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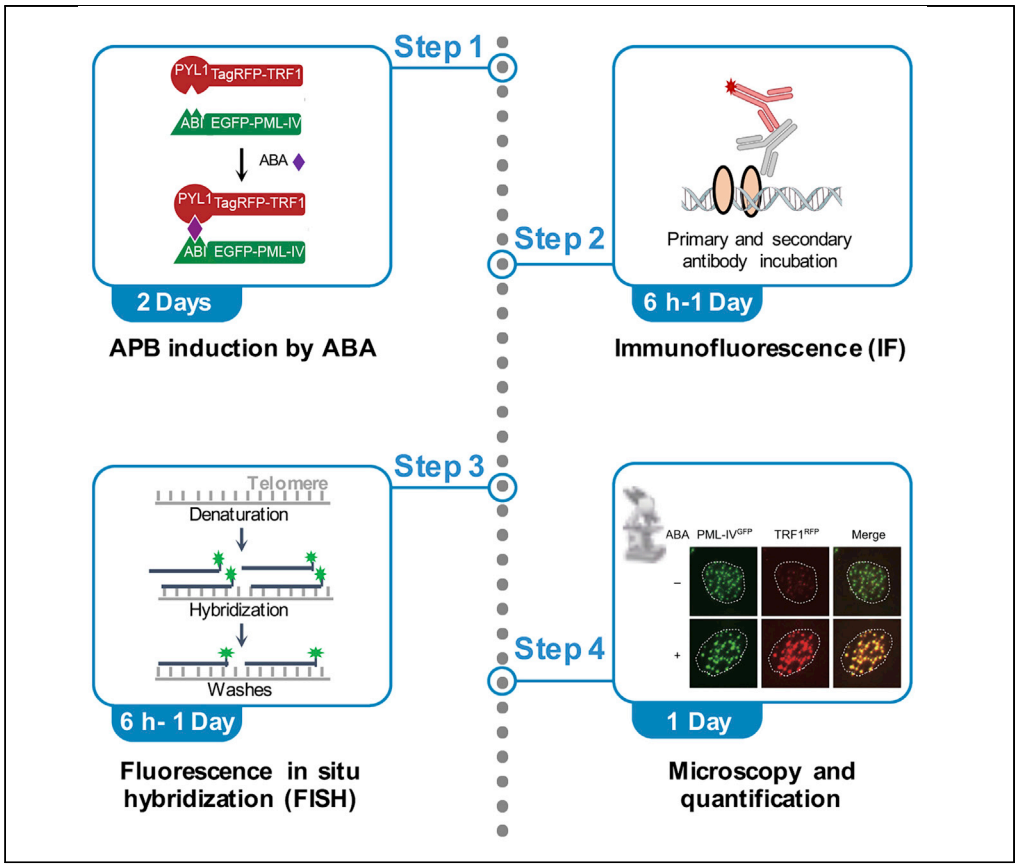
Protocol to stimulate and delineate alternative lengthening of telomeres in human U2OS cells



Alternative lengthening of telomeres (ALT) is a telomerase-independent but recombination-dependent pathway that maintains telomeres. Here, we describe a protocol to stimulate the formation of ALT-associated PML bodies (APBs) and ALT activity by tethering PML-IV to telomeres in human U2OS cells. Through immunofluorescence, *in situ* hybridization, and microscopy, we analyze dynamics of telomere clustering, visualize recruitment of DNA repair proteins to APBs, and measure telomere DNA synthesis during ALT. This protocol provides a unique approach to delineate the ALT pathway.

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Highlights

An ABA-inducible system to tether PML-IV to telomeres and promote APB formation

Visualization of proteins and DNA synthesis in APBs by immunofluorescence

Detection of telomeres by fluorescence *in situ* hybridization

Quantification of proteins and DNA synthesis at telomeres in APBs

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Protocol to stimulate and delineate alternative lengthening of telomeres in human U2OS cells

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SUMMARY

Alternative lengthening of telomeres (ALT) is a telomerase-independent but recombination-dependent pathway that maintains telomeres. Here, we describe a protocol to stimulate the formation of ALT-associated PML bodies (APBs) and ALT activity by tethering PML-IV to telomeres in human U2OS cells. Through immunofluorescence, *in situ* hybridization, and microscopy, we analyze dynamics of telomere clustering, visualize recruitment of DNA repair proteins to APBs, and measure telomere DNA synthesis during ALT. This protocol provides a unique approach to delineate the ALT pathway.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2021).

