Supplementary figure legends.

Figure S1. Generation of the Rif1^{FH} allele and normal viability of Rif1^{FH} homozygous mice. (**A**) Targeting strategy for the insertion of the FlagHA tag in the endogenous Rif1 locus. Schematic diagram of the mouse Rif1 locus (WT), the targeting construct used for homologous recombination-mediated allele introduction and of the targeted Rif1 locus prior (targeted allele) and after (Rif1^{FH} allele) Flpase (Flp)-mediated recombination. Red triangles represent FRT sites and grey rectangle a Neomycin (Neo) selection marker gene as indicated. Restriction sites for NdeI and BamHI as well as the respective Southern fragments detected by a 5' probe are shown (*black line). (**B**) Southern blot validation of Rif1^{FH} targeted ESCs used for blastocyst injection. (**C**) Rif1^{FH} is a functional allele. Rif1^{FH} homozygous mice derived from heterozygous intercrosses are born in normal mendelian ratios. (**D**) Rif1^{FH} and untagged Rif1 proteins display identical intranuclear localization. Immunofluorescence staining of untagged (anti-Rif1 1240, red) and FlagHA-tagged (anti-HA, green) Rif1 protein in Rif1$^{FH/+}$ MEF.

Figure S2. Definition of the 6 S-phase substages by EdU staining (green) on pMEFs nuclei (DAPI, blue). S1-S3 represent early S-phase stages, where replication of euchromatin occurs and is characterized by a diffuse pan-nuclear staining. In S1 single replication foci are distinguishable as separated EdU dots, in S2 replication is excluded from the nucleoli (dark regions in the nuclei, not stained by DAPI), while in S3 EdU shows a diffuse nuclear staining. Mid S-phase comprises S4 and S5, the time of replication of pHC. In S4 replication occurs at the outer chromocenter borders (brighter DAPI-stained patches), giving rise to characteristic "rings". During S5 replication occupies the inner area of the chromocenter. S6 represents late replication.

