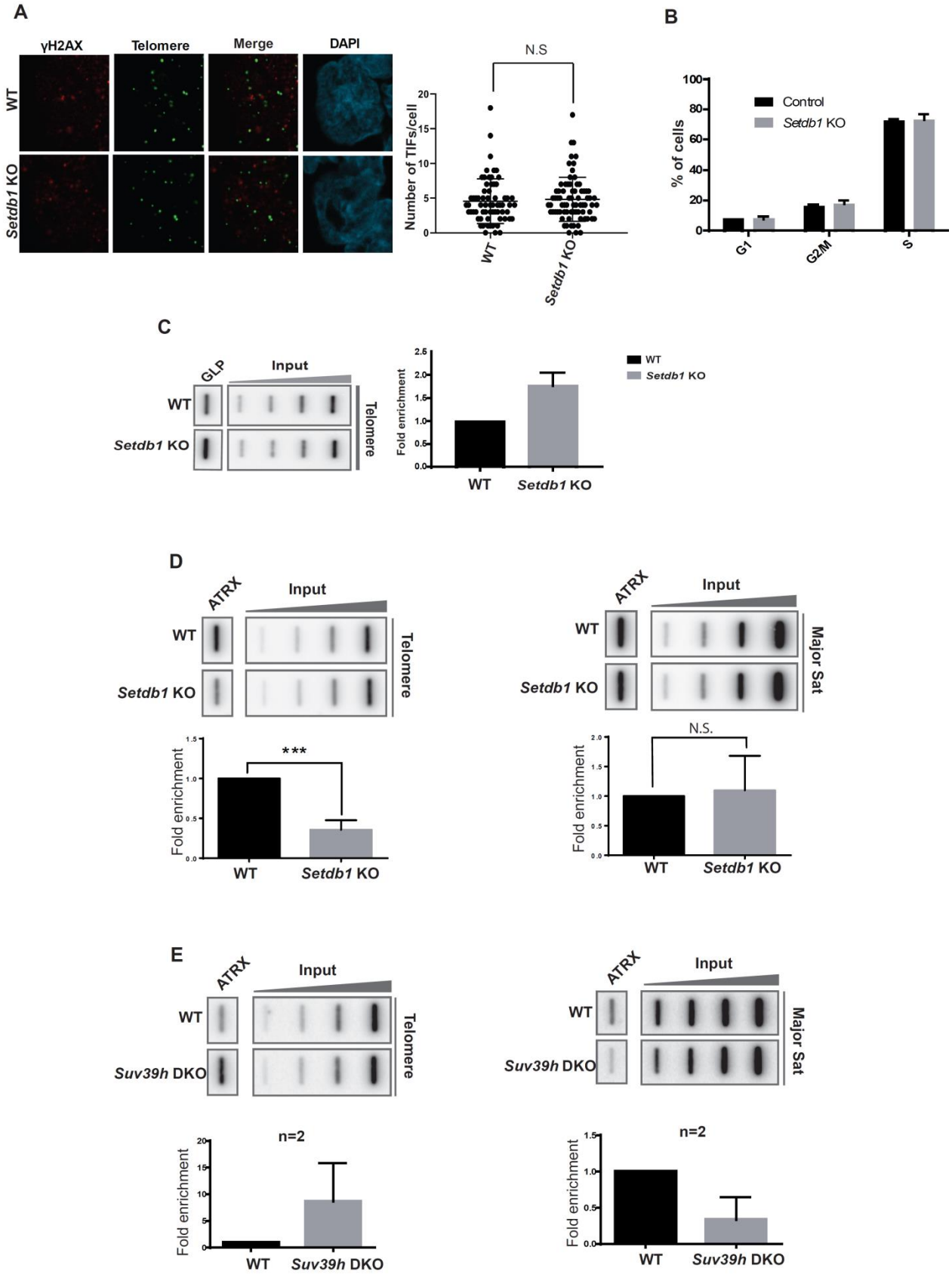

A
γH2AX
Telomere
Merge
DAPI
WT
Setdb1 KO
N.S
Number of TIFs/cell
WT
Setdb1 KO
B
Control
Setdb1 KO
% of cells
G1
G2M
S
C
GLP
Input
WT
Setdb1 KO
Telomere
Fold enrichment
WT
Setdb1 KO
D
ATRX
Input
WT
Setdb1 KO
Telomere
***
Fold enrichment
WT
Setdb1 KO
Major Sat
N.S.
E
Suv39h DKO
n=2

