Supplemental Information

Cell lines

HEK293 and VA13/WI38 cells were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) supplemented with 10% FBS and 1% Pen-Strep.

Synchronization of cells

To synchronize HeLa cells at G1/S phase, cells were cultured in medium containing 2.5 mM thymidine for 16 h. Cells were washed and cultured in fresh medium for 8 h and then cultured in medium containing thymidine for another 16 h.

Transfections

Small interfering RNA (siRNA)-mediated knockdown in HeLa cells was performed with GeneSolution siRNAs (Qiagen) siTTF1-1 (SI04207294), siTTF1-2 (SI04247040), siTIM-3 (SI00745017), siTIM-5 (SI04140297), siTIM-6 (SI04142194), siTIM-7 (SI04282880) or siCTRL (1027280), and HiPerFect transfection reagent (Qiagen). OriP vectors were transfected to 293E cells with TransIT 2020 (Mirus). Transfection of shRNA expression vectors into mouse ES cells was with Xfect mESC reagent (Takara Bio). All transfections were according to the manufacturers' instructions.

Plasmids

To obtain the Sal-box fragments T1-2-R1, R2 and R3 (Supplemental Figure S1E-G), the PCR fragment amplified with primer set YA794/5 was divided by StyI that cleaves at the 3′ end of each R repeat. Sal-box fragments T1-5, T1-2 and T3-5 were obtained by PCR from the cloned Sal-box T1-2-R1 transcription terminator sequence with primer sets YA847/51, YA847/8, and YA849/51, respectively. The pyrimidine-rich fragment R1 Py-rich was isolated from the R1 DNA fragment by SalI cleavage in Sal-box T5 and StyI. These fragments were cloned into the Hind III site of the OriP vector and their sequence and orientation of insertion were verified by sequencing analysis.

Western blot

Total cell lysate was prepared in lysis buffer (50mM Tris-HCl pH8.0, 2mM EDTA, 150mM NaCl, 1% Triton-X), separated by SDS-PAGE and then transferred to a PVDF membrane. Used as primary antibodies were: anti-TTF-1 A302-361A (Bethyl-Laboratories), anti-TIM ab50943 (Abcam) and anti-alpha Tubulin ab4074 (Abcam).

RT-PCR

Total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen) and cDNAs were synthesized from mRNA primed with oligo (dT) and Super Script III (Life Technologies). PCR was performed with primer sets YA657/8 for human *TTF-1*, YA640/1 for human *ACTB*, YA693/4 for mouse *Ttf-1* and YA743/4 for mouse *Actb*.

Supplemental Table

PCR primers used in this study

Name	Sequence (5′ to 3′)
YA592	TATTGAAAGTCAGCCCTCGACA
YA593	GAGGAGCGAGGAGGAAGGAC
YA640	AGAAAATCTGGCACCACACC
YA641	GGGGTGTTGAAGGTCTCAAA
YA646	GTTGCCATGGTAATCCTGCT
YA647	ACCCAGAAGCAGGTCGTCTA
YA650	GAACCGCAGGTTCAGACATT
YA651	ACCCAGAAGCAGGTCGTCTA
YA657	GCCATGATGGAAGAAGGTGT
YA658	CATCGGCATCTCCTGAATCT
YA693	ACCACTTCTGCCCACAAGAA
YA694	TTTCACGCACTTTTGCTGAC
YA743	GATCTGGCACCACACCTTCT
YA744	GGGGTGTTGAAGGTCTCAAA
YA780	ATGGGATCGGCCATTGAACA
YA781	TCAGAAGAACTCGTCAAGAA
YA794	TCGGTACCCGGGGATCCGTCCTTCCTCCTCGCTCCT
YA795	GACTCTAGAGGATCGCAGAGGCAGCAGCAGCAGC
YA847	CGTCCTTCCTCCTCGCTCCT
YA849	TGAGACTCAGCCGGCGTCTC
YA851	AGTGACTCCCTCTTAAAAGT
YA872	GATATTCAGAATGTTTCATTCCTAC
YA873	ACGAACTAAACCTGACTACG
YA879	GTCCGTCCTTCCGTTCGTCTT
YA914	GACGAGATCCTCGCCGTC
YA915	TGAACAAGATGGATTGCACG