BEFORE YOU BEGIN

ALT-associated PML bodies (APBs) are unique nuclear structures containing the promyelocytic leukemia (PML) protein, telomeric and DNA damage factors, and telomeric DNA, and are critical for alternative lengthening of telomeres (ALT) (Yeager et al., 1999; Zhang et al., 2019). PML-IV is the main PML variant functionally supporting APBs and ALT telomere synthesis during ALT. To tether PML-IV to telomeres in U2OS cells, we have established a U2OS-derivative cell line expressing PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV (Zhang et al., 2021). In the presence of abscisic acid (ABA) (Liang et al., 2011), PYL1-TagRFP-TRF1 interacts with ABI-EGFP-PML-IV in cells, leading to a robust induction of APBs and ALT activity. Before you start using the protocol, you need to have the PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV-expressing U2OS cell line and ABA in place. If you plan to test specific proteins for their localization to APBs and functions in ALT, you need antibodies and siRNAs to detect and deplete these proteins. If you need to induce APB formation and ALT activity in other ALT$^+$ cell lines, PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV-expressing viruses can be used to generate derivative cell lines. One needs to obtain the PYL-1-TagRFP-TRF1 and ABI-EGFP-PML-IV plasmids and propagate virus to establish such cell lines.

Generation of plasmids

Timing: 1 week

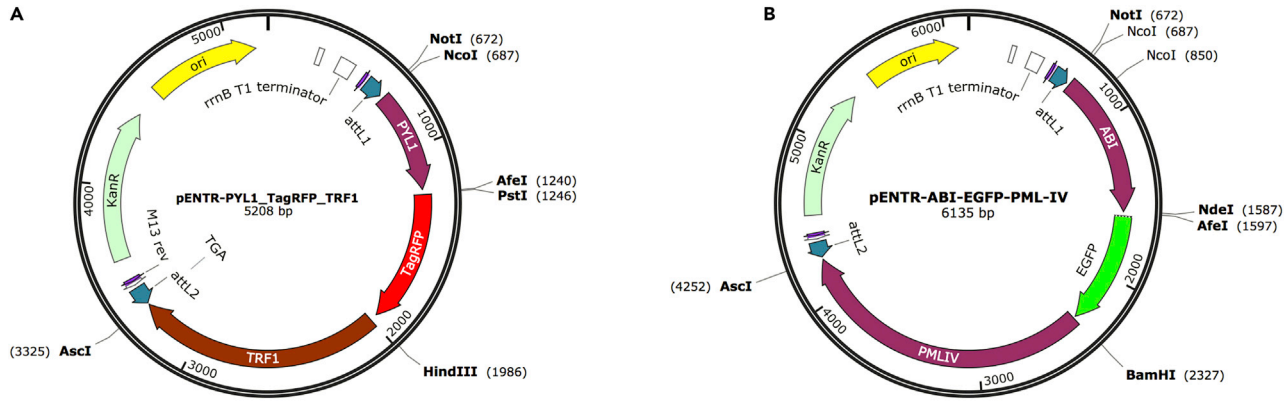

Figure 1. Plasmid map of pENTR-PYL1-TagRFP-TRF1 and pENTR-ABI-EGFP-PML-IV

(A and B) Multiple restriction enzymes important for further cloning are shown in pENTR-PYL1-TagRFP-TRF1 (A) and pENTR-ABI-EGFP-PML-IV (B).

1. The DNA fragments encoding PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV were cloned between NcoI and AscI sites of the Gateway entry vector pENTR, generating the plasmids pENTR-PYL1-TagRFP-TRF1 and pENTR-ABI-EGFP-PML-IV (Figure 1).
2. Using pLenti PGK Puro DEST (w529-2) (Addgene plasmid #19068) and pLenti PGK Neo DEST (w531-1) (Addgene plasmid #19067) as destination vectors, we generate lentiviral expression plasmids pLenti PGK PYL1-TagRFP-TRF1(Puro) and pLenti PGK-ABI-EGFP-PMLIV(Neo) through LR recombination reaction.

Note: These plasmids can be modified to express other proteins for ABA-inducible interaction or tethering by replacing TRF1 and PML-IV (Figure 1). It's recommended to firstly do cloning on pENTR vector instead of lentiviral expression plasmid, considering the availability of usable restriction enzymes and stability of lentiviral expression plasmid.

⚠ CRITICAL: Destination vectors (pLenti PGK Puro DEST and pLenti PGK Neo DEST) can be propagated in DB3.1 strain. To keep the stability of the plasmids, DB3.1 strain can be cultured at 30°C. For the propagation of lentiviral expression plasmids, NEB Stable Competent E. coli is highly recommended.