Figure S3

Fig. S3. ATRX binds heterochromatic telomeres. (**A**) (Right) γH2AX immunostaining (red) combined with telomere fluorescence in situ hybridization staining (green) to identify DNA damage at telomeres. (Left) Quantification of telomeres and γH2AX co-localization forming TIFs per cells in wild-type and *Setdb1* KO mESC. (**B**) Histogram showing the cell cycle distribution of *Setdb1* KO cells 4 days after knockout induction with tamoxifen. (**C**) (Left) ChIP experiments using an antibody raised against GLP on wild-type and *Setdb1*-negative chromatin from ESCs. 20% of the immunoprecipitated DNA was blotted and probed with a telomere-specific probe. (Right) Quantifications of the fold enrichment of GLP normalized by the input in *Setdb1* KO relative to the WT. (**D**) (Top) ChIP experiments using ATRX antibody on wild-type and *Setdb1* KO chromatin. 20% of the immunoprecipitated DNA was blotted and probed with a telomere or a pericentromere (Major Sat)-specific probe. (Bottom) Quantifications of the fold enrichment of ATRX normalized by the inputin *Setdb1* KO relative to the wild-type.. (**E**) (Top) ChIP experiments using ATRX antibody on WT and *Suv39h* KO chromatin. 20% of the immunoprecipitated DNA was blotted and probed with a telomere or a pericentromere (Major Sat)-specific probe. (Bottom) Graph of quantifications of the fold enrichment of ATRX normalized by the input in *Suv39h* KO relative to the wild-type.

