

Supporting Information

Extrachromosomal Telomeres Derived from Excessive Strand Displacements

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Figure S1, related to Figure 1. 4SET assay, a simple and efficient method for detecting single-stranded extrachromosomal telomeric DNA. (A) Comparison of genomic DNA purification between DNeasy Blood & Tissue Kit (Qiagen, 69504) and our 4SET method in U2OS and Saos2 cells. (B) Comparison between Dig-labeled strand-specific probes and standard end-labeling probes. (C) Comparison of 4SET results with DNA dissolved at 4°C overnight vs 55°C for 2 hours, followed by 4°C overnight. Observed smear signals potentially indicate the presence of nicked or gapped DNA. The graph to the right displays the quantified signals potentially indicative of nicked or gapped DNA (mean \pm SD; unpaired t-test). (D) Western blot analysis of the whole, chromatin (Chr.), and soluble using anti-Histone H3, Lamin A/C, Emerin, GAPDH, and beta-actin for the validation of cell fractions. (E) SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 1C. (F) Western blot analysis of the whole, chromatin (Chr.), and soluble fractions using PML antibody. (G) Western blot analysis of Saos2 Wild-type and PML Knock-out cells using PML antibody, along with a Ponceau image as a loading control. (H) 4SET assay for SaoS2 Wild-type and PML Knock-out cells after fractionation into Whole and soluble fractions. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA staining of gel image. (I) C-circle assay using DNA in (H) (top). Quantification of C-circle assay (mean \pm SD; unpaired t-test) (bottom). (J) 4SET assay conducted using a nucleotide ladder (RiboRuler) to measure the size of C-rich single-stranded DNA in SaoS2 soluble fraction, analyzed on both 0.5% and 1% agarose gels. (K) 4SET assay for SaoS2 treated with Zeocin (100 μ g/ml), Etoposide (ETO) (10 μ M), or Camptothecin (CPT) (0.25 μ M) for 24 hrs. (L) Quantification of the C-circle assay in Fig. 1E (SaoS2 cells). Relative amount of C-circle assay products (mean \pm SD; unpaired t-test). (M) Cell fractionation using E1 and E2 buffers to separate chromatin, nucleoplasm, and cytoplasm. (N) Western blot analysis conducted using the fractionated DNAs in (M) using anti-Histone H3, Lamin A/C, GAPDH, and PML for the validation of cell fractions. (O) 4SET assay for fractionated DNAs in (M).

Figure S2, related to Figure 2. The presence of C-rich telomeric single-stranded DNA. (A) S1 nuclease assay on U2OS and SaoS2 soluble DNA fractions. (B) Lambda exonuclease assay on U2OS soluble DNA fraction. (C) Telomere-FISH with TelG (G-rich) and TelC (C-rich) PNA probes for chromatin (denatured FISH) and soluble fractions (native FISH).

Figure S3, related to Figure 3. MRE11 nuclease activity suppresses the generation of C-rich ssDNA. (A) SYBR DNA image for G-rich probe and C-rich probe gels in Fig. 3D. (B) 4SET assay for HeLa LT cells with or without Mirin treatment. Samples were fractionated into soluble DNA fraction or whole DNA. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. SYBR DNA image for G-probe gel. (C) 4SET assay for SaoS2 cells with Mirin, PFM01, PFM39 or mock (control) treatments. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image for G-rich probe and C-rich probe gels. (D) Western blot of SaoS2 post-transfection with siLuciferase

(siLuc) or siMRE11s. **(E)** 4SET for SaoS2, with and without Mre11 treatment post siLuc or siMRE11s transfection. The graph to the right presents the quantification of relative amount of C-rich ssDNAs (mean \pm SD; unpaired t-test). **(F)** 4SET for SaoS2 WT and SaoS2 PML KO with/without Mirin treatment. **(G)** Quantification of 4SET from F, showing relative C-rich ssDNA (whole) levels (mean \pm SD). **(H)** Native IdU pulldown was conducted using IdU (3D4) antibody for Saos2 WT and Saos2 PML KO after IdU incorporation. IgG served as a negative control. Chromatin DNA was also included to control for DNA quantity. **(I)** Quantification of the native IdU-pulldown assay in H; Relative amount of nascent C-rich ssDNAs (mean \pm SD; unpaired t-test).

Figure S4, related to Figure 4. C-rich ssDNAs are derived from lagging strand during the Okazaki fragment processing. **(A)** Relative amount of DNA2 mRNA levels measured by quantitative-PCR in U2OS cells transfected with siRNAs targeting DNA2 or control (siLuc). **(B)** SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 4C. **(C)** Relative amount of FEN1 mRNA levels measured by quantitative-PCR in U2OS cells transfected with siRNAs targeting FEN1 or control (siLuc). **(D)** Illustration depicting 5' flap processing at lagging strand telomeres by DNA2-RPA or FEN1-PCNA. **(E)** SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 4E. **(F)** 4SET assay for U2OS cells with PARPi (Talazoparib), Mirin, or mock (control) treatments. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA images for G-rich probe and C-rich probe gels. **(G)** 4SET assay for SaoS2 cells after transfection of siRNAs targeting PRIMPOL, or control (siLuc) with or without Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image for G-rich probe. **(H)** 4SET assay for U2OS PRIMPOL KO cells after transfection of PRIMPOL WT, S255D, or control cDNA plasmid with or without Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA images for G-rich probe and C-rich probe gels.

Figure S5, related to Figure 5. ATRX suppresses the generation of C-rich ssDNAs. **(A)** SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 5D. **(B)** SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 5G. **(C)** Relative amount of FANCM mRNA levels measured by quantitative-PCR in U2OS cells transfected with siRNAs targeting FANCM or control (siLuc). **(D)** Relative quantification of R-loops at telomeres using the 7p TERRA primer following DNA/RNA hybrid immunoprecipitation with the S9.6 antibody or IgG in U2OS cells transfected with siRNAs targeting FANCM or control (siLuc). **(E)** 4SET assay for U2OS cells after transfection of siRNAs targeting FANCM or control (siLuc) with Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image for G-rich probe gel. **(F)** Western blot analysis of U2OS RAD51AP1 KO (C1) or control. **(G)** 4SET assay for U2OS RAD51AP1 KO (C1) or control with Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image for G-rich probe gel. **(H)** 4SET assay for HeLa LT TERC KO or HeLa LT TERC KO cells expressing shRNA targeting ATRX gene (ATR_X 590) with or without Mirin or PARPi

treatment. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. SYBR DNA image for G-rich probe gel.

Figure S6, related to Figure 6. CST complex-mediated priming and subsequent strand displacements by DNA polymerase delta generates C-rich ssDNAs. (A) Relative amount of STN1 mRNA levels measured by quantitative-PCR in U2OS and SaoS2 cells expressing shRNA targeting STN1 gene or control (shNT). **(B)** SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 6D. **(C)** 4SET assay for U2OS cells expressing shRNAs targeting STN1, or non-targeting (NT) control. Cells were treated with Mirin (50 μ M, 48 hr). SYBR DNA images for G-rich probe and C-rich probe gels. **(D)** SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 6F. **(E)** 4SET assay for Mirin treated U2OS cells along with aphidicolin, CD437 or control. Samples were fractionated into soluble DNA fraction or whole DNA. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image for G-rich probe gel. **(F)** 4SET assay for Mirin treated U2OS WT or BLM KO cells. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image for G-rich probe gel. **(G)** Quantification of the 4SET assay in F; as the relative amount of C-rich ssDNA (whole). (mean \pm SD; unpaired t-test). **(H)** SYBR DNA images for G-rich probe and C-rich probe gels in Fig. 6G.