Supplemental figure legends

Supplemental Figure 1

Cartoons illustrate the strategy determining the direction of replication fork arrest at an RFB site. Y-arc signal is seen in 2D-gel analysis when an examined fragment replicates passively from one end to the other end. The apex of Y-arc, the slowest migration position in the second dimension, corresponds to Y-fork of half-replicated fragment that is structure of the maximal deviating from linearity. (I) The replication fork progresses on a fragment from right to left direction. Signal that represents Y-fork at RFB site (black dot) shifts in a counterclockwise direction on the Y-arc when a restriction enzyme shortens the unreplicated stem of Y-fork. (II) In the case that replication fork progresses the opposite direction, the restriction cut shortens already-duplicated branches of Y-fork. Thus the corresponding signal (gray dot) shifts in a clockwise direction on the Y-arc.

Supplemental Figure 2

(A) Schematic diagram of the initiation of replication on the origin (OriP) of the Eppstein-Barr virus, as controlled by the viral protein EBNA-1. For episomal replication, a vector containing the OriP was used. The viral protein EBNA-1 binds to the FR (family of repeats) and DS (dyad symmetry) elements within OriP, recruits the cellular replication machinery and initiates replication. One of the replication forks is blocked by the FR-EBNA-1 complex, so that, eventually, replication of the plasmid proceeds unidirectionally. (B) Strategy of OriP/EBNA-1 episomal replication assay. 293E cells stably expressing EBNA-1 protein were transfected with the OriP-containing plasmids (OriP vector). The transfected cells were incubated for six days to allow OriP vectors to replicate and then their DNA was isolated. (C) The OriP vector became resistant to DpnI-cleavage after multiple rounds of replication during six days incubation. Empty OriP vector recovered from 293E cells were digested with NcoI alone (DpnI -) or together with DpnI (+). The NcoI fragments containing multiple DpnI restriction sites were visualized by Southern blotting with the oriP-2 probe. (D) Replication intermediates of empty OriP vector as analyzed by neutral/neutral 2D-gel–electrophoresis. The XbaI-ClaI fragments

were visualized by hybridization with the oriP-1 probe. (E) Schematic diagram of dissection of the transcription terminator region. The dissected fragments 1 to 7 were inserted into the OriP vector at the HindIII site in the head-on direction. (F) 2D-gel analyses of the replication of OriP vectors containing the fragments in (E). ApaLI-NdeI fragments were visualized by Southern blotting with the oriP-1 probe. Red arrowheads indicate accumulation of Y-forks representing RFB activity. All the Sal-box containing fragments (no. 1, 2, 3, 4, 6 and 7, but not 5), exhibited RFB activities in the head-on direction. Fragments 1 and 4, which contain Sal-box elements T1 to T5, caused the accumulation of two different Y-fork intermediates. These could be resolved after splitting fragment 4 into fragments 6 and 7, which separated Sal-box T1 to T2 and T3 to T5, respectively. Thus Sal-box regions T1 to T2 and T3 to T5 can both act as an independent RFB site. (G) 2D-gel analyses of replication intermediates on OriP vectors with fragments cloned in the co-direction. The undissected Class I fragment was in the BstZ17I site (cf. with panel on the right in Fig. 2B), the other fragments were inserted into the HindIII site (cf. with corresponding panels in F). Red arrowheads indicate accumulation of Y-forks representing replication fork arrests. Asterisks mark cross-hybridization with non-replicating molecules. The Sal-box regions T1 to T5 (fragment 4) and T3 to T5 (fragment 7) caused Y-fork accumulation, whereas the Sal-box region with T1 and T2 (fragment 6) was inactive. The whole fragment (Class I) gave rise to three arrested replication forks, which is explained by the presence of Sal-box elements T4 and T5 in each of the three repeats.