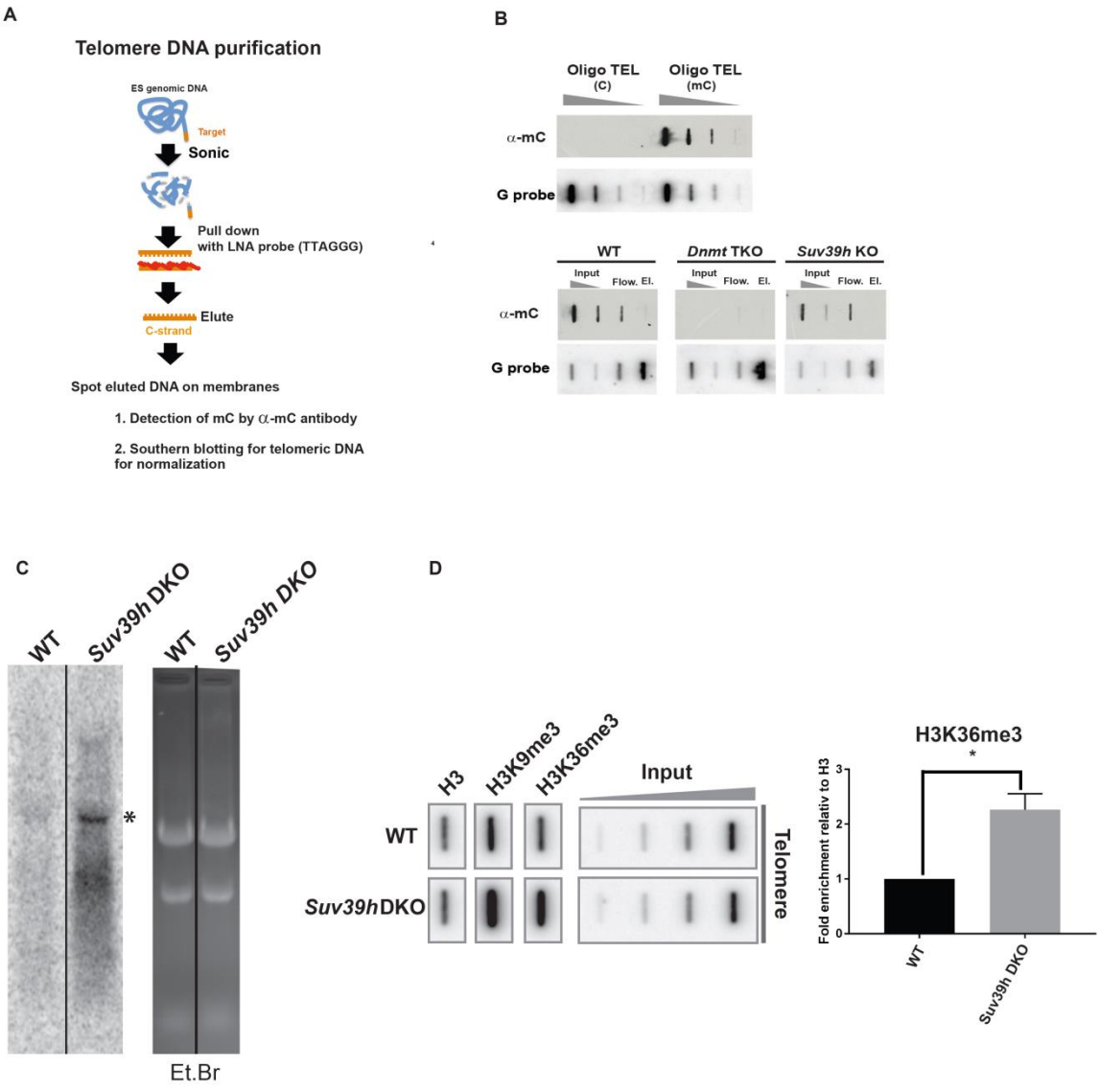

A
Telomere DNA purification
ES genomic DNA
Target
Sonic
Pull down
with LNA probe (TTAGGG)
Elute
C-strand
Spot eluted DNA on membranes
1. Detection of mC by α-mC antibody
2. Southern blotting for telomeric DNA
for normalization
B
Oligo TEL
(C)
Oligo TEL
(mC)
α-mC
G probe
WT
Dnmt TKO
Suv39h KO
Input
Flow.
El.
α-mC
G probe
C
WT
Suv39h DKO
WT
Suv39h DKO
*
Et.Br
D
H3
H3K9me3
H3K36me3
Input
WT
Suv39hDKO
Telomere
H3K36me3
*
Fold enrichment relativ to H3
0
1
2
3
WT
Suv39h DKO

Figure S4

Fig. S4. Telomeric heterochromatin stimulates transcriptional elongation. (**A**) Strategy used to measure asymmetric DNA methylation at telomeres. Genomic DNA from WT, *Dnmt* triple KO (having virtually no DNA methylation) and *Suv39h* KO (having a strong DNMT3 enrichment at telomeres) was sheared and telomeric DNA was enriched with telomere PICh probes. The enriched DNA was eluted and spotted on nitrocellulose filters and assayed for its content in methyl-Cytosine (mC) using an anti 5MeC antibody. (**B**) (Top) Titration of the mC signal using known amounts of a (CCCTAA)4 oligo (Oligo Tel C) or a (CCmCTAA)4+(mCCCTAA)4 oligo (Oligo Tel mC). (Bottom) mC signal at purified telomeric DNA of WT, *Dnmt* TKO as negative control or *Suv39h* KO cells. (**C**) TERRA Northern blot showing TERRA levels in WT and *Suv39h*KO cells. (**D**) (Left) ChIP experiments using H3, H3K9me3 and H3K36me3 antibodies on WT and *Suv39h*KO chromatin. 20% of the immunoprecipitated DNA was blotted and probed with a telomere specific probe. (Right) Quantifications of the fold enrichment at *Suv39h* KO telomeres of H3K36 methylation normalized by the total H3 signal and to the input relative to WT.