Figure S1

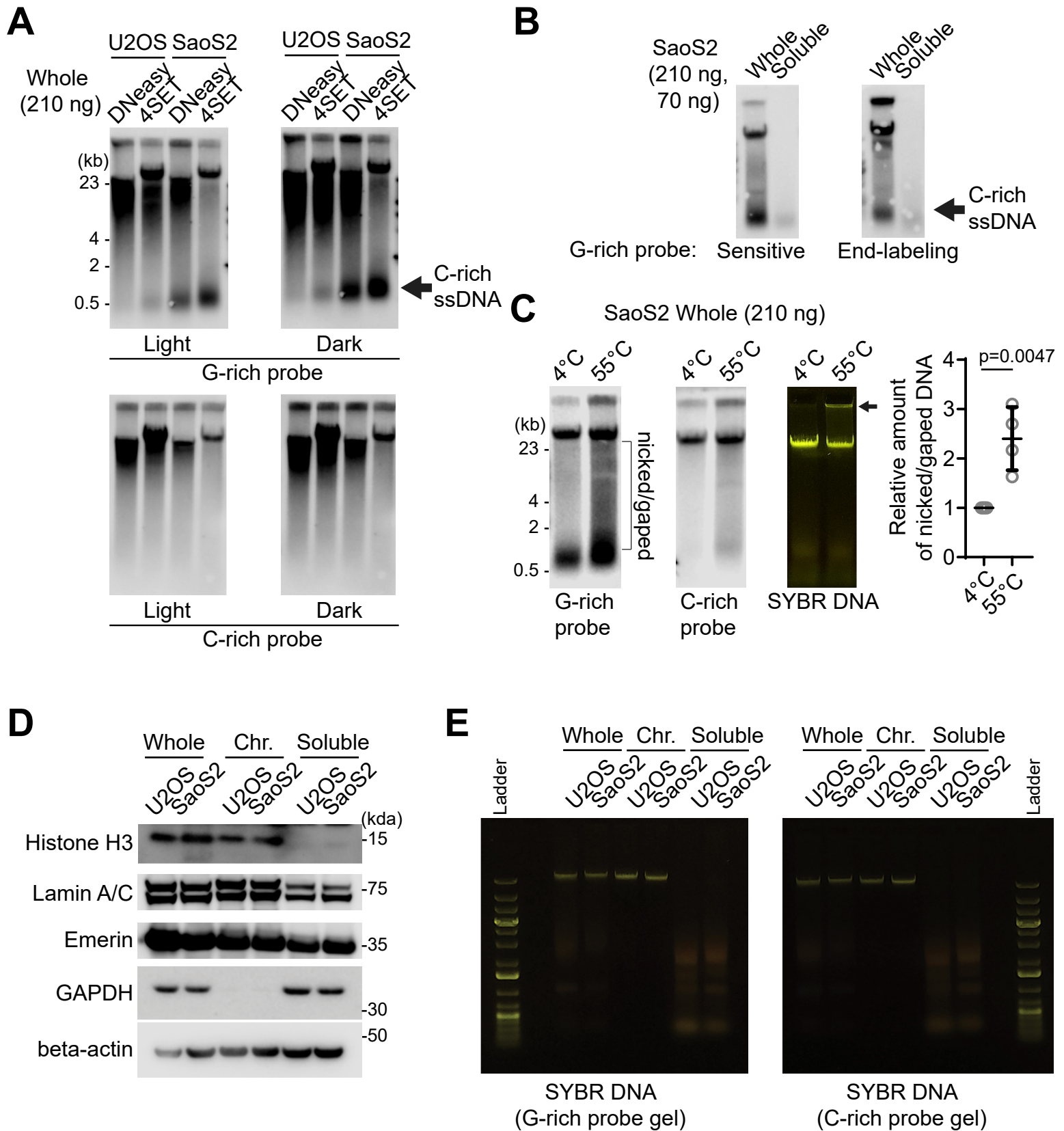


Figure S1

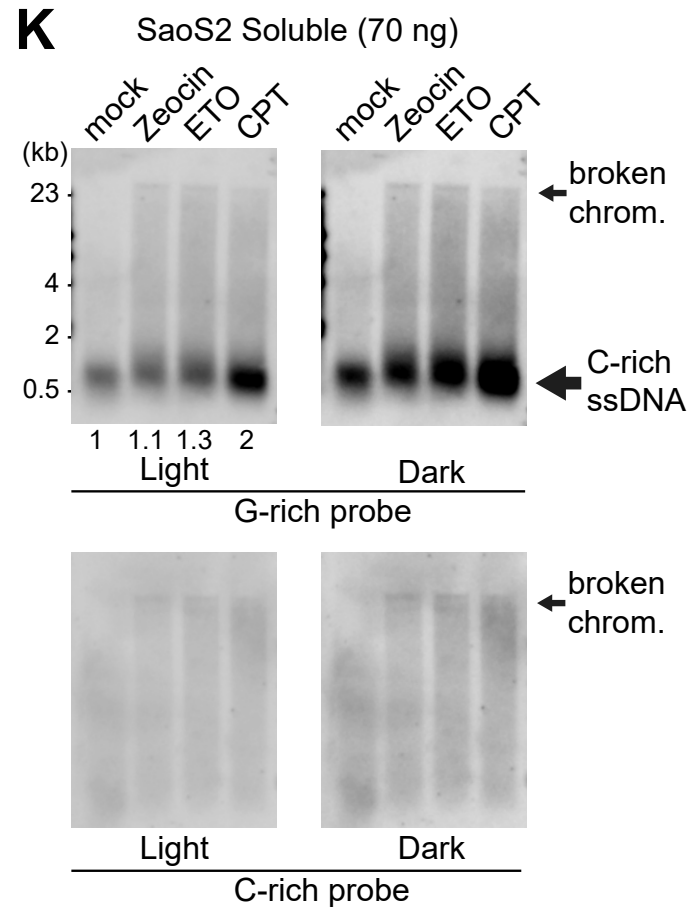
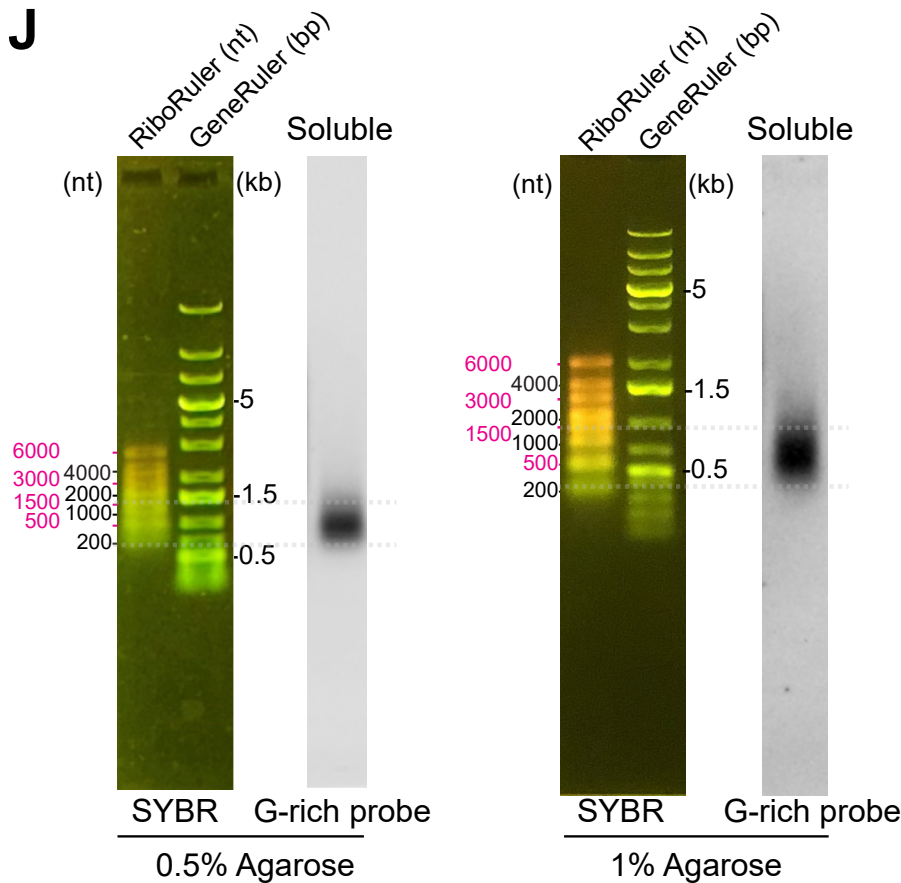
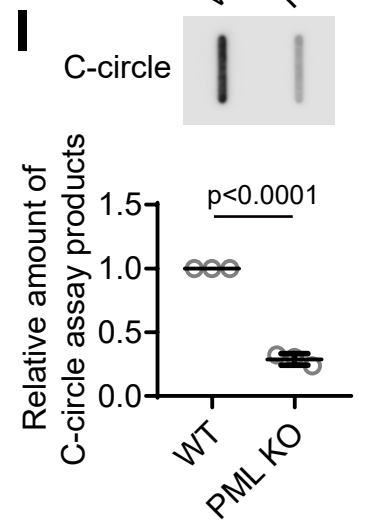
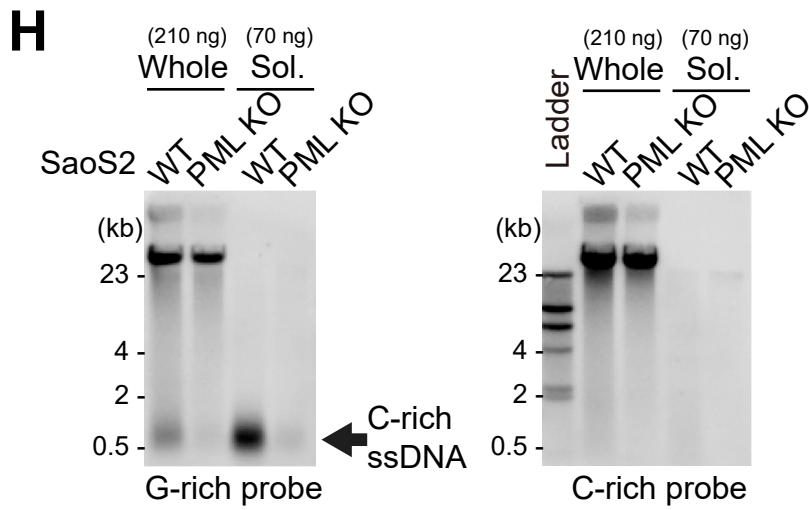
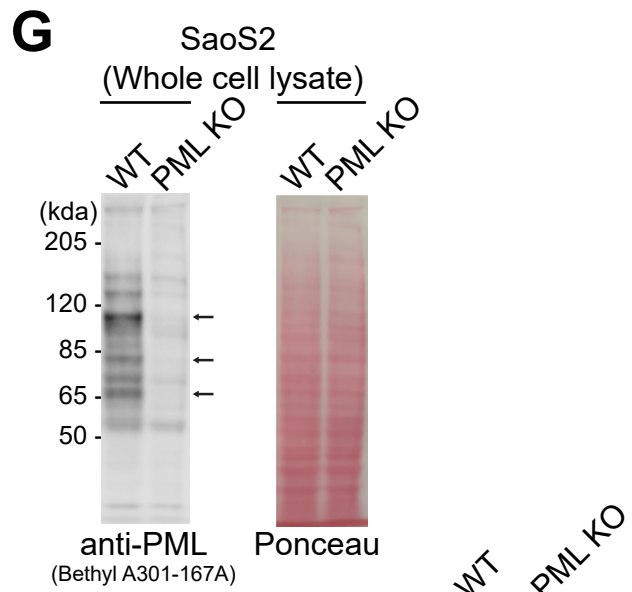
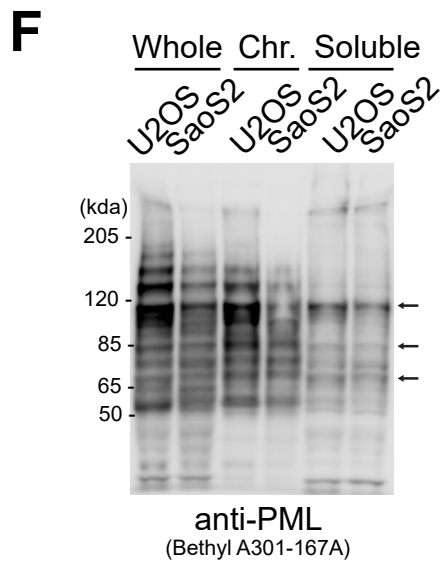
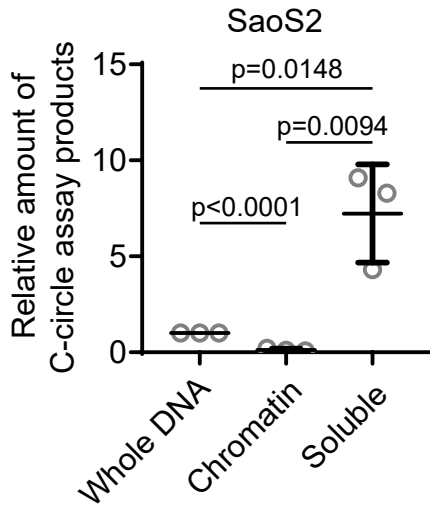
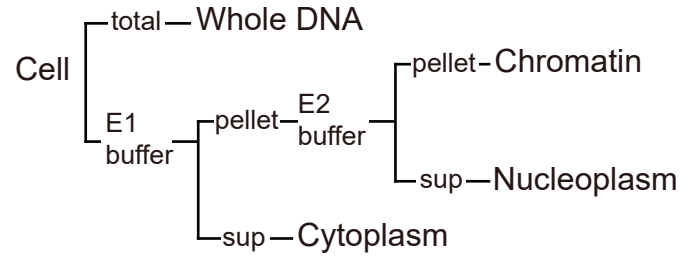