Generation of cell lines expressing PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV

Timing: 2 weeks

3. Viruses for expression of PYL1- TagRFP-TRF1 and ABI-EGFP- PML-IV are packaged in 293T cells, following the protocol "shRNA/sgRNA/ORF Low Throughput Viral Production (10 cm dish/6 well)" from Broad Institute (https://portals.broadinstitute.org/gpp/public/resources/protocols).
4. U2OS cells are infected with both viruses together (each virus: 1/4 volume of the final culture media, final MOI around 1, polybrene: 8 μg/mL), and selected by 1 μg/mL Puromycin and 1 mg/mL Geneticin for 1 week.

Note: For different cells, the concentration of antibiotics should be tested.

5. U2OS cells expressing PYL1- TagRFP-TRF1 and ABI-EGFP-PML-IV were enriched by sorting GFP+ RFP+ U2OS cells by BD FACSAria™ Fusion cell sorter, and cultured for frozen stock, or further experiments.

Cell culture

6. U2OS cells expressing PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV are cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamine and 1% penicillin/ streptomycin in a 37°C incubator at 5% CO2.

Preparation of coverslips

Timing: 1 h

7. Place 18-mm round glass coverslips in 12-well tissue culture plates, and sterilize them in 1 mL 75% ethanol for 10 min. Troubleshooting 2 and 4.

Note: As an alternative, autoclaved coverslips can be used without further sterilization by 75% ethanol.

Note: The thickness of the coverslips should be suitable for 60× imaging. If coverslips from alternative brand are chosen, make sure to use coverslips with low fluorescent background.

Note: For suspension cells, coverslips can be further coated to help cell attachment.

8. Remove ethanol with aspiration as much as possible and let coverslips air dry in a tissue culture hood for 0.5 h with UV light on.

Note: Sterilized coverslips can be kept in covered tissue culture plates at 20°C–22°C until use, with caution for contamination.

Preparation of unique reagents

Timing: 1 h

9. Stock solution of 200 mM ABA: dissolve 50 mg (+)-Abscisic Acid (ABA) in 946 μL Dimethyl Sulfoxide (DMSO) in biosafety cabinet. Aliquot the stock and store at −20°C. The maximum time for storage is 1 year.
10. Stock solution of 10 mM EdU: dissolve 10 mg EdU (5-ethynyl-2′-deoxyuridine) in 4 mL DMSO in biosafety cabinet. Aliquot the stock and store at −20°C. The maximum time for storage is 1 year.
11. Stock solution of 1 mM Azide-PEG3-biotin: Prepare 10 mM stock by dissolving 10 mg Azide-PEG3-biotin in 2.25 mL DMSO, and then dilute to 1 mM with DMSO. Aliquot the stock and store at −20°C. The maximum time for storage is 1 year.
12. 3% PFA preparation: mix 400 mL milli-Q H2O, 15 g Paraformaldehyde, 30 μL 10 N NaOH together, heat to 60°C and stir until the complete dissolution of PFA, add 50 mL 10× PBS, Stir and filter the solution, add 10 g sucrose, and add milli-Q H_2O to the final volume 500 mL, stir it until the complete dissolution of sucrose. Aliquot the stock and store at −20°C. The maximum time for storage is 1 year.

Note: PFA is toxic. Prepare the solution in fume hood.

13. 200 mg/mL BSA solution: dissolve 200 mg in 1 mL milli-Q H2O, sterilize with 0.45 μm filter. Aliquot the stock and store at −20°C. The maximum time for storage is 1 year.
14. Stock solution of 50% dextran sulfate: Dissolve 0.5 g dextran sulfate in 600 μL milli-Q H2O, mix by pipetting slowly until dextran sulfate is completely dissolved, and bring the volume to 1 mL with milli-Q H2O. Aliquot the stock into 1.5 mL tubes with 20 μL each, and store at −20°C. This stock solution is stable for up to 1 year.