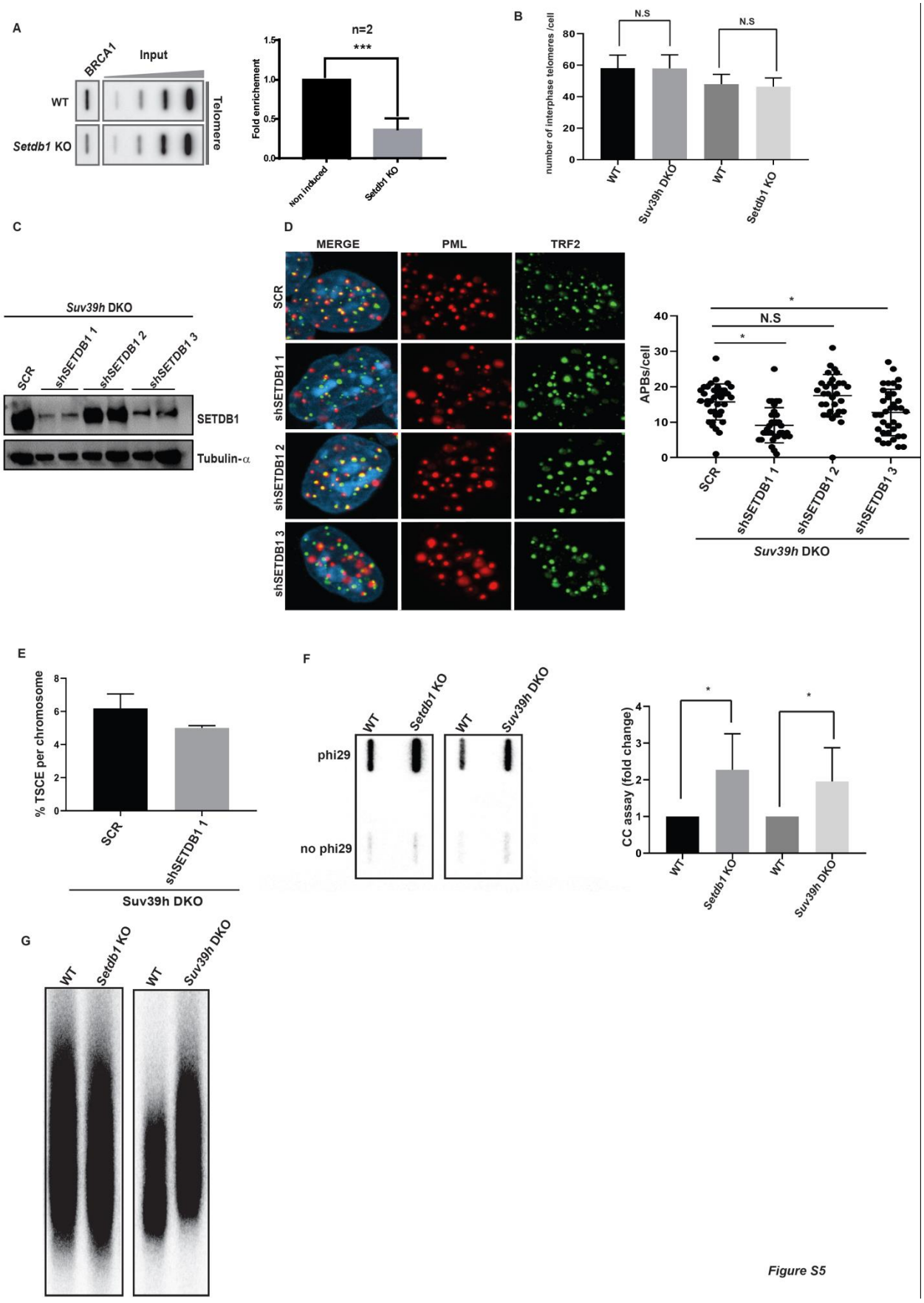
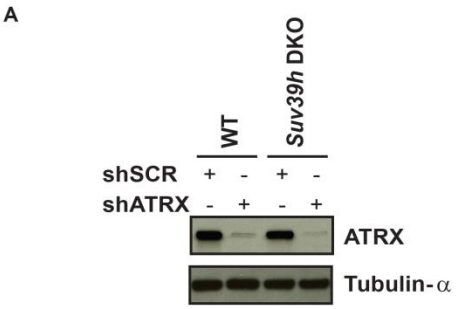
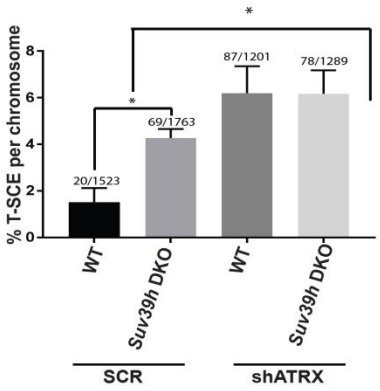
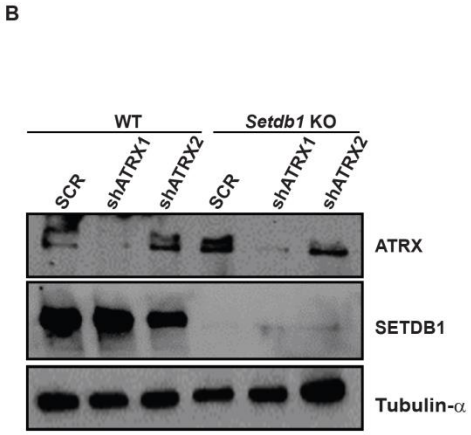

A
BRCA1
Input
WT
Setdb1 KO
Telomere
n=2
***
Fold enrichement
Non induced
Setdb1 KO
B
N.S
N.S
number of interphase telomeres /cell
WT
Suv39h DKO
WT
Setdb1 KO
C
Suv39h DKO
SCR
shSETDB1 1
shSETDB1 2
shSETDB1 3
SETDB1
Tubulin-α
D
MERGE
PML
TRF2
SCR
shSETDB1 1
shSETDB1 2
shSETDB1 3
APBs/cell
N.S
SCR
shSETDB1 1
shSETDB1 2
shSETDB1 3
Suv39h DKO
E
% TSCE per chromosome
SCR
shSETDB1 1
Suv39h DKO
F
WT
Setdb1 KO
WT
Suv39h DKO
phi29
no phi29
CC assay (fold change)
WT
Setdb1 KO
WT
Suv39h DKO
G
WT
Setdb1 KO
WT
Suv39h DKO

Figure S5

Fig. S5. SETDB1-dependent heterochromatin promotes the recruitment of recombination factors and the appearance of ALT features. (**A**) (Left) ChIP experiment using an anti BRCA1 antibody in wild-type non induced and *Setdb1* KO mESCs. 20% of the immunoprecipitated DNA was blotted and probed with a telomere specific probe. (Right) Quantification of the fold enrichment of BRCA1 at telomere normalized by the input relative to the wild-type. (**B**) Quantification of the number of interphase telomere signal per cell from FISH experiment of the wild-type and *Suv39h* or *Setdb1* negative mESCs. (**C**) Immunoblotting against SETDB1 or TUBULIN-α as a loading control in *Suv39h* negative cells transiently expressing a shRNA control or a shRNA against Setdb1 for knock-down. (**D**) (Left) Immunostaining of PML (red) or TRF2 (green) in *Suv39h* negative cells knock-down for Setdb1. (Right) Quantification of the number of co-localizations of telomeres with PML associated Bodies per cell. (**E**) Quantification of the percentage of the number of T-SCE per chromosome in *Suv39h* KO cells knockdown with an shRNA against SETDB1. (**F**) (Left) C-circle assay in wild-type and *Setdb1* or *Suv39h* negative mESCs. (Right) Quantification of the fold change of C-circle accumulation relative to wild-type in *Setdb1* and *Suv39h* negative cells. (**G**) Terminal Restriction Fragment analysis of telomeres in wild-type and *Setdb1* or *Suv39h* negative cells.