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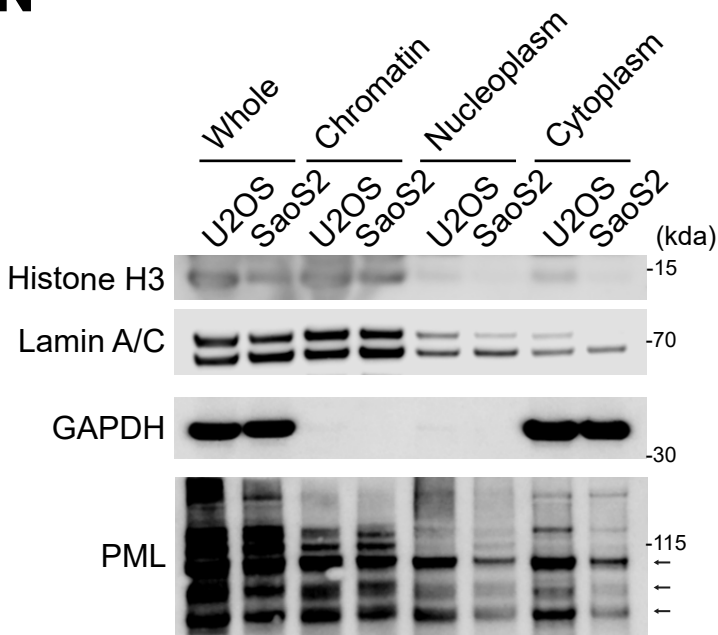
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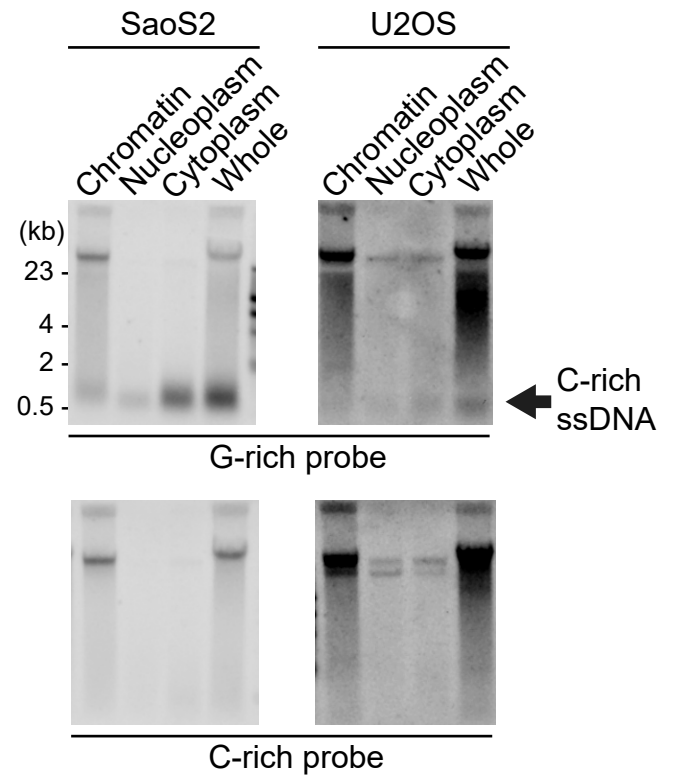
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N