Supplemental Figure 3

(A) Schematic diagram of the transcription terminator in mouse rDNA containing two repeated segments, mR1 and mR2, consisting of Sal-box T1 to T5 and T6 to T10, respectively. (B) Alignment of mR1 and mR2 repeats with a sequence identity of 86 % between mR1 (13424 to 13711 in BK000964, GenBank) and mR2 (13827 to 14103). (C) Ku70 is dispensable for the arrest of replication forks at RFB sites. DNAs from $Ku70^{-/-}$ and wild-type cells were digested with EcoRI and AseI, separated by 2D-gel electrophoresis and hybridized to the mouse 28S probe.

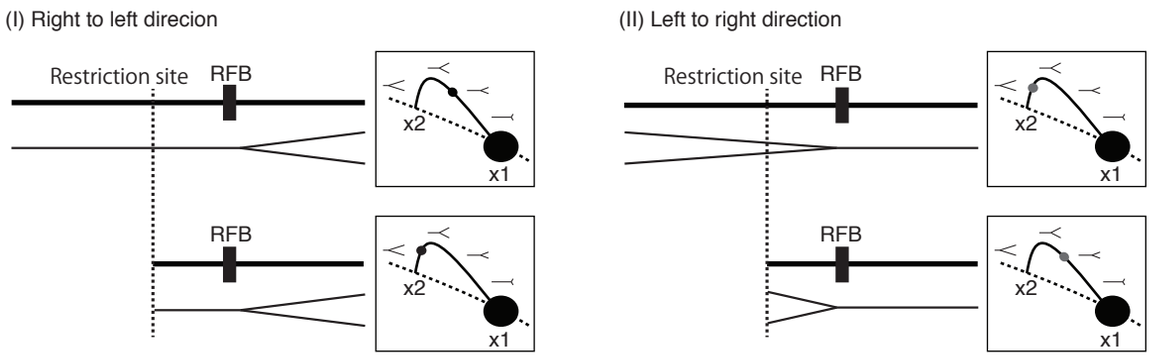
Supplemental Figure 4

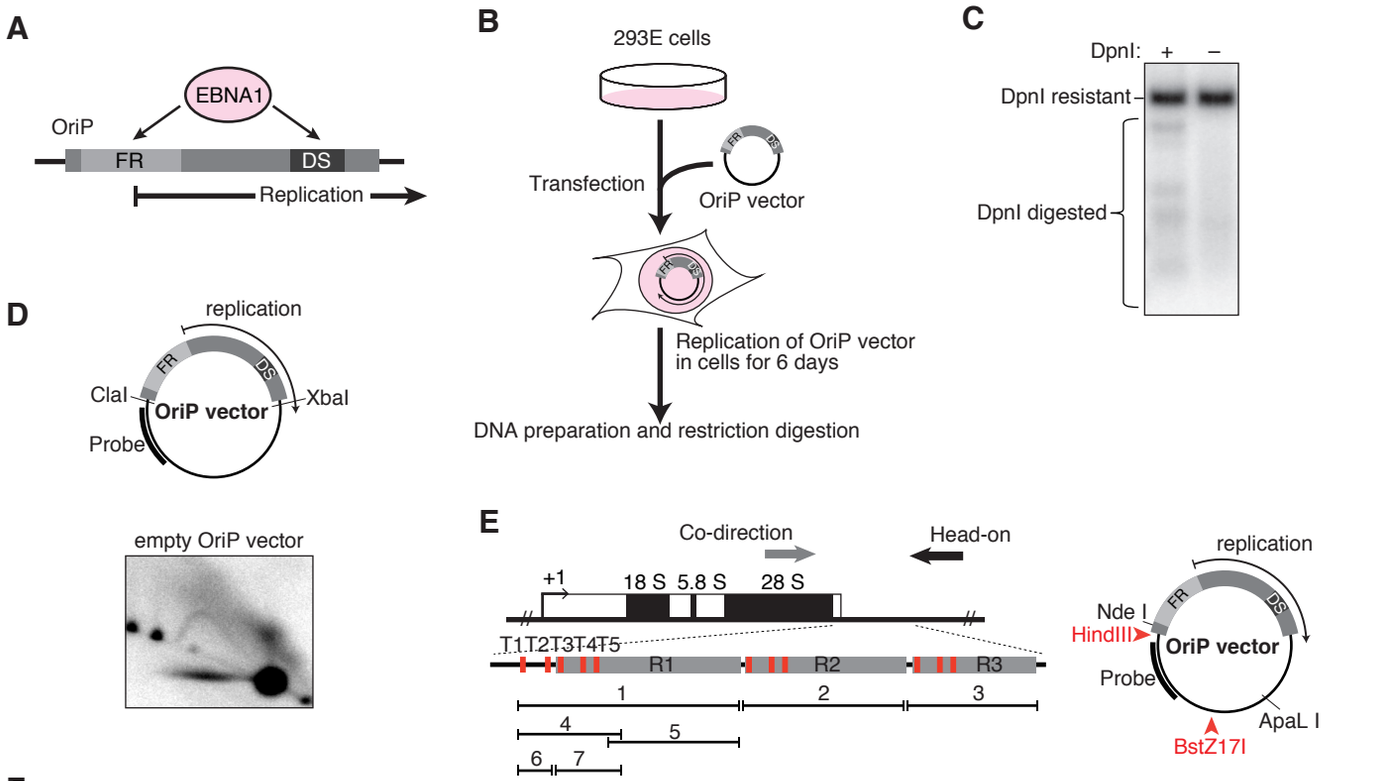
(A) SacII resistance of the transcription terminator regions in HeLa, HEK293 and VA13/WI38 cells. Genomic DNA, digested by AflII with or without SacII, was hybridized to the 28S probe. Each of these cell lines showed an individual pattern of the length heterogeneity of AflII fragments, which is due to the number of R-repeats. For quantification of the signals, serial dilutions (1, 0.5, 0.25, 0.125μg) of digested DNA were loaded alongside. (B) Quantification of SacII-resistant fractions, using