A
WT
Suv39h DKO
shSCR + - + -
shATRX - + - +
ATRX
Tubulin-α


% T-SCE per chromosome
0
2
4
6
8
*
*
20/1523
69/1763
87/1201
78/1289
WT
Suv39h DKO
WT
Suv39h DKO
SCR
shATRX


B
WT
Setdb1 KO
SCR
shATRX1
shATRX2
SCR
shATRX1
shATRX2
ATRX
SETDB1
Tubulin-α

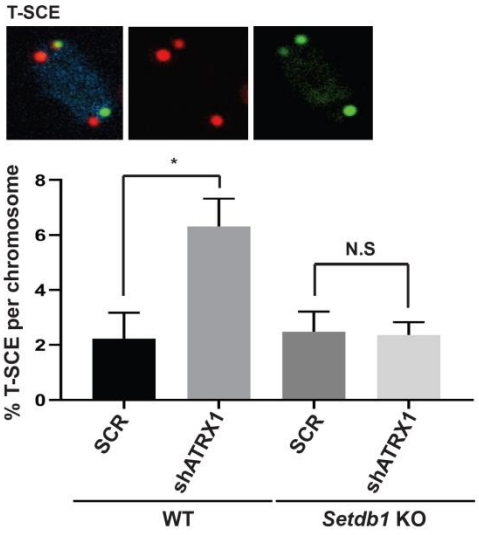

C
T-SCE
% T-SCE per chromosome
0
2
4
6
8
*
N.S
SCR
shATRX1
SCR
shATRX1
WT
Setdb1 KO

Figure S6

Fig. S6. ATRX prevents recombination when telomeres are heterochromatic. (**A**) Immunoblotting against ATRX or TUBULIN-α as loading control in wild-type or *Suv39h* negative mESCs expressing an shRNA against ATRX. (**B**) Quantification of % of T-SCE per chromosome upon scramble or Atrx knockdown in wild-type or *Suv39h* negative mESCs. (**C**) Immunoblotting against ATRX, SETDB1 or α-TUBULIN as a loading control in wild type or *Setdb1* negative mESCs expressing an shRNA scramble or an shRNA against ATRX. (**D**) Visualization of T-SCE with the reciprocal exchange of the leading and lagging strands. The C-rich strand is in green and the G-rich strand is in red. Quantification of the % of T-SCE per chromosome in wild-type or *Setdb1* negative mESCs knockdown for ATRX.