O



Whole - 140ng
Chromatin&Nucleoplasm&Cytoplasm - 70ng

Figure S2

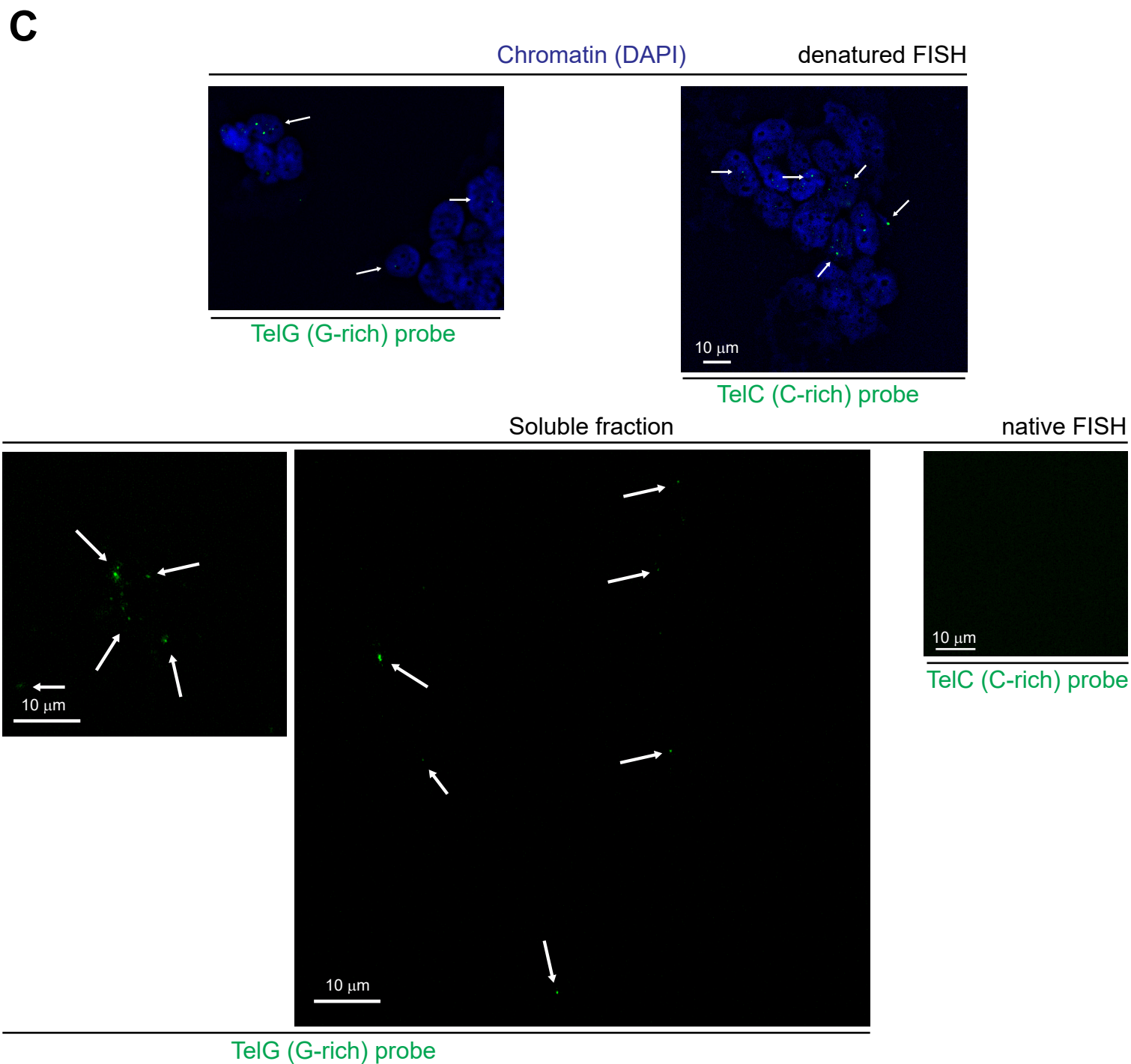
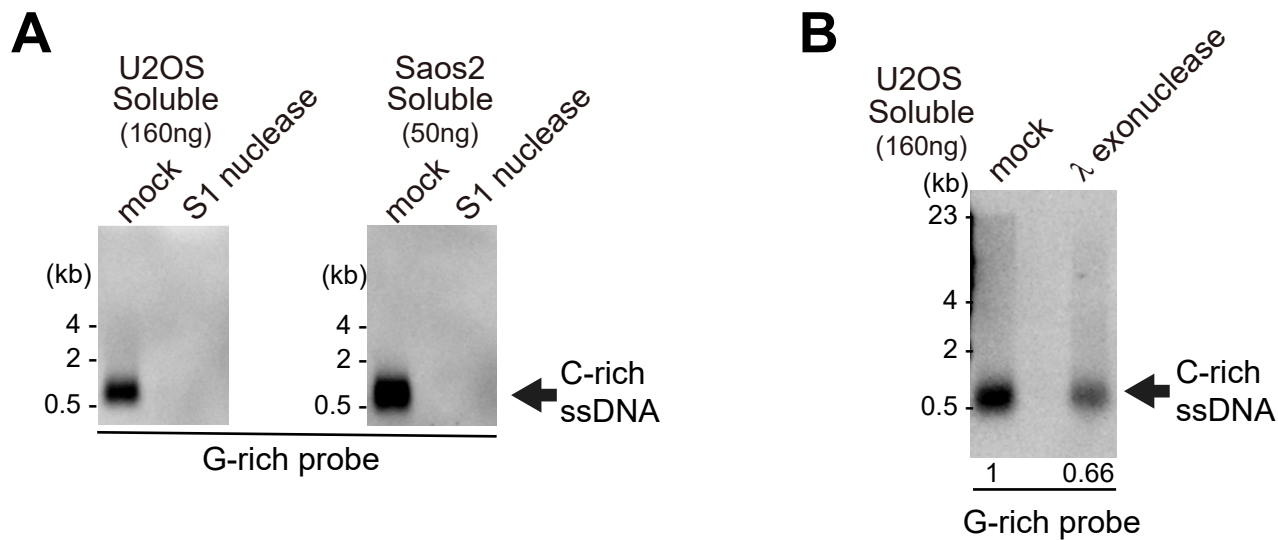