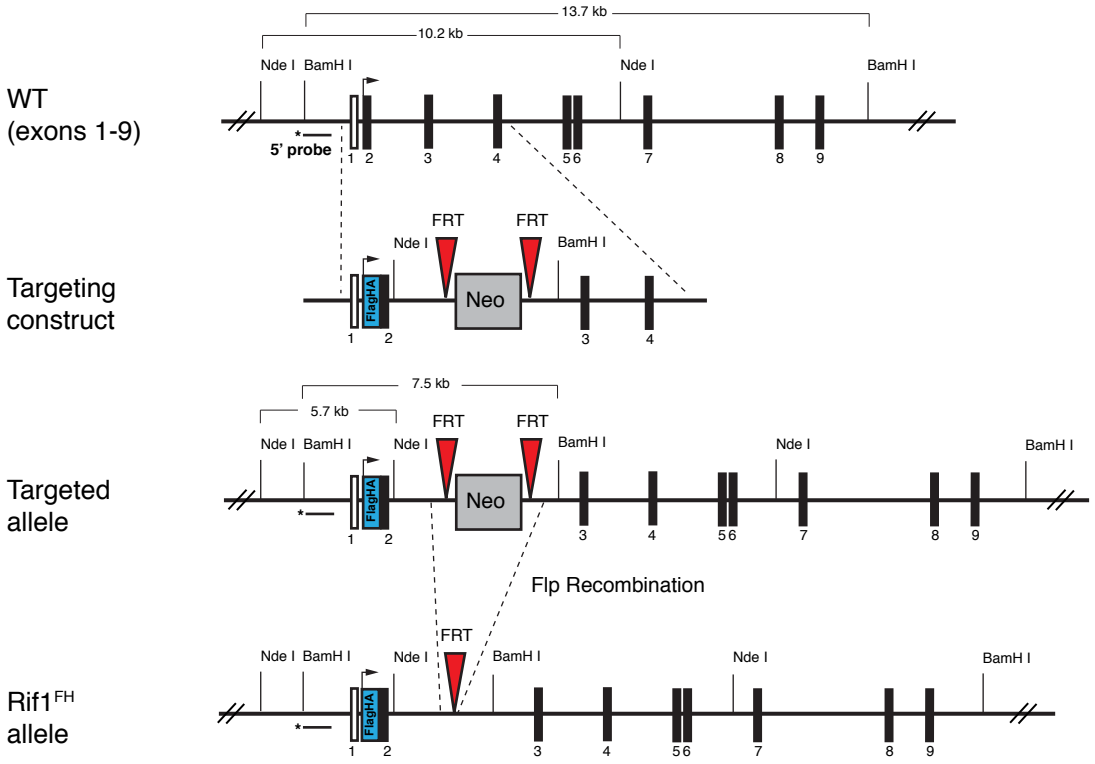
Figure S3. (**A**) Western blot for Rif1 on either $Rif1^{F/F}$ or $Rif1^{+/+}$ pMEF infected with a retrovirus encoding the CRE recombinase. Three independent $Rif1^{F/F}$ and $Rif1^{+/+}$ cell lines (A-C) are shown. Loading control: unspecific signal. Anti-Rif1 antibody 1240 was used. (**B** and **C**). The replication profiles of biological replicates from Fig. 3D and Fig. 3E are shown. Two independent $Rif1^{-/-}$ (red) compared to two $Rif1^{+/+}$ (black) pMEFs. (**D**) Microarray analyses of gene expression upon Rif1 deletion. Two $Rif1^{+/+}$ and two $Rif1^{F/F}$ pMEF independent lines have been analyzed upon infection with CRE-recombinase (CRE) or Empty Vector (EV). Each dot represents one transcript of 28,853 genes screened. Scatter plots showing average raw intensity values of transcript levels between $Rif1^{+/+}$+CRE versus +EV (left) and $Rif1^{F/F}$ +CRE versus +EV (right). The outer green lines demarcate the 2.0 fold change boundaries. No gene showed a statistically significant change in transcription (P-value <0.05).

Figure S4. (**A**) Immunofluorescence for trimethylated Lys9 histone H3 (H3K9me3), and HP1α (green) in $Rif1^{+/+}$ and $Rif1^{F/F}$ pMEF +CRE ($Rif1^{-/-}$). EdU is in red and DAPI in blue. (**B**) $Rif1^{-/-}$ cells show normal levels of total H3K9me3, trimethylated Lys27 histone H3 (H3K27me3), trimethylated Lys20 histone H4 (H4K20me3), HP1α and MeCP2 as revealed by immunoblots of serial dilutions of whole cells extracts of two $Rif1^{+/+}$ and $Rif1^{F/F}$ +CRE ($Rif1^{-/-}$) cell lines (A-B), respectively. (**C**) The same membrane used for the Southern-Western Blot in Fig. 5B probed for Southern with an anti-major satellite probe. U= enzymatic units (**D**) Western blot for Rif1 from $Rif1^{+/+}$; $Rosa26^{CreERT2/+}$ and $Rif1^{F/F}$; $Rosa26^{CreERT2/+}$($Rif1^{-/-}$) treated with 4-hydroxytamoxifen at 13, 15 and 17 hours upon release from G0. Since Rif1 levels in cells arrested in G0 are lower than in G1 (LG vs. G0 and (Xu & Blackburn, 2004), to fully appreciate the efficiency of the CRE-mediated deletion the first G1/S timepoints are shown. LG = logarithmically growing. Smc1=loading control.