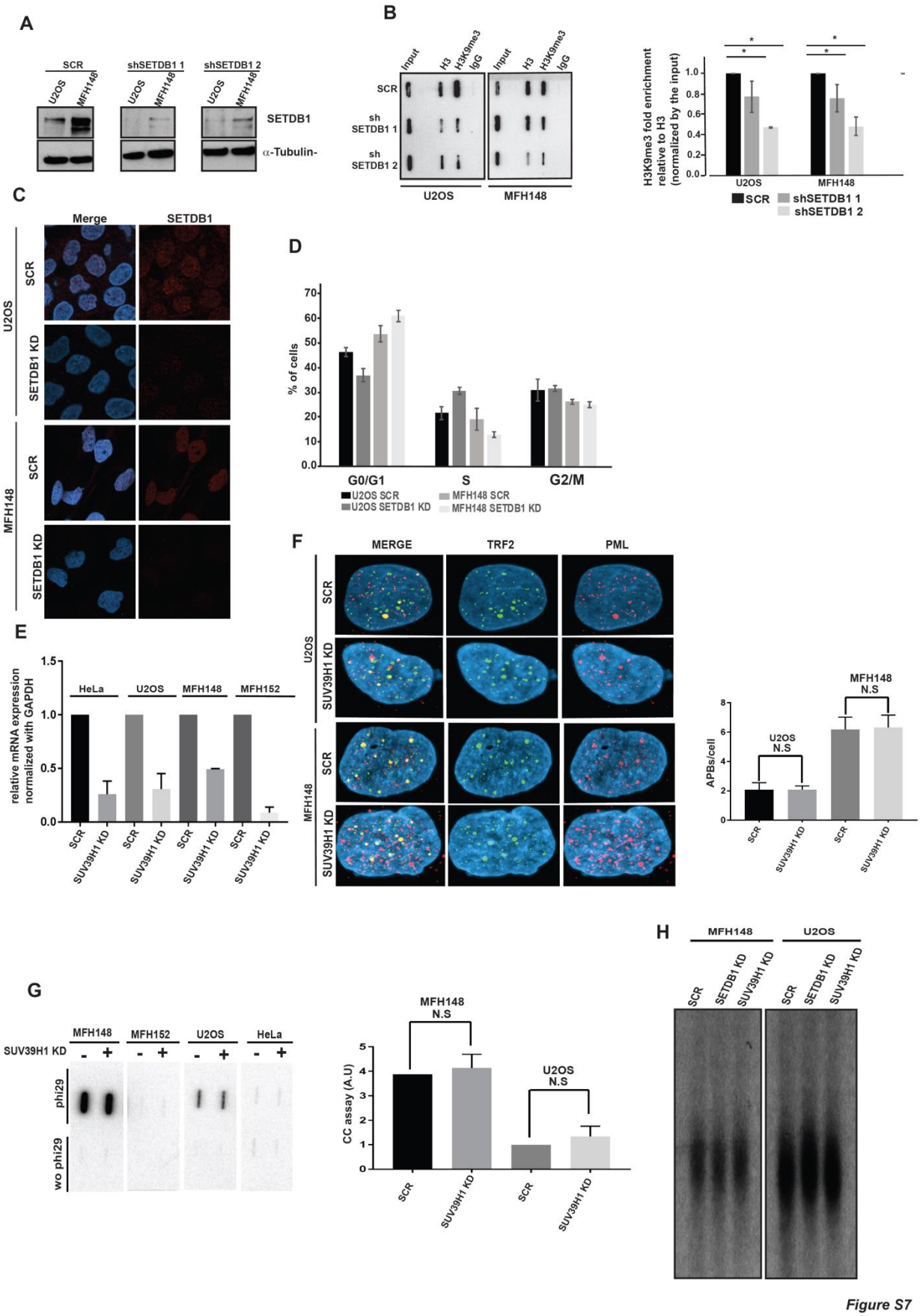

A
SCR
shSETDB1 1
shSETDB1 2
U2OS
MFH148
SETDB1
α-Tubulin-
B
Input
H3
H3K9me3
IgG
SCR
sh SETDB1 1
sh SETDB1 2
U2OS
MFH148
H3K9me3 fold enrichment relative to H3 (normalized by the input)
SCR
shSETDB1 1
shSETDB1 2
C
Merge
SETDB1
U2OS
MFH148
SETDB1 KD
D
% of cells
G0/G1
S
G2/M
U2OS SCR
U2OS SETDB1 KD
MFH148 SCR
MFH148 SETDB1 KD
E
relative mRNA expression normalized with GAPDH
HeLa
U2OS
MFH148
MFH152
SUV39H1 KD
F
MERGE
TRF2
PML
APBs/cell
U2OS N.S
MFH148 N.S
G
MFH148
MFH152
U2OS
HeLa
SUV39H1 KD
phi29
wo phi29
CC assay (A.U)
MFH148 N.S
U2OS N.S
H
MFH148
U2OS
SCR
SETDB1 KD
SUV39H1 KD

Figure S7

Fig. S7. Loss of SETDB1 and not of SUV39H promotes ALT features. (**A**) Immunoblotting against SETDB1 or TUBULIN-α as a loading control in ALT cell line U2OS or MFH148 transiently expressing a scramble or a Setdb1 shRNA for knock-down. (**B**) (Left) ChIP experiment using a H3K9me3 antibody in ALT cells U2OS or MFH148 knockdown for Setdb1. The immuno-precipitated DNA was plotted and probed with telomeric probes (Right) ChIP quantification of the fold enrichment of H3K9me3 normalized by H3 and the input relative to non-targeting scramble shRNA. (**C**) Immuno-staining analysis of SETDB1 in two ALT cell line U2OS and MFH148 using siRNA to induce the knock-down of Setdb1. (**D**) Cell cycle distribution analysed by FACS of U2OS and MFH148 upon the knock-down of Setdb1 by siRNA. (**E**) RT-qPCR analyses of the expression of Suv39h1 mRNA upon its knockdown by siRNA in HeLa, U2OS, MFH148 and MFH152 cells relative to the non-targeting scramble knock-down. . Data are normalized to GAPDH mRNA. (**F**)- (Left) Co-immunostaining of TRF2 (green) with PML (red) to measure ALT-associated PML Body formation upon Suv39h1 knockdown in two ALT cell lines U2OS and MFH148. (Right) quantification of number of telomeres associated PML bodies (APBs) per cells. (**G**) (Left) C-circle assay in two ALT positive cell line MFH148 and U2OS and ALT negative cell line MFH152 and HeLa upon a scramble or SUV39H1 knockdown (Right) Quantification of C-circle signal in MFH148 and U2OS upon the knockdown of SUV39H1. (**H**) Telomere Restriction Fragment of two ALT cell line MFH148 and U2OS upon the knock-down of Setdb1 or SUV39H1 by siRNA.