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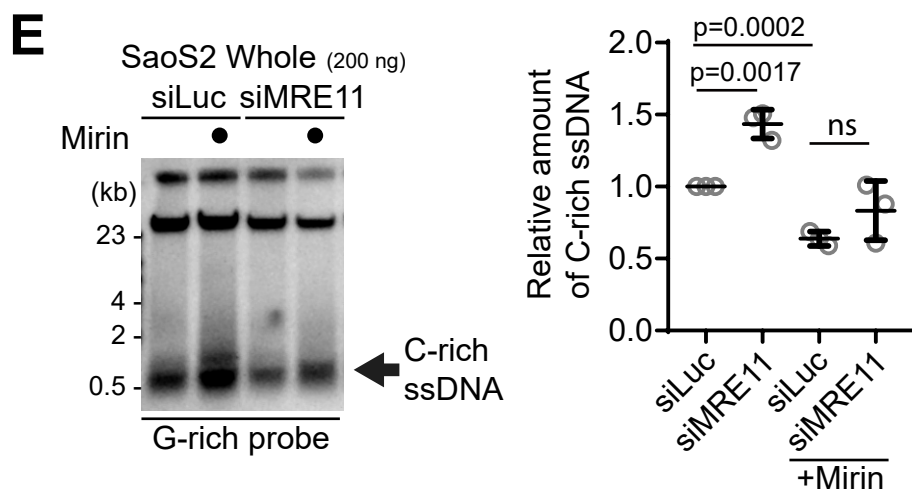
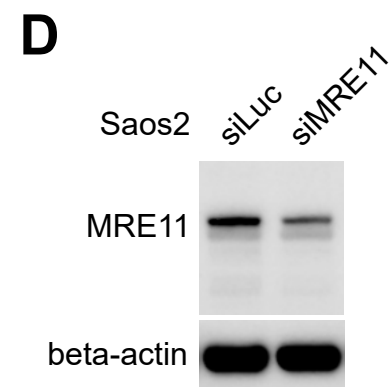
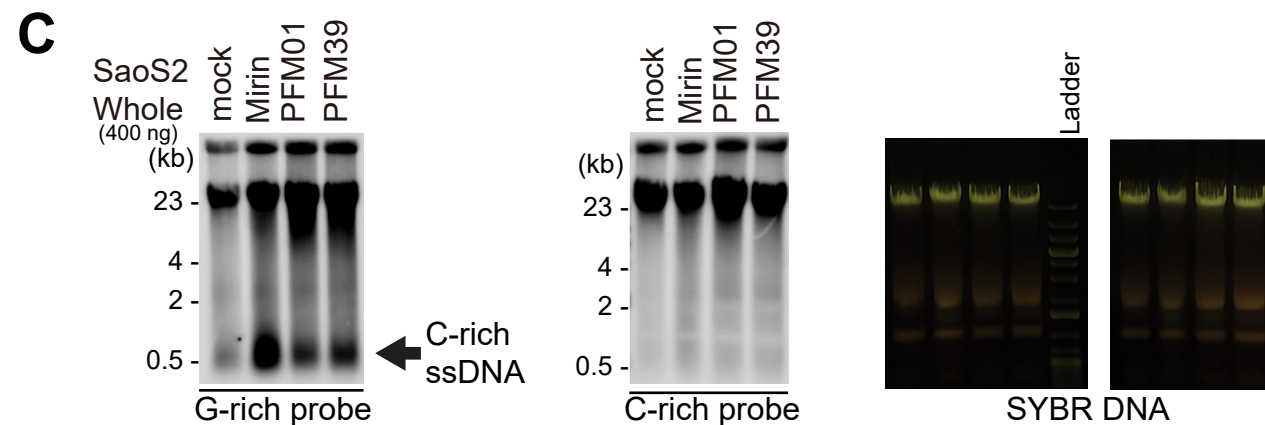
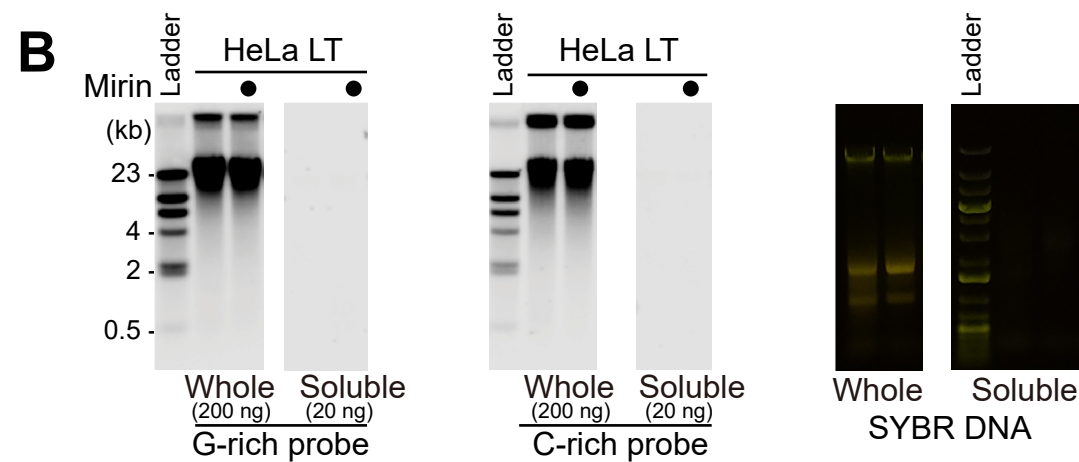
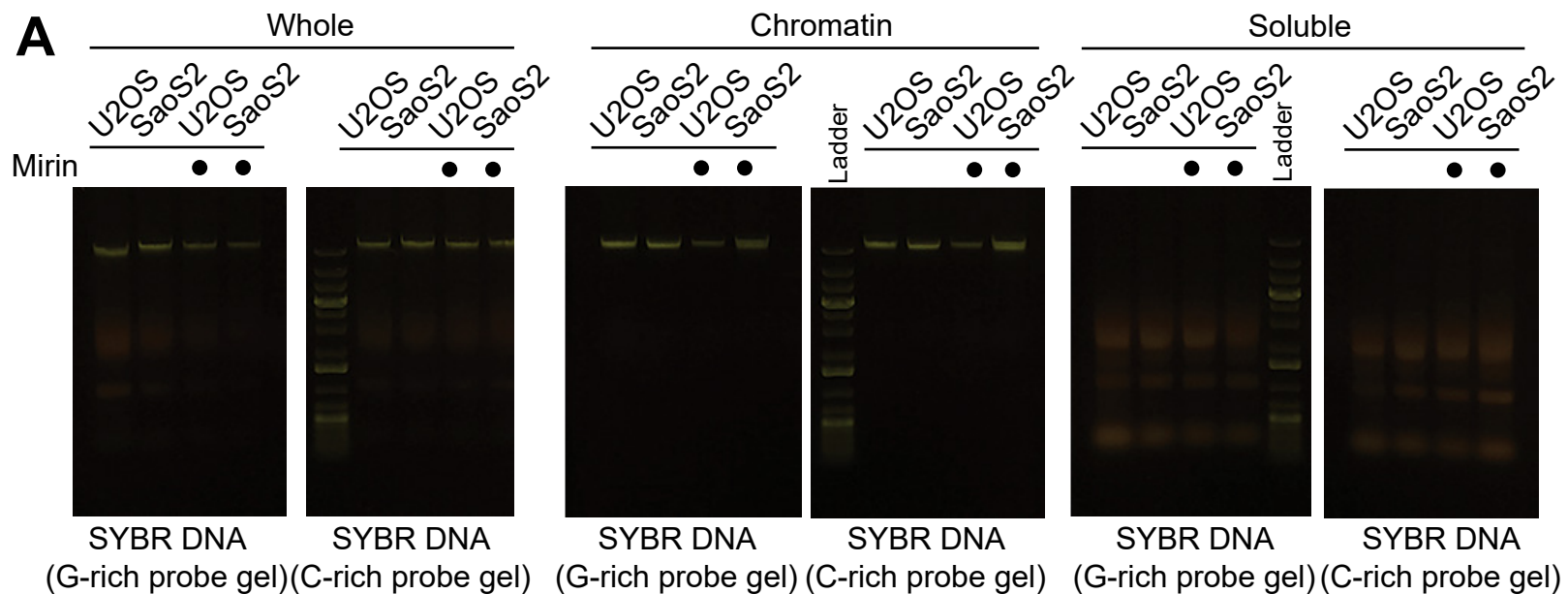
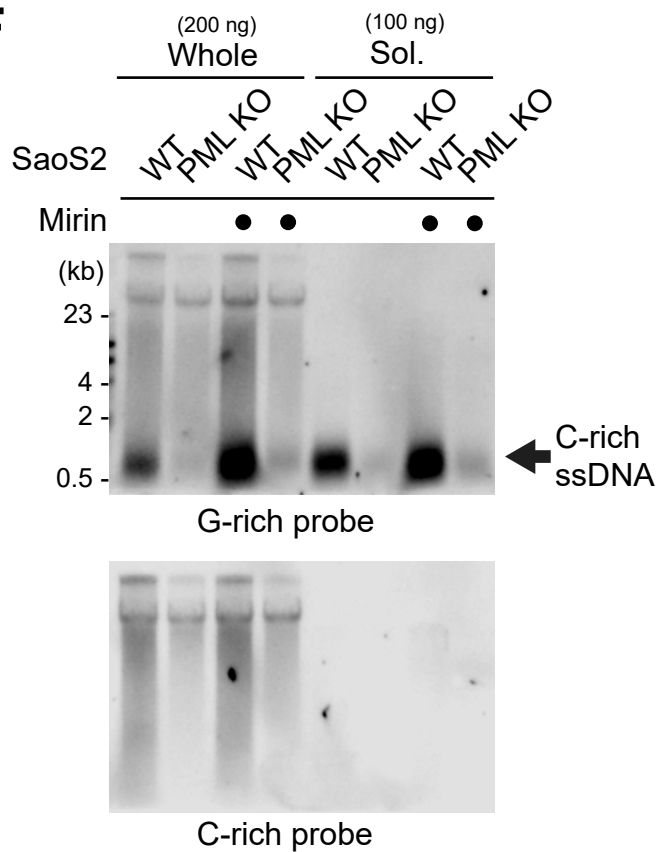
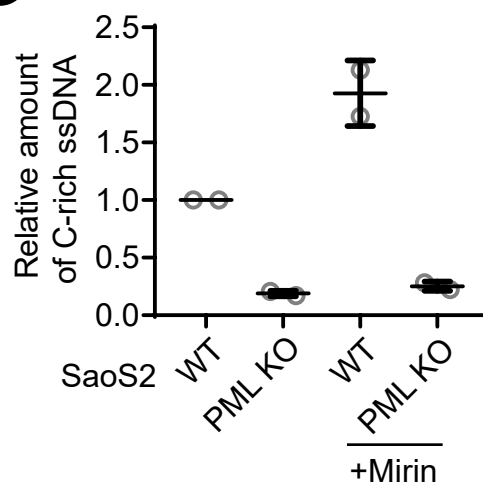


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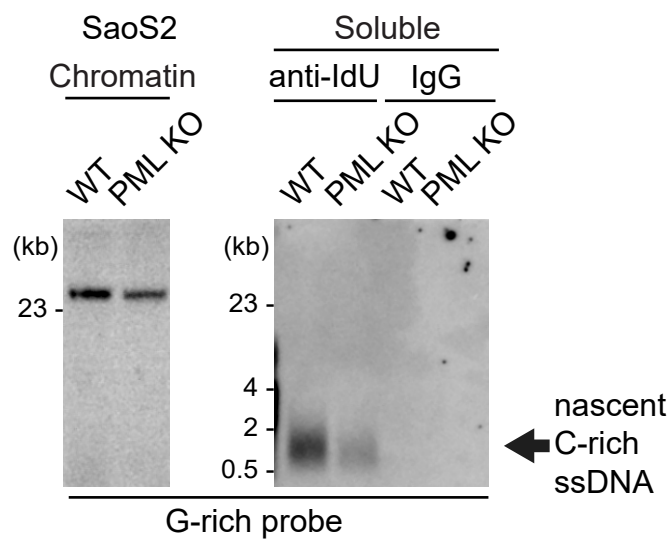
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G