Note: 50% dextran sulfate is very viscous. It takes some time for dextran sulfate to dissolve in H2O by pipetting.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Biotin (1:1000)	Abcam	Cat#ab53494; RRID: AB_867860
Anti-BLM (1:1000)	Bethyl	Cat#A300-110A; RRID: AB_2064794
Alexa Fluor® 594 AffiniPure Donkey Anti-Rabbit IgG (H+L) (1:500)	Jackson ImmunoResearch	Cat#711-585-152; RRID: AB_2340621
Chemicals, peptides, and recombination proteins		
Polybrene	Merck Millipore	Cat#TR-1003-G
Puromycin	Thermo Fisher Scientific	Cat#A1113802
Geneticin	Thermo Fisher Scientific	Cat#10131027
(+)-Abscisic Acid	Cayman Chemical	10073; CAS: 21293-29-8
EdU (5-ethynyl-2′-deoxyuridine)	Thermo Fisher Scientific	Cat#A10044
Dimethyl Sulfoxide (DMSO)	Sigma	D2650; CAS: 67-68-5
Paraformaldehyde (PFA)	Sigma	158127; CAS: 30525-89-4
Bovine Serum Albumin (BSA)	Sigma	B2064; CAS: 9048-46-8
20× SSC	Thermo Fisher Scientific	Cat#AM9763
Azide-PEG3-biotin	Sigma	762024; CAS: 875770-34-6
Dextran Sulfate sodium	Sigma	D8906; CAS: 9011-18-1
Formamide	Sigma	47671; CAS: 75-12-7
TelC-FITC	PNA BIO	Cat#F1009
DAPI	Sigma	D9542; CAS:28718-90-3
VECTASHIELD Antifade Mounting Medium	Vector Laboratories	Cat#H-1000-10
Critical commercial assays		
Gateway™ LR Clonase™ II Enzyme mix	Thermo Fisher Scientific	11791100
Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit	Thermo Fisher Scientific	C10337
Experimental models: Cell lines		
Human: 293T	ATCC	N/A
Human: U2OS expressing PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV	Zhang et al. (2021)	N/A
Experimental models: Organisms/strains		
NEB Stable Competent E. coli	NEB	Cat#C3040H
Recombinant DNA		
pENTR-PYL1-TagRFP-TRF1	Zhang et al. (2021)	N/A
pENTR-ABI-TagRFP-PML-IV	Zhang et al. (2021)	N/A
pLenti PGK Puro DEST (w529-2)	Campeau et al., 2009	Addgene Plasmid #19068
pLenti PGK Neo DEST (w531-1)	Campeau et al., 2009	Addgene Plasmid #19067
pLenti PGK PYL1-TagRFP-TRF1(Puro)	Zhang et al. (2021)	N/A
pLenti PGK-ABI_EGFP_PMLIV(Neo)	Zhang et al. (2021)	N/A
dR8.2	Laboratory of Lee Zou	N/A
VSV-G	Laboratory of Lee Zou	N/A
Software and algorithms		
FociLab2.0	Laboratory of Li-Lin Du	https://github.com/bottom618/FociLab2.0_Li-Lin-Du-lab
GraphPad Prism 7	GraphPad Software, Inc.	https://www.graphpad.com/scientific-software/prism/
CellProfiler	Broad Institute	https://cellprofiler.org/
Other		
18-mm round glass coverslips	Fisher	12-546-P
Nikon 90i	Nikon	N/A
VWR Standard Heatblock	American Laboratory Trading	21435

MATERIALS AND EQUIPMENT

Pre-extraction buffer (0.1% Triton X-100, 20 mM HEPES-KOH pH 7.9, 50 mM NaCl, 300 mM sucrose)		
Reagent	Final concentration	Amount
1 M HEPES-KOH pH 7.9	20 mM	2 mL
5 M NaCl	50 mM	1 mL
10% Triton X-100	0.1%	1 mL
Sucrose	300 mM	10.3 g
Milli-Q Water	Fill to 100 mL	
Total		100 mL
This solution can be kept at 4°C for up to 1 month.		

Permeabilization buffer (1×PBS containing 0.5% Triton X-100)		
Reagent	Final concentration	Amount
1× PBS	1×	95 mL
10% Triton X-100	0.5%	5 mL
Total		100 mL
This solution can be kept at 4°C for up to 1 month.		

PBST (1× PBS containing 0.05% Tween- 20)		
Reagent	Final concentration	Amount
1× PBS	1×	99.5 mL
10% Tween-20	0.05%	0.5 mL
Total		100
This solution can be stored at 20°C–22°C for 1 month.		

PBS containing 3% bovine serum albumin (BSA)		
Reagent	Final concentration	Amount
1× PBS	1×	10 mL
BSA	3%	0.3 g
Total		10 mL
This solution is prepared freshly, can be kept for 24 h at 4°C.		

Blocking solution (1× PBS containing 0.05% Tween-20 and 3% BSA)		
Reagent	Final concentration	Amount
1× PBST	1×	10 mL
BSA	3%	0.3 g
Total		10 mL
This solution is prepared freshly, can be kept for 24 h at 4°C.		

Critical reagent: Hybridizing solution (70% formamide, 2× SSC, 2 mg/mL BSA, 10% dextran sulfate)		
Reagent	Final concentration	Amount
Formamide	70%	35 µL
20× SSC	2×	5 µL
200 mg/mL BSA	2 mg/mL	0.5 µL
50% Dextran sulfate	10%	10 µL
Total		50.5 µL
This solution is prepared freshly.		

Wash solution 1 (2× SSC, 70% formamide)		
Reagent	Final concentration	Amount
Formamide	70%	35 mL
20× SSC	2×	5 mL
Milli-Q Water	–	10 mL
Total		50 mL
This solution is prepared freshly.		