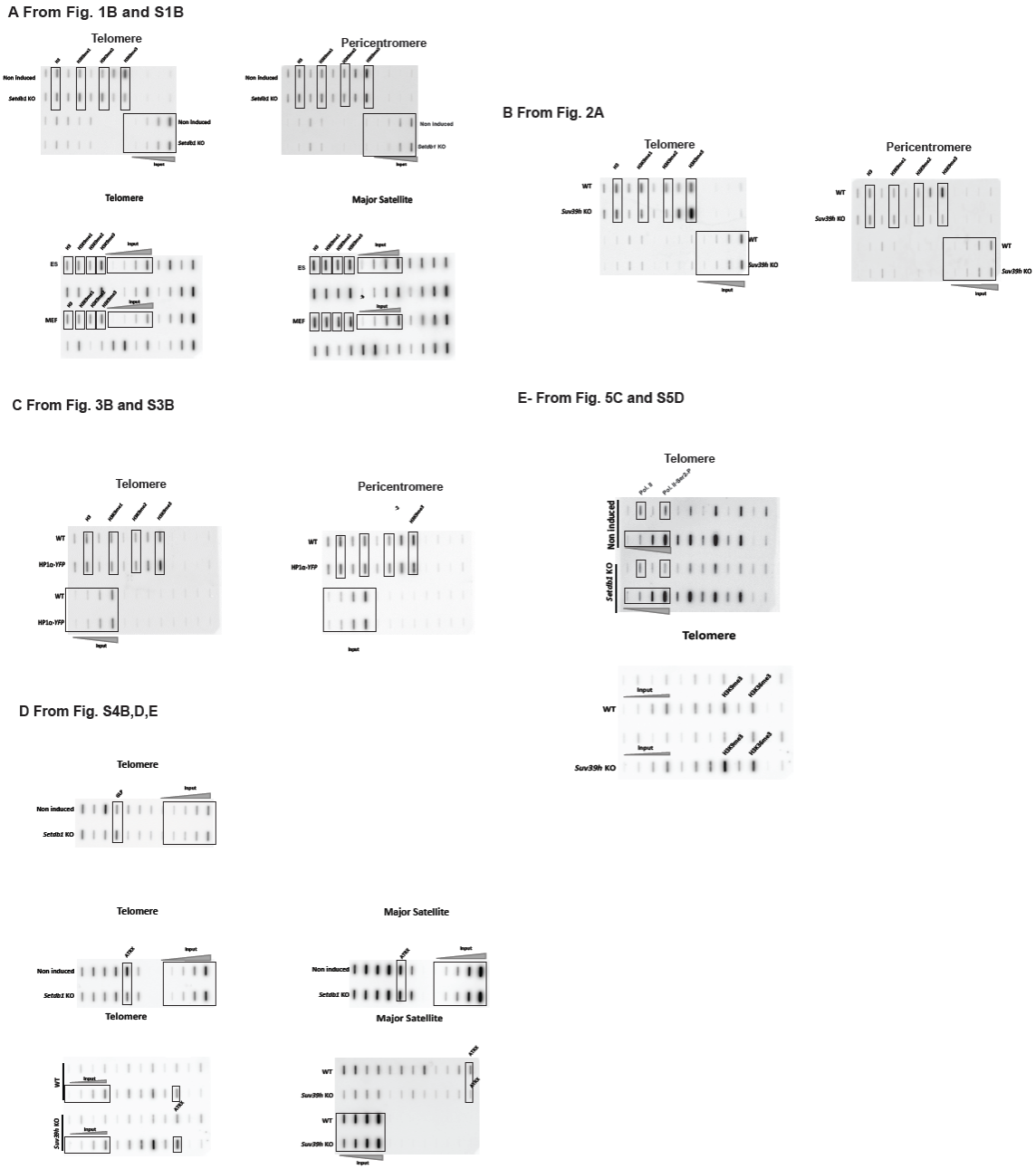


Figure S8

Fig. S8. Full membranes from which ChIP figures were prepared.

Table S1. List of factors enriched in the telomere PICh performed with wild-type ESCs.

Table S2. List of factors enriched in the telomere PICh performed with wild-type mouse embryonic fibroblast cells.