H



I

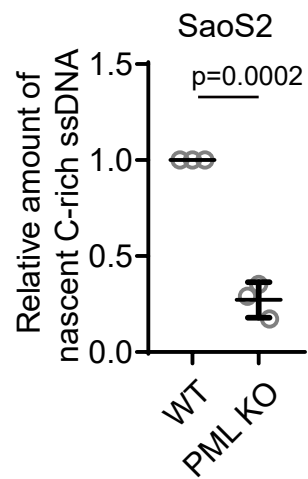
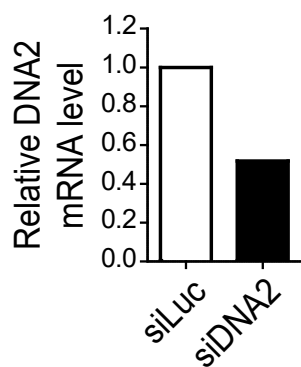
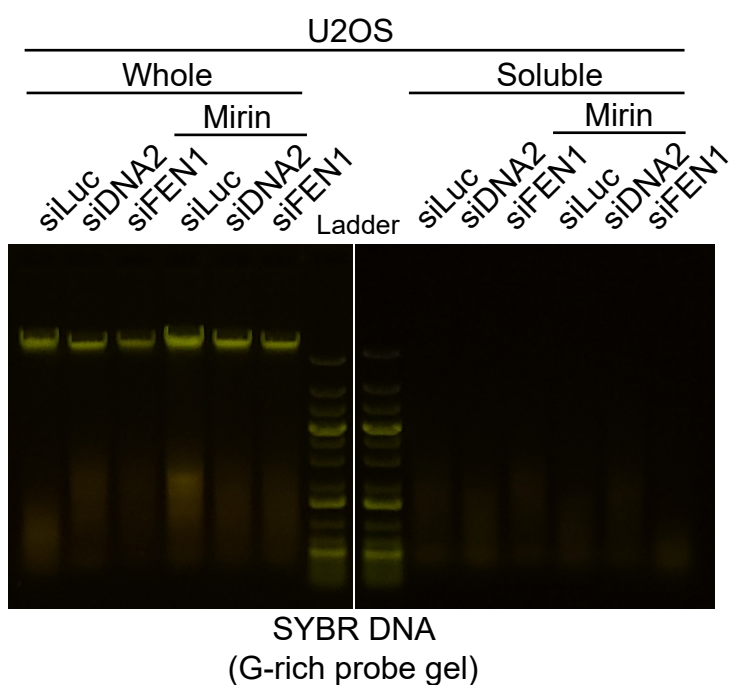


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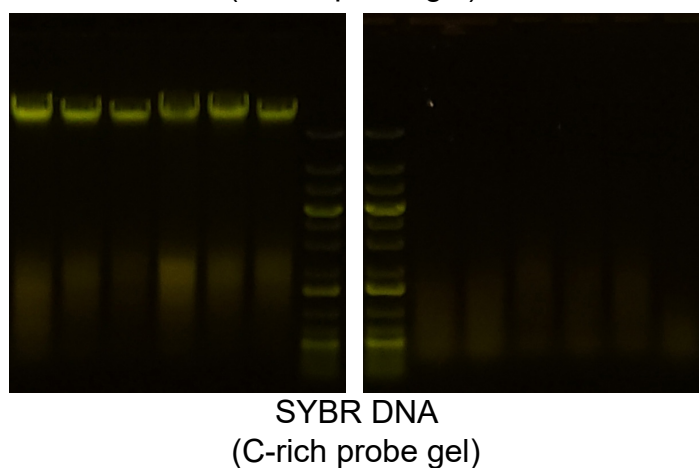
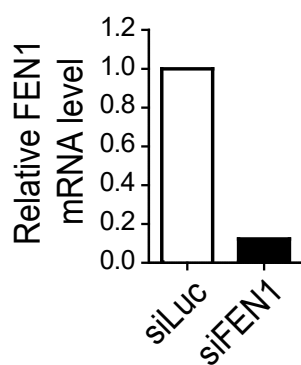
A



B

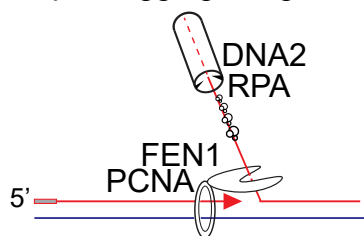


C



D

5' flap in lagging daughter strand (C-rich)



E

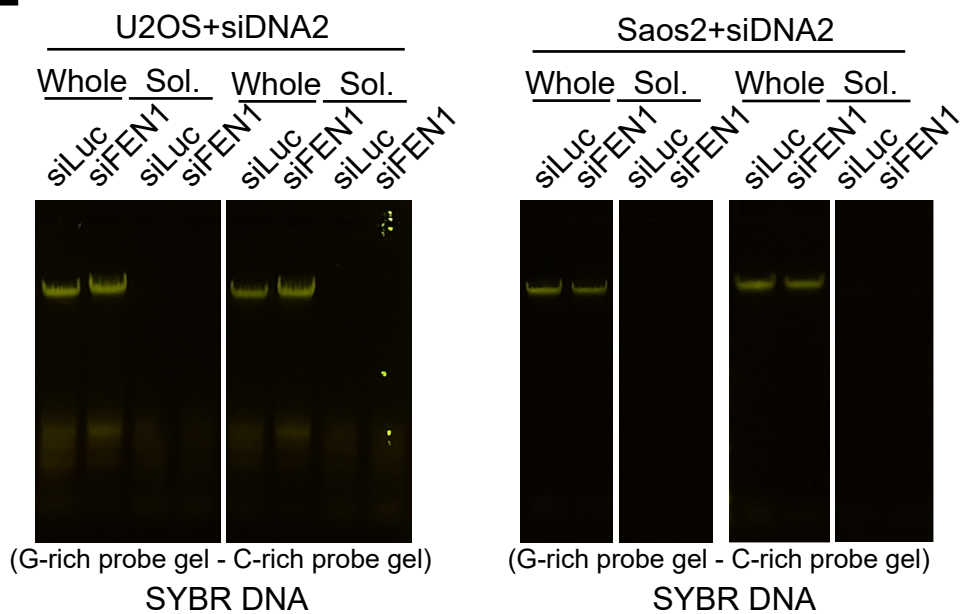
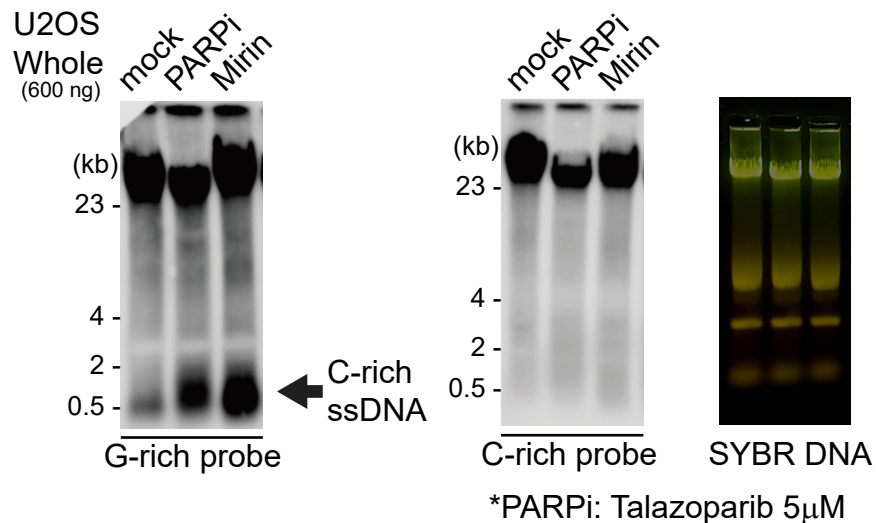
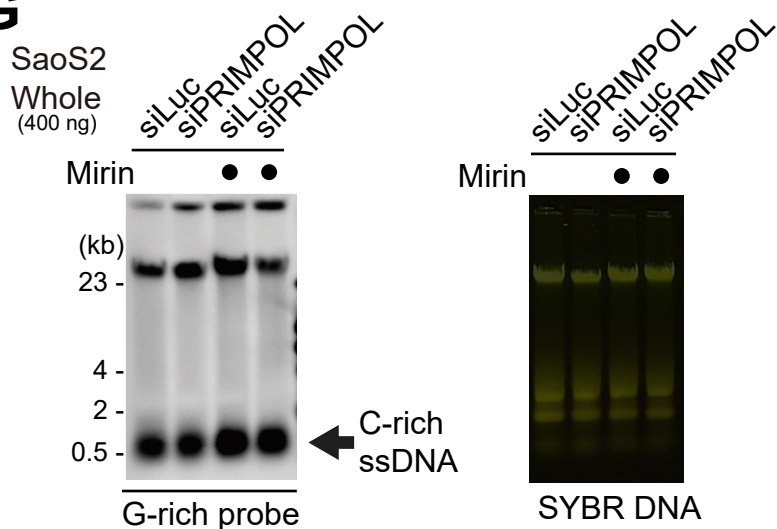