the serial dilutions as the standards. The extent of CpG methylation within the AflII fragments also differed between the cell lines. Note that the fraction of VA13/W138 DNA containing a single R-repeat was more than 100 % resistant to SacII, which is likely due to overlapping SacII-digested bands derived from Class I or II DNAs. (C) 2D-gel analyses of replication intermediates in HeLa, HEK293 and VA13/WI38 cells. The AflII fragments were visualized with the 28S probe. Images for the AflII/SacII double digestion are shown with normal (top) and enhanced contrast (bottom). SacII treatment erased the signals of replication forks arrested at RFBs. In VA13/WI38 cells, only the SacII resistant Y-arc was detectable, which did not exhibited accumulation of Y-forks corresponding to RFB activity. Thus, as in the case of HeLa cells, RFBs are only functional in non-methylated rDNA copies.

Supplemental Figure 5

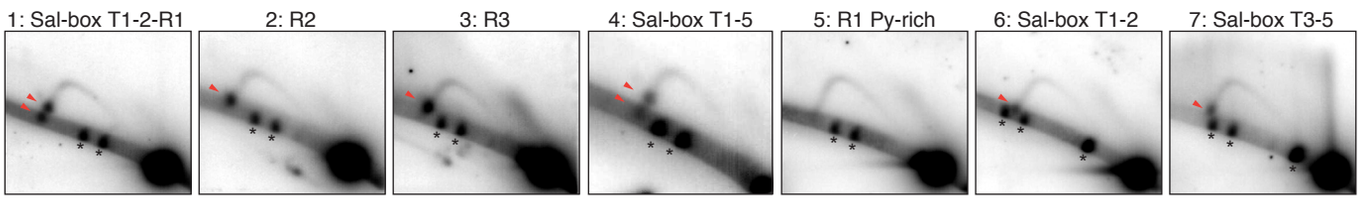
2D-gel analyses of replication intermediates in cells transfected with siTTF-1. MboI fragments were visualized with the 3′ETS probe. Red arrowheads indicate Y-fork accumulation at RFB^{R1} and RFB^{T1}.