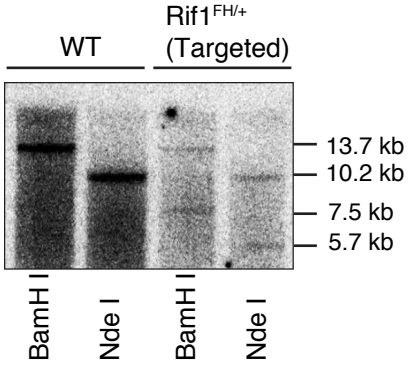
Figure S5. (**A**) Rif1 deletion by CRE infection decreases the percentage of $BrdU^+$ cells. 30' BrdU pulse was given to six independent $Rif1^{+/+}$ or $Rif1^{F/F}$ pMEF lines

infected with a retrovirus either encoding for CRE recombinase (CRE) or empty (EV). BrdU content was analyzed by FACS. The changes were quantified by calculating the percentage reduction of $BrdU^+$ cells as a ratio of EV/CRE. (**B**) Table summarizing the percentage of cells in G1 and G2, as quantified by FACS analysis of six independent $Rif1^{+/+}$ or $Rif1^{F/F}$ pMEF lines infected with a retrovirus either encoding for CRE recombinase (CRE) or empty (EV). (**C**) The percentage of G1 and G2 cells monitored by FACS analysis from the experiment in Fig. 8B was plotted versus time. An average of three independent pMEF lines for each genotype is presented. (**D**) Western blot for p21 on either $Rif1^{F/F}$ or $Rif1^{+/+}$ cycling pMEF infected with a retrovirus encoding the CRE recombinase. Three independent $Rif1^{F/F}$ and $Rif1^{+/+}$ cell lines (A-C) are shown. Loading control: alpha tubulin. (**E**) MEFs were FACS sorted on the basis of their DNA content, as measured by Hoechst 33342, in three fractions, G0/G1, S and G2/M. The purity of each of them was judged by re-analyzing part of the sorted cells by FACS. Each histogram shows the peak of the main fraction and the relative contamination from other fractions. G2/M shows high contamination from the S-phase fraction (42%). Protein extracts from these cells are shown in Fig. 8B.