Supplementary Materials

REAGENT and RESOURCE

Antibodies	***Reference***	***Description***
Anti-SETB1 (5H6A12)	Abcam # ab107225	Mouse monoclonal
Anti-ATRX (H300)	Santa # Cruz sc-15408	Rabbit polyclonal
Anti-ATRX (Clone 39f)	Millipore #MABE1798	Mouse monoclonal
Anti-TRF1 and TRF2	Titia de Lange Lab	Rabbit polyclonal
Anti-PML (PG-M3)	Santa Cruz # sc-966	Mouse monoclonal
Anti-PML (H238)	Santa Cruz # sc-5621	Rabbit polyclonal
Anti- γH2A.X (JBW301)	Millipore # 05-636	Mouse monoclonal
Anti-HP1α (2616)	Cell signaling # 2616	Rabbit polyclonal
Anti-HP1β (1MOD 1A9)	Euromedex #1MOD 1A9	Mouse monoclonal
Alexa Fluor 568	Thermo Fisher Scientific # A-11004	Goat anti-mouse
Alexa Fluor 568	Thermo Fisher Scientific # A-11011	Goat anti-rabbit
Alexa Fluor 488	Thermo Fisher Scientific # A-11029	Goat anti-mouse
Alexa Fluor 488	Thermo Fisher Scientific # A-11008	Goat anti-rabbit

ChIP antibodies

Antibody	***Reference***	***Description***	***Dynabead***
Anti-RNA pol II (N-20)	Santa Cruz # sc-899	Rabbit polyclonal	Protein A
Anti-RNA pol II CTD pSer2	Active Motif #61083	Rat monoclonal	Protein G
Anti-ATRX (H-300)	SantaCruz # sc-15408	Rabbit polyclonal	Protein A
Anti-BRCA1	Satoshi Namekawa lab	Rabbit polyclonal	Protein A
Anti-GLP	Abcam # Ab41969	Rabbit polyclonal	Protein A
Anti-H3 (PAN)	Abcam # ab1791	Rabbit polyclonal	Protein A
Anti- H3K9me1	Abcam # Ab9045	Rabbit polyclonal	Protein A
Anti-H3K9me2	Abcam # Ab1220	Mouse monoclonal	Protein A
Anti-H3K9me3	Abcam # ab8898	Rabbit polyclonal	Protein A
Anti-H3K36me3	Abcam # ab9050	Rabbit polyclonal	Protein A
Anti-IgG Rabbit	SantaCruz # sc-2027	Rabbit IgG	Protein A
Anti-IgG Mouse	SantaCruz # sc-2025	Mouse IgG	Protein G

Western Blot

Antibody	***Reference***	***Description***	***Dilution***
Anti-SETDB1	Abcam # ab107225	Mouse monoclonal	1:500
Anti-DNMT3B	Abcam # ab16049	Rabbit polyclonal	1:500
Anti-ATRX (H-300)	SantaCruz # sc-15408	Rabbit polyclonal	1:500
Anti-DAXX	Upstate # 07 471	Rabbit polyclonal	1:500

Anti-TRF1	T. de Lange # 1449	Rabbit polyclonal	1:1000
Anti-TRF2	T. de Lange # 1255	Rabbit polyclonal	1:1000
Anti-SPT 16	Upstate # 07225	Rabbit polyclonal	1:500
Anti-XRN2	Proteintech # 112671AP	Rabbit polyclonal	1:500
Anti-H3 (PAN)	Abcam # ab1791	Rabbit polyclonal	1:10000
Anti-H3.1/2	Millipore # ABE154	Rabbit polyclonal	1:500
Anti-H3.3 (MO1)	Abnova # HOC00321	Mouse monoclonal	1:200
Anti-H3K9me3	Abcam # ab8898	Rabbit polyclonal	1:1000
Anti-H3K36me3	Abcam # ab9050	Rabbit polyclonal	1:500
Anti-H3K4me3	Abcam # ab8580	Rabbit polyclonal	1:500
Anti-H3K27me3	Active Motif # 61017	Mouse monoclonal	1:300
Anti-HP1α/β (C7F11)	Cell Signaling # 2623	Rabbit monoclonal	1:1000
Anti-HP1α (2616)	Cell signaling # 2616	Rabbit polyclonal	1:1000
Anti-HP1β (1MOD 1A9)	Euromedex #1MOD 1A9	Mouse monoclonal	1:1000

Sequence based reagents

Commercial FISH probe

Telomere TelC-Cy3 PNA probe	Panagene	F1002
Telomere TelG-A488 LNA probe	Exiqon	

- Telomeric probe:
  (CCCTAA)6
- Major Satellite probe:
  TATGGCGAGGAAAACTGAAAAAGGTGGAAAATTTAGAAATGTCCACTGTAG
  GACGTGGAATATGGCAAG

Chemicals

Chemical	***Source***	***Reference***
(Z)-4-Hydroxytamoxifen	Sigma-Aldrich	H7904
Protein A Dynabeads	Life Technologies	10004D
Protein G Dynabeads	Life Technologies	10002D
Positively charged nylon Hybond XL membrane	GE Healthcare	45-001-151
PerfectHyb Plus	Sigma	051M6009
Streptavidin, Alexa Fluor 488 conjugate	Invitrogen	S11223
TRIzol reagent	Invitrogen	15596026

Critical Commercial Assays	***Source***	***Reference***
NorthernMax Kit	ThermoFisher Scientifique	AM1940
SilverQuest	Invitrogen	45-1001
Polynucleodtide Kinase kit	NEB	M0201S
Prime-a-Gene kit	Promega	U1100