Supplemental Figure 1

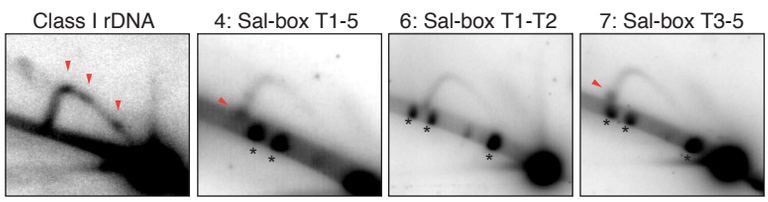




F Head-on direction

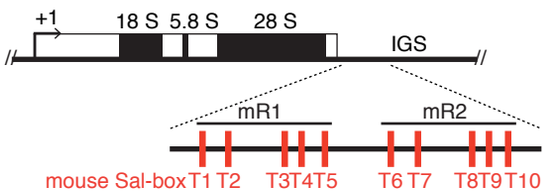


G Co-direction



Supplemental Figure 3

A



B

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                T1
13424: GGAGGTCGACCAGTACTCCGGGCGACACTTTG---TTTTTTTTTTTTCCCCCGATGCTGG
       ********************************   ************** ****** ***
13827: GGAGGTCGACCAGTACTCCGGGCGACACTTTGTTTTTTTTTTTTTTTTCCACCGATGATGG
       T2 T6
13481: AGGTCGACCAGATGTCCGAAAGTGTCCCCCCCCCCCCCCCCCCCCCCCGGCGCGGAGCG
       ************************    ***  ******** ******  ****  ***
13887: AGGTCGACCAGATGTCCGAAAGTGT----CCCGTCCCCCCCCTCCCCCCCCCGCGACGCG
       T7
13541: GCGGGGCCACCCCGGACCCCTTTTTTTTTTTTTTTTTTTTTTTAAATTCCTGGAACCTT
       *****  *** * *** * ****************************** **********
13943: GCGGGCTCACTCTGGA-CTCTTTTTTTTTTTTTTTTTTTTTTTAAATTTCTGGAACCTT
          T3                                          T4
13601: TAGGTCGACCAGTTGTCCGTCTTTTACTCCTTCATATAGGTCGACCAGTACTCCGGGTGG
        ********************** **** ****************          *****
14002: AAGGTCGACCAGTTGTCCGTCTTTCACTCATTCATATAGGTCGACC---------GGTGG
        T8                                          T9
                                        T5
13661: TACTTTGTCTTTTTCTGAAAATCCCAGAGGTCGACCAGATATCCGAAAGTC
       ********************** ********************* ** *******
14053: TACTTTGTCTTTTTCTGAAAATCGCAGAGGTCGACCAGATGTCAGAAAGTC
                                        T10
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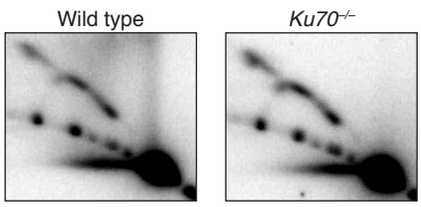
matching 86% between R1 and R2