A



B

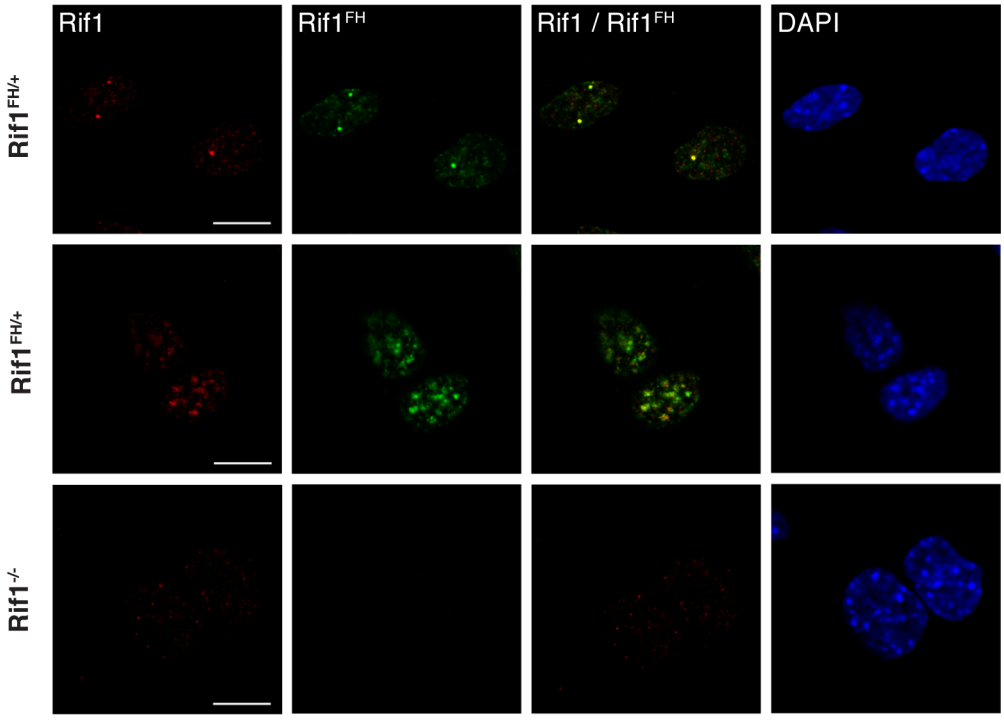


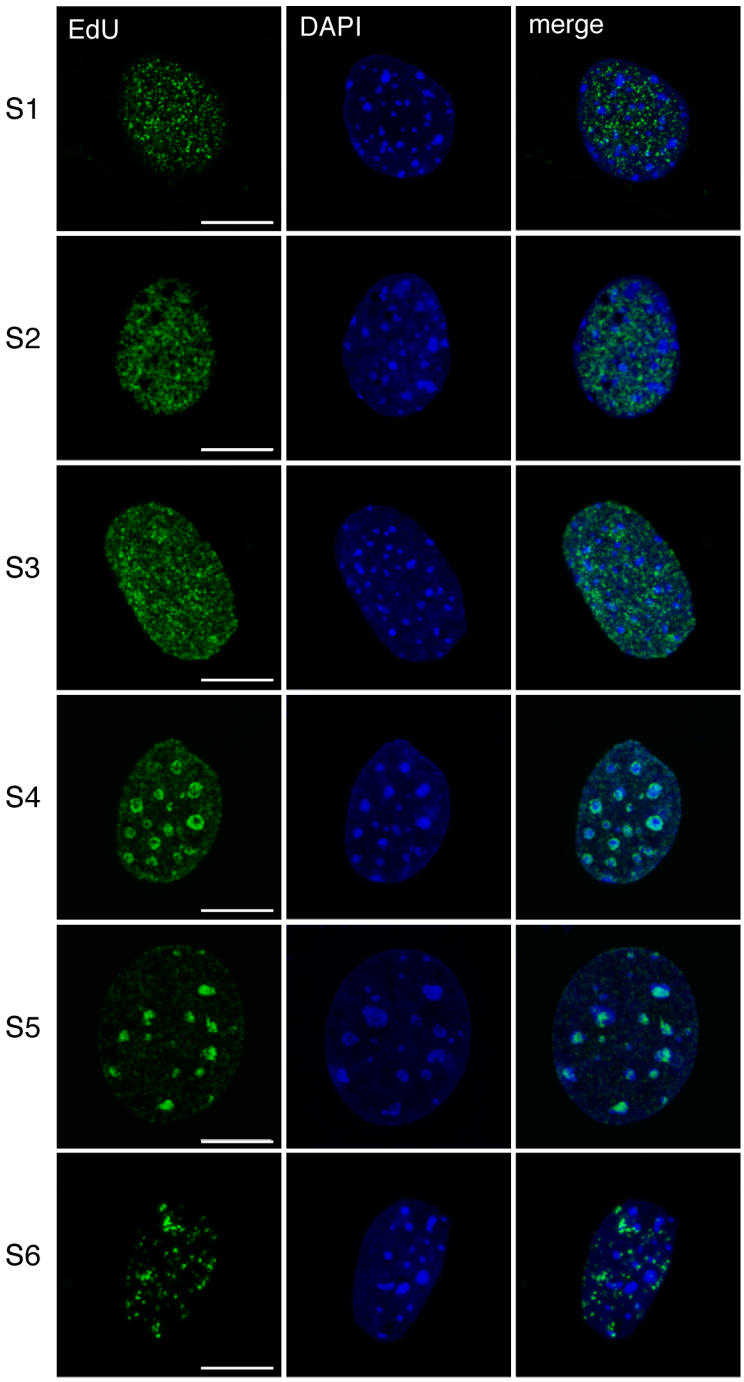
C