Wash solution 2 (2× SSC, 0.1% Tween-20)		
Reagent	Final concentration	Amount
20× SSC	2×	5 mL
10% Tween-20	0.1%	0.5 mL
Milli-Q Water	–	44.5 mL
Total		50 mL
This solution can be stored at room temperature for 1 month.		

STEP-BY-STEP METHOD DETAILS

Induction of PML-IV tethering to telomeres by ABA

Timing: 2 days

To induce the tethering of PML-IV to telomeres and stimulate ALT activity, U2OS cells expressing PYL1- TagRFP-TRF1 and ABI-EGFP-PML-IV are treated with ABA to induce dimerization of the PYL1 and ABI tagged proteins (Figure 2A).

1. U2OS cells expressing PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV are seeded with 1 mL culture medium in coverslips-containing 12-well plates at a confluency of 30%–50% (0.6 × 10^5–1 × 10^5 cells), and cultured for 24 h.

   Note: To monitor the dynamics of telomere clustering, 4 wells of U2OS (0, 4, 8, 12 h) are required. To visualize the proteins or EdU signal at telomeres, 2 wells of U2OS are required.

   Note: If larger culture vessels are desired, scale reagent volumes relative to the vessel surface area (see: https://www.thermofisher.com/au/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html for surface areas of various culture vessels).

2. To monitor the dynamics of telomere clustering, visualize the enrichment of various proteins at telomeres, and telomeric DNA synthesis, treat the cells with 200 μM ABA or DMSO as following.
   a. To monitor the dynamics of telomere clustering, treat the cells with 200 μM ABA by adding 1 μL of 200 mM ABA stock solution to 1 mL of cell media, and incubate at 37°C for 4, 8, and 12 h. DMSO is used for ABA no-treated control.
   b. To visualize the enrichment of various proteins at telomeres, treat the cells with 1 μL of 200 mM ABA or DMSO for 14 h.
   c. To visualize telomeric DNA synthesis, treat the cells with 1 μL of 200 mM ABA or DMSO for 18 h, and then incubate the cells with 20 μM 5-ethynyl-2′-deoxyuridine (EdU) at 37°C for 1 h by adding 2 μL 10 mM EdU stock solution to 1 mL of cell media.

Detection of proteins and DNA synthesis in APBs by immunofluorescence (IF)