Figure S4

F



G



H

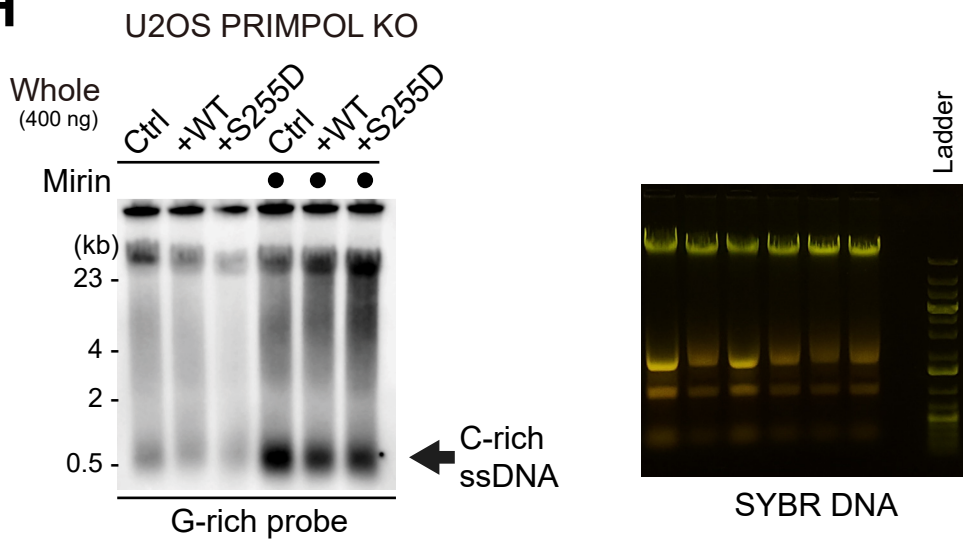


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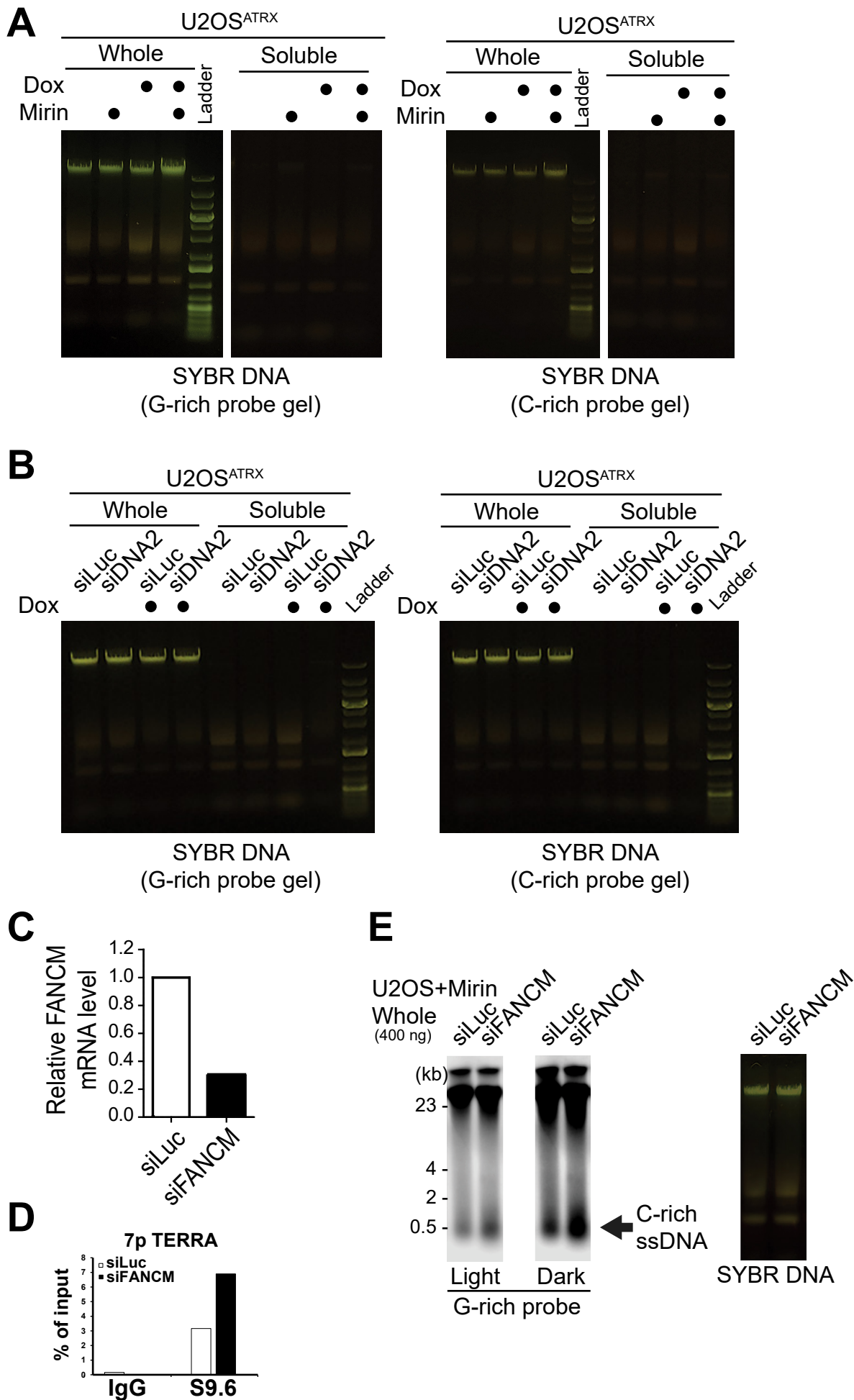
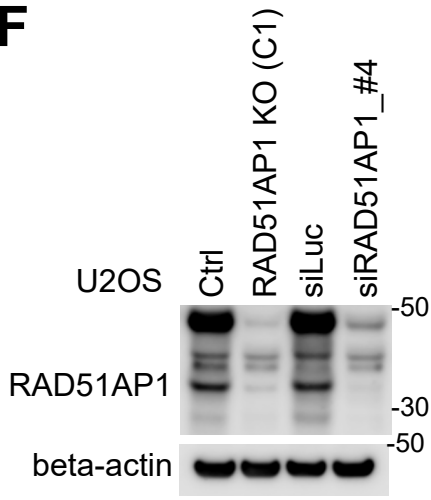
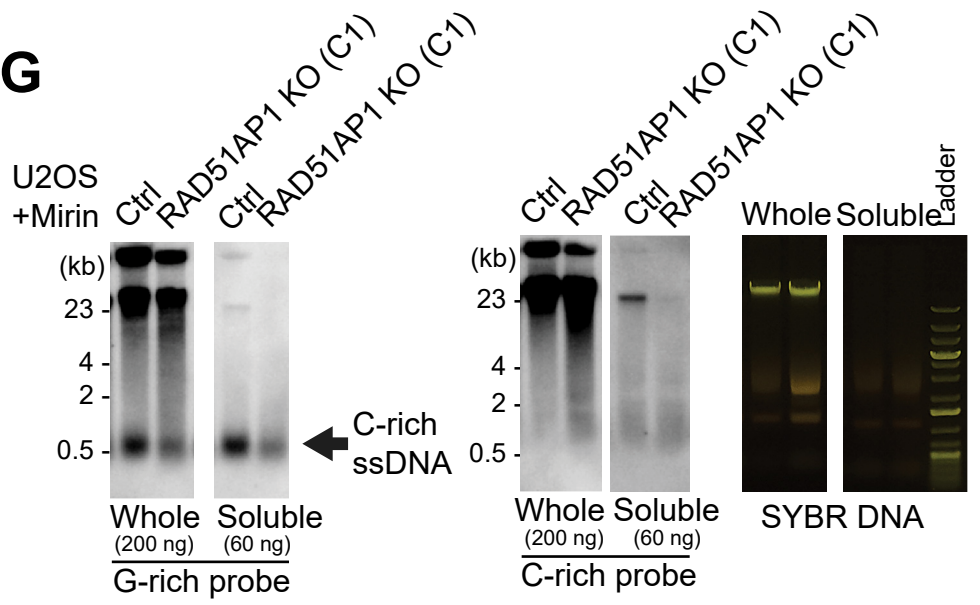