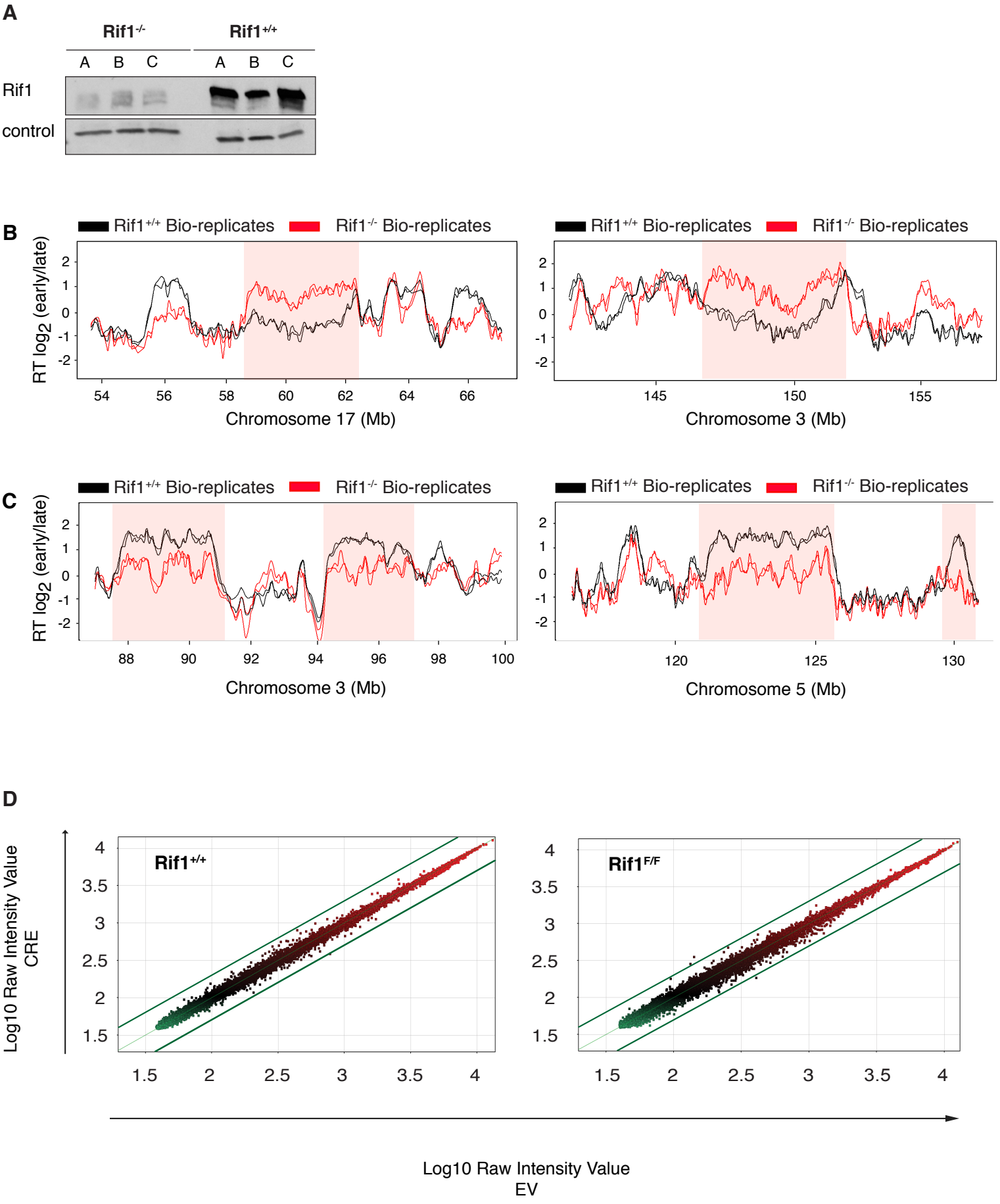
live pups at weaning C57B6 Rif1$^{FH/+}$ xRif1$^{FH/+}$