Timing: 6 h–1 day

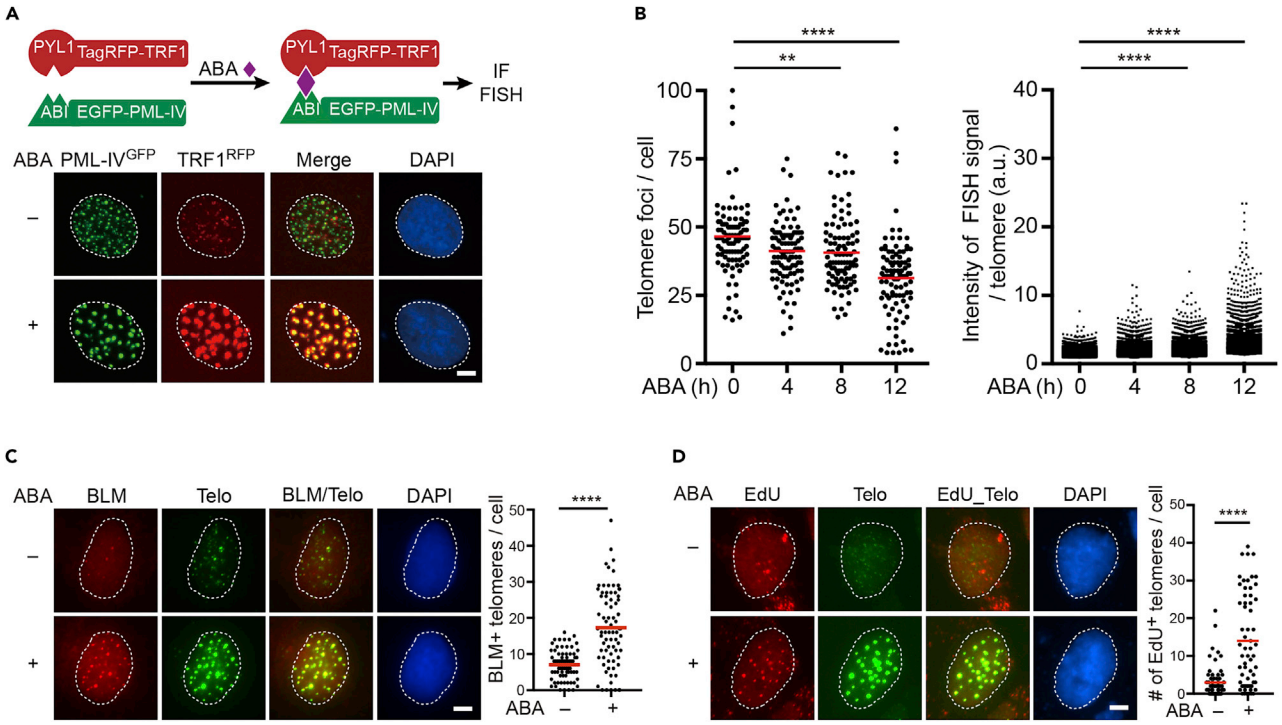

Figure 2. ABA-dependent tethering of PML-IV to telomeres induces telomere clustering, enrichment of DDR factors at telomeres and telomere synthesis

(A) Schematic showing the ABA-induced tethering of PML-IV to telomeres (up). U2OS cells expressing TRF1^{RFP} and PML-IV^{GFP} were treated with DMSO or ABA for 18 h. Fluorescence of PML-IV^{GFP} and TRF1^{GFP} was directly analyzed (down). Scale bar, 5 μm.

(B) U2OS cells expressing TRF1^{RFP} and PML-IV^{GFP} were exposed to ABA as indicated. Telomeres were detected by FISH, and the numbers of telomere foci (left) in individual cells (n=100) and FISH intensity (right) of individual telomeres (n=2,000) were quantified. Red lines: mean numbers of telomere foci per cell. **p<0.001, ****p < 0.0001, determined by unpaired Student's t test.

(C) U2OS cells expressing TRF1^{RFP} and PML-IV^{GFP} were exposed to DMSO or ABA for 14 h. BLM was analyzed by IF, and telomeres were detected by FISH. Representative images showing the enrichment of BLM at telomeres after ABA treatment (left). Scale bar: 5 μm. The numbers of BLM$^+$ telomeres in individual cells (n>100) were quantified (right). Red lines represent the mean number of BLM$^+$ telomeres per cell. ****p < 0.0001, determined by unpaired Student's t test.

(D) U2OS cells expressing TRF1^{RFP} and PML-IV^{GFP} were treated with DMSO or ABA for 18 h, and then labeled with EdU for 1 h. The EdU and telomeres in non-S phase cells were analyzed by IF and FISH. Representative images showing the induction of telomere synthesis after ABA treatment (left). Scale bar: 5 μm. The numbers of EdU$^+$ telomeres in individual cells (n=61) were quantified (right). Red lines represent the mean number of EdU$^+$ telomeres per cell. ****p < 0.0001, determined by unpaired Student's t test.

APBs enrich numerous DNA repair and replication proteins. BLM is one of the well-known DNA repair factor localized at APBs, and it is critical for ALT telomere DNA synthesis at APBs (Zhang et al., 2019). So we use BLM as an example for IF, and we monitor ALT telomeric DNA synthesis by detecting the incorporation of EdU at APBs by IF. The cells on coverslips are subjected to pre-extraction, fixation, permeabilization, and then stained with primary and secondary antibodies for Immunofluorescence (IF).

3. Cool the plate with cells on ice, rinse once with 1 mL ice-cold 1 × phosphate buffered saline (PBS). Troubleshooting 5.
4. Treat cells with 1 mL ice-cold pre-extraction buffer (0.1% Triton X-100, 20 mM HEPES-KOH pH 7.9, 50 mM NaCl, 300 mM sucrose) for 5 min on ice, and then rinse once with 1 mL ice-cold 1× PBS.

Note: The pre-extraction step helps to remove proteins that are not chromatin-bound.

⚠ CRITICAL: The incubation time of pre-extraction buffer is important. Longer time incubation may lead to weaker IF signal.

5. Remove the plate from ice, and fix cells with 0.5 mL 3% paraformaldehyde (PFA) for 15 min at 20°C–22°C, and rinse the cells once with 1 mL 1× PBS. Troubleshooting 1.

⚠ CRITICAL: Freshly prepared or thawed PFA of good quality is important for the experiment.

Note: PFA is toxic. PFA should always be handled under a fume hood.

Pause point: The fixed cells can be kept in 1× PBS at 4°C up to one week.

6. Permeabilize cells in 0.5 mL permeabilization buffer (1×PBS containing 0.5% Triton X-100) for 5 min. Rinse cells once with 1 mL 1×PBS. Troubleshooting 1.
7. If EdU staining is not detected, go to step 9. For monitoring telomeric DNA synthesis by EdU staining, rinse cells with 0.5 mL PBS containing 3% bovine serum albumin (BSA). Prepare click-iT reaction reagents as following. Troubleshooting 1.