Figure S5

F



G



H

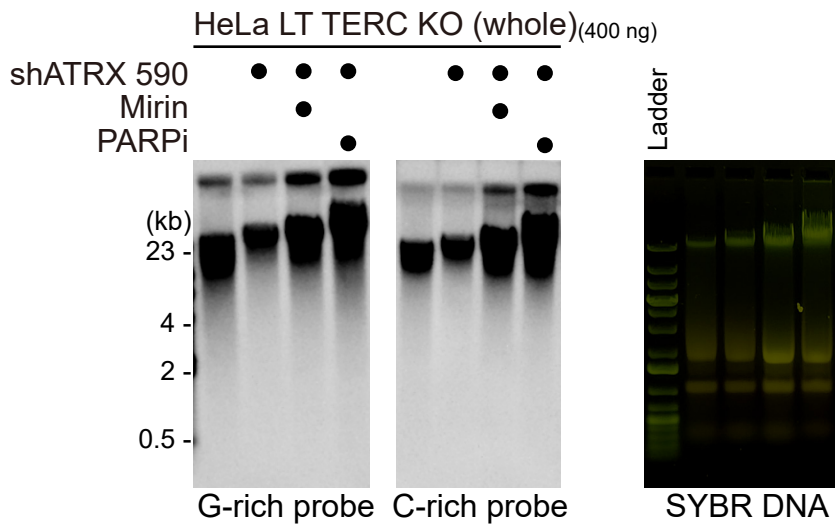
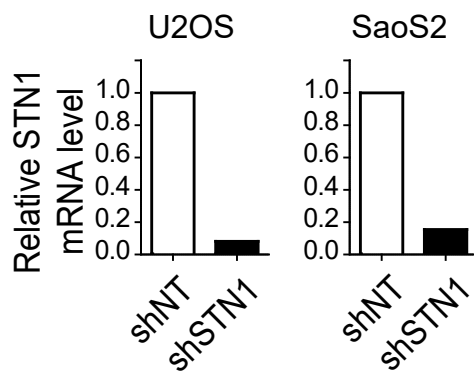
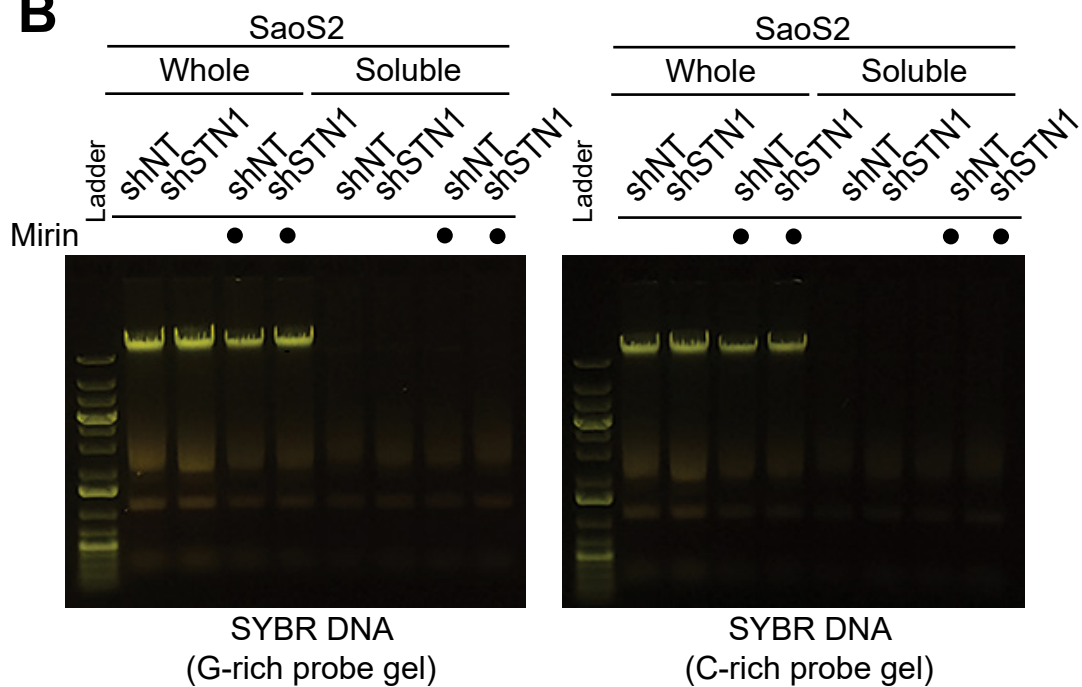


Figure S6

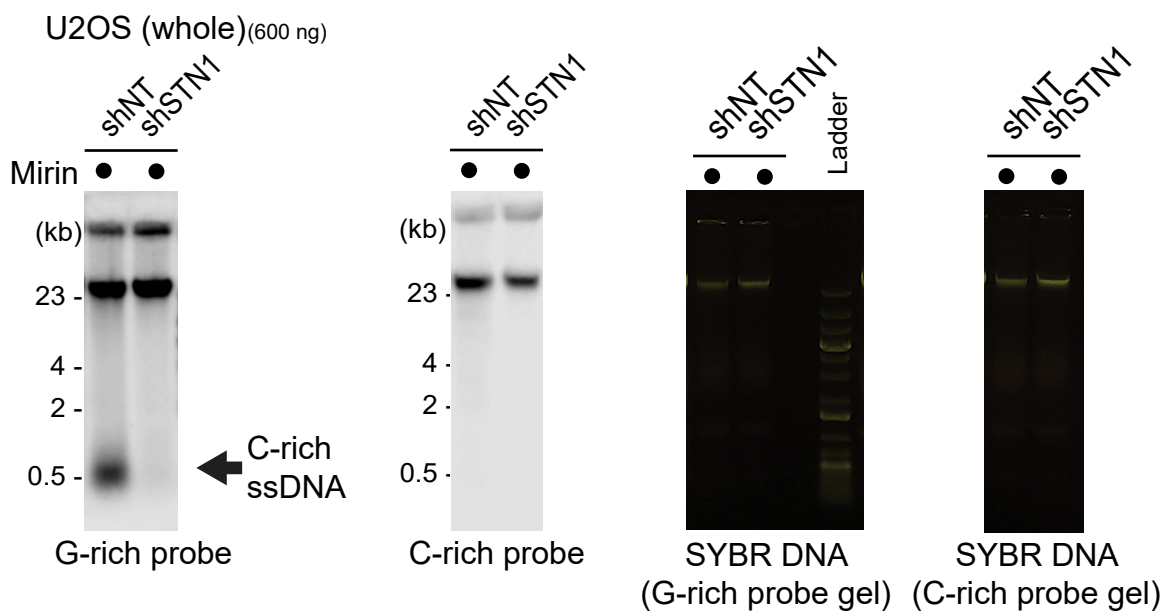
A



B



C



D

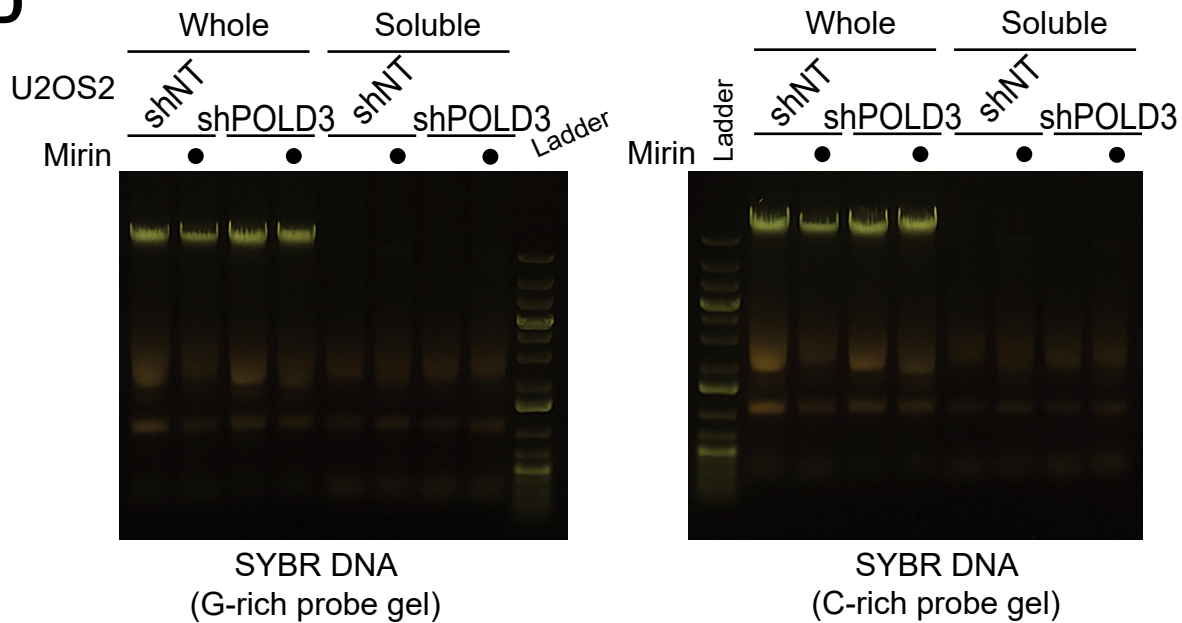


Figure S6 **E**