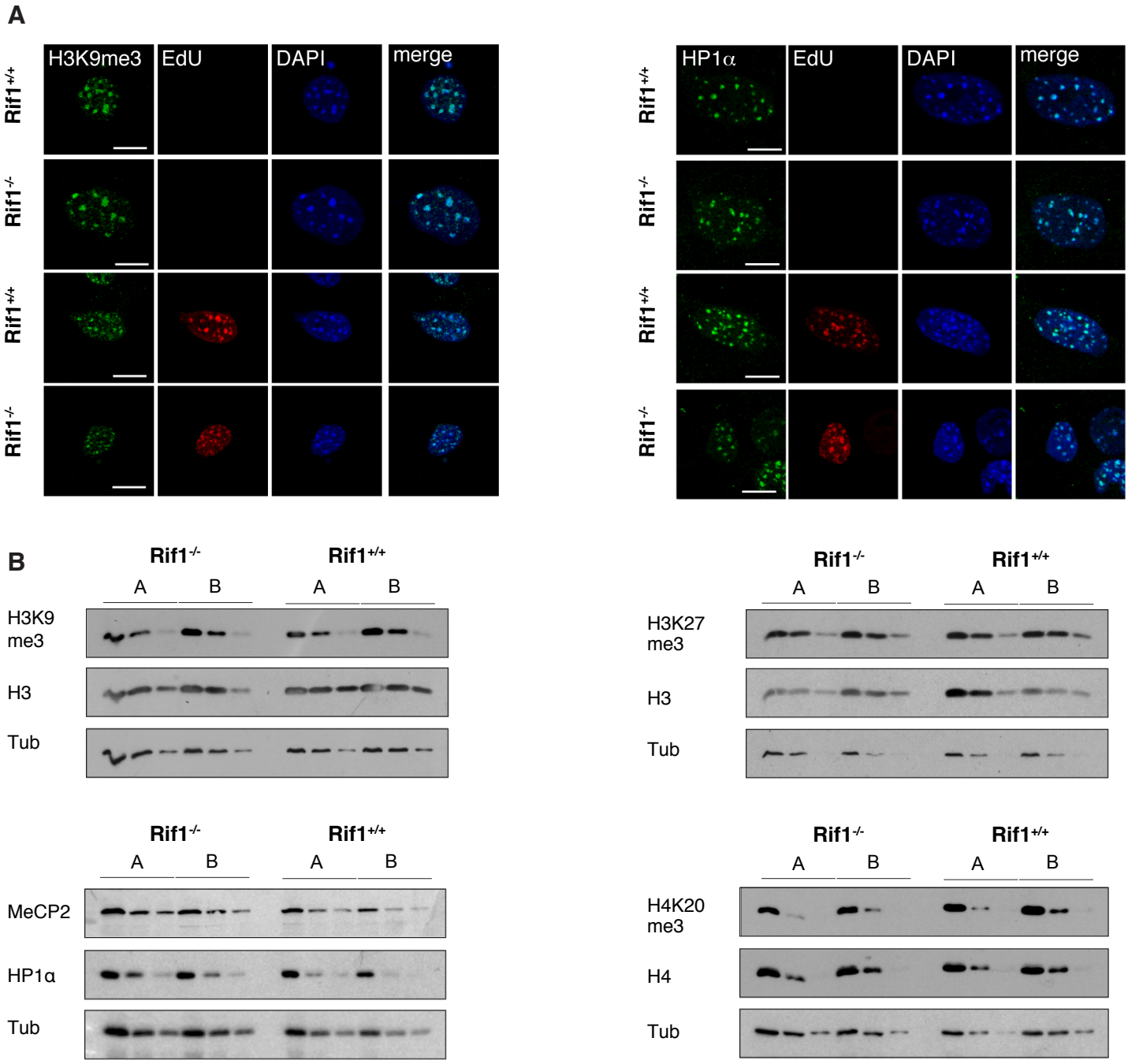
	+/+	FH/+	FH/FH
observed	29	45	27
expected	25	50	25