Click-iT reaction mix components	Total: 50 μL for 2 coverslips
1× Click-iT reaction buffer	42 μL
$CuSO_4$ (100 mM)	2 μL
Azide-PEG3-Biotin (1 mM)	1 μL
Reaction buffer additive (1×)	5 μL

⚠ CRITICAL: The Click-iT reaction mixture should always be prepared freshly and used immediately.

8. Immediately incubate coverslips with 25 μL reaction mixture on parafilm, with the cell-carrying side facing down. The incubation is done at 20°C–22°C in the dark for 0.5 h.

Note: Steps 7 and 8 is only required for detecting EdU signal in cells. Through the click-iT reaction, biotin is covalently conjugated to EdU. Thus, in the following steps, the signal of EdU can be detected with anti-biotin antibody.

Note: To keep coverslips moisturized, it is recommended to put the coverslips on parafilm in a covered container with wet filter paper or a humidified black box.

⚠ CRITICAL: Make sure to remove the residual buffer on coverslips before incubation. Keep the incubation time same for each coverslip.

⚠ CRITICAL: It is important to keep the samples protected from light during incubations.

9. Incubate cells in freshly prepared 0.5 mL blocking solution (1× PBS containing 0.05% Tween-20 and 3% BSA) at 20°C–22°C for 1 h. Troubleshooting 2.
10. Incubation with primary antibody against target proteins. Troubleshooting 1 and 2.
    a. Dilute the primary anti-BLM antibody (for detecting BLM, 1:1000) or anti-biotin antibody (for detecting EdU click it-reacted with Azide-biotin, 1:1000) in blocking solution (the dilution factor for different antibodies should be optimized by titration, 25 μL for each sample).
    b. Place parafilm in a flat container and put 25 μL diluted antibody solution on parafilm for each coverslip.
    c. Pick up the coverslips from 12-well plates, and remove the remanent buffer on the coverslips by tapping them on filter paper.
    d. Overlay the coverslips on the antibody solution with the cell-carrying side facing down.
    e. Incubate at 20°C–22°C for 2 h or at 4°C for 12 h–24 h.

Note: Place wet filter paper in the container to prevent drying.

Note: BLM is a protein that accumulates at telomeres in ALT$^+$ cells. Anti-biotin antibody is used to detect the EdU conjugated with biotin by click-iT reaction. An anti-rabbit secondary antibody conjugated to 594 will be used to visualize BLM and biotin.

Pause point: The coverslips can be incubated with primary antibody for 24 h.

11. Move coverslips back into 12-well plates containing 1 mL PBST (1× PBS containing 0.05% Tween- 20) in each well.

Note: Make sure that the cell-carrying side of coverslips faces up.

12. Wash coverslips three times (5 min each) with 1 mL PBST (1× PBS containing 0.05% Tween-20) under gentle agitation.
13. Incubate coverslips with anti-rabbit secondary antibodies conjugated to 594 fluorophores (Alexa Fluor® 594 AffiniPure Donkey Anti-Rabbit IgG (H+L), 1:500) in the blocking solution for 1 h in dark at 20°C–22°C. For the incubation with secondary antibody, repeat steps 10b–d.
14. Move the coverslips back into 12-well plates, and wash the coverslips three times (5 min each) with 1 mL PBST (1× PBS containing 0.05% Tween-20).

⚠ CRITICAL: For all the steps after the incubation with secondary antibody, it is important to shield the samples from light.

Detection of telomeres by fluorescence *in situ* hybridization (FISH)

Timing: 6 h–1 day

The cells on coverslips are fixed again, dehydrated and hybridized with a peptide nucleic acid (PNA) probe of telomeres.

15. Fix cells again with 0.5 mL 3% PFA at 20°C–22°C for 15 min. Troubleshooting 1.

⚠ CRITICAL: This fixation step is important for maintaining the IF signal.

16. Dehydrate cells in 0.5 mL 70%, 85%, and 100% ethanol for 2 min each. Troubleshooting 6.
17. Take coverslips out of 12-well plates and put them on the covers of 12-well plates to air dry completely.

Note: Use the inner side of covers, and don't let coverslips to lie completely flat (otherwise it will be difficult to separate coverslips from the cover after drying). Keep the 12-well plates for subsequent washing steps.

18. Prepare the hybridizing solution (70% formamide, 2× SSC, 2 mg/mL BSA, 10% dextran sulfate) freshly as following. 10 μL hybridizing solution is required for each sample. Troubleshooting 3.

Hybridization solution	Stock solution	Total: 50.5 μL
70% formamide	100% formamide	35 μL
2× SSC	20× SSC	5 μL
BSA 2 mg/mL	200 mg/mL	0.5 μL
10% dextran sulfate	50%	10 μL

Note: Formamide is toxic, should always be handled under a fume hood.