C



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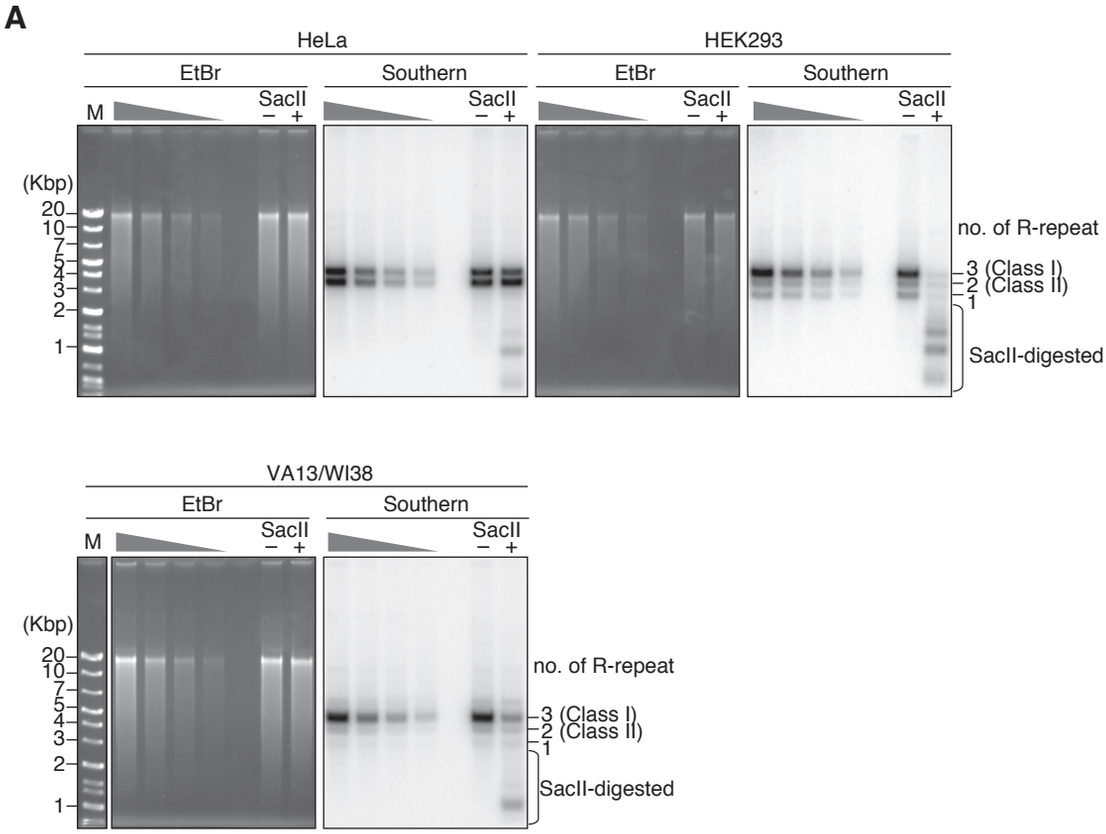
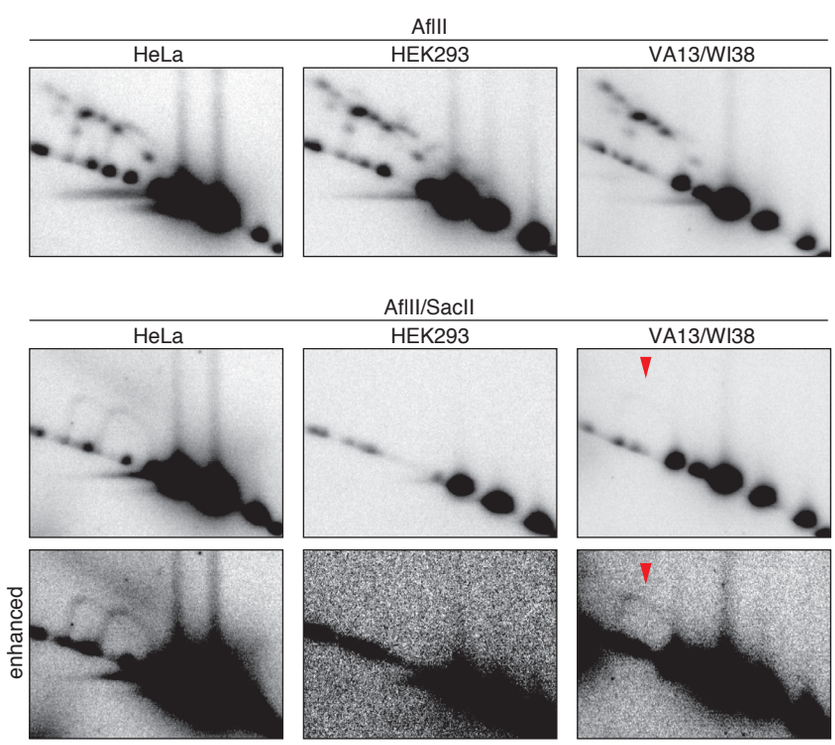


B

% of SacII resistant

no. of R-repeat	HeLa	HEK293	VA13/WI38
3	68.65%	5.49%	29.12%
2	96.97%	15.54%	96.46%
1	–	20.03%	104.09%

C



Supplemental Figure 5