D

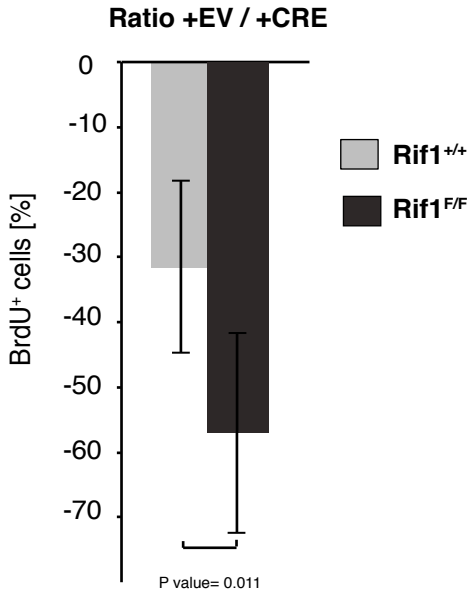



EdU
DAPI
merge
S1
S2
S3
S4
S5
S6


A
Rif1-/-
Rif1+/+
A B C
A B C
Rif1
control
B
Rif1+/+ Bio-replicates
Rif1-/- Bio-replicates
RT log2 (early/late)
Chromosome 17 (Mb)
Chromosome 3 (Mb)
C
Chromosome 3 (Mb)
Chromosome 5 (Mb)
D
Rif1+/+
Rif1F/F
Log10 Raw Intensity Value CRE
Log10 Raw Intensity Value EV


A
H3K9me3
EdU
DAPI
merge
HP1α
Rif1+/+
Rif1-/-
B
Rif1-/-
Rif1+/+
A
B
H3K9 me3
H3
Tub
H3K27 me3
MeCP2
HP1α
H4K20 me3
H4
C
U MNase
0.06
0.08
0.2
SB: MajSat
D
synchronous
LG
G0
13
15
17
h
Rif1
Smc1

A

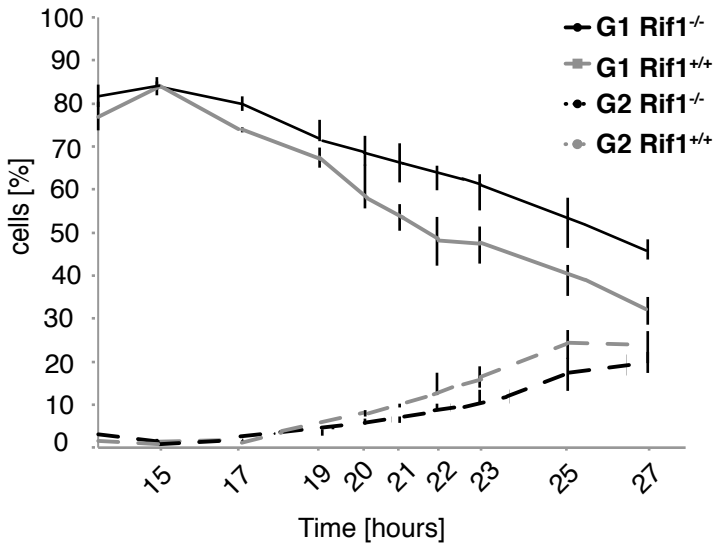


B

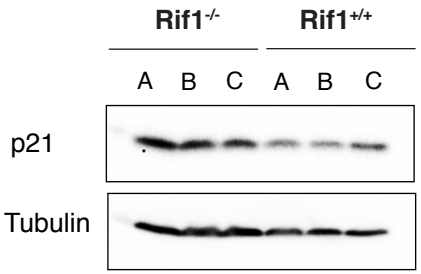
G1			
EV	CRE	**P value**	
57	63	0.004	Rif1$^{F/F}$
56	59	0.4	Rif1$^{+/+}$

G2			
EV	CRE	**P value**	
25	26	0.4	Rif1$^{F/F}$
27	28	0.6	Rif1$^{+/+}$

C



D



E