19. For each sample, mix 0.2 μL of 5 μM PNA telomere probe (TelC-FITC, F1009, PNA BIO) in 10 μL hybridization buffer to a final concentration of 100 nM. Vortex and centrifuge at 1000 g for 30 s.

Note: TelG probe can also be used as alternative.

⚠ CRITICAL: It's important to prepare a master mix for the same batch of samples.

20. Heat up the hybridizing solution containing PNA probe at 85°C for 2 min. Meanwhile, prewarm the coverslips and slides (75 × 25 mm) at 85°C on a metal heat block for 2 min. Troubleshooting 4.

⚠ CRITICAL: Preheating the coverslip and PNA probe is critical for minimizing the background.

21. Add 10 μL the hybridization solution with PNA probe to each coverslip. Cover the coverslip with a prewarmed slide quickly, and make sure the hybridization solution is evenly distributed between coverslip and slide.
22. Heat the slide with the coverslip for 10 min at 85°C on a metal heat block. Troubleshooting 3 and 6.

Note: To heat the slide with even temperature, it will be better to invert the cube of heat block, and let the flat surface up.

⚠ CRITICAL: make sure to heat the slides that need to be compared for exactly the same time.

23. Place the slide in a closed container at 20°C–22°C for 2 h–24 h for hybridization. Troubleshooting 3.

Note: Put wet filter paper in the container to prevent drying. keep the container in dark.

⚠ CRITICAL: Longer incubation time gives brighter FISH signal.

Pause point: The coverslips can be incubated with hybridization solution with PNA probe for 24 h.

24. Immerse coverslips in wash solution 1 (2× SSC, 70% formamide), and then put coverslip back to the 12-well plate.
25. Wash coverslips with 1 mL wash solution 1 (2× SSC, 70% formamide) three times for 5 min each. Troubleshooting 4.
26. Wash coverslips with 1 mL wash solution 2 (2× SSC, 0.1% Tween-20) three times for 5 min each. During the second wash, cells were stained with 1 μg/mL DAPI for 5 min.
27. Prepare the slides for microscope with 6 μL VECTASHIELD Antifade mounting medium, and seal the slides with nail polish.
28. The images were captured with a Nikon 90i microscope (objective:60×; channels: DAPI, 488, 594; No z-stack). Adjust the exposure time of each channel to make sure the signal not saturated. Same imaging parameters must be used for the samples to be compared.

Note: Other widefield or confocal fluorescence microscope can be used as alternatives.

Note: Z-stack analysis of 3D images is a more accurate method to analyze colocalization, and it can be used to obtain additional information of the spatial organization of proteins in APBs.

Note: Since the fluorescence of PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV is largely abolished during the dehydration and hybridization steps, the fluorescence signals of 594 and FITC can be detected without interference from GFP and RFP. Troubleshooting 6.

⚠ CRITICAL: The samples need to be compared should be imaged together with the same imaging parameters, to make sure all the conditions are the same.

EXPECTED OUTCOMES

After U2OS cells expressing PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV are treated with ABA, the number of telomere foci gradually decreases, whereas telomere FISH intensity increases, indicating that telomeres are clustered upon the induction of APBs by ABA (Figure 2B). BLM, a protein critical for ALT, is colocalized with telomeres after 14 h ABA treatment (Figure 2C). Telomere DNA synthesis in non-S phase cells is enhanced by ABA (Figure 2D). All these outcomes suggest that the ABA-induced tethering of PML-IV to telomeres drastically stimulates ALT activity in U2OS cells.

This assay can be used to study the localization of proteins of interest to APBs. For example, using this assay, we showed that RAD52 is localized to the APBs induced by tethering of PML-IV to telomeres (Zhang et al., 2021). This assay can also be used to analyze the specific ALT events occurring in APBs. For example, we showed that RPA and phosphorylated RPA accumulate in induced APBs, suggesting that replication stress is increased by ALT in APBs. Finally, this assay can be used to test the contributions of specific proteins or their activities to ALT telomere synthesis in APBs. For example, we showed that the helicase activity of BLM is important for the ALT activity in APBs. Similar experiments are applicable to other proteins that may regulate or participate in ALT. The hyperactivation of ALT in this inducible system allows us to characterize the molecular events involved in ALT within 24 h, but it should be noted that prolonged ALT hyperactivation may alter cell cycle progression (Zhang et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. To analyze the signal of IF and FISH, the colocalization and intensity of these signals were quantified together using the FociLab 2.0 software developed by Dr. Li-Lin Du's lab. FociLab 2.0 is used to find the nucleus from DAPI channel, quantify the area and intensity of telomere foci from FITC channel, and quantify the area and intensity of BLM or EdU foci from 594 channel, and then analyze the co-localization of foci between FITC and 594 channels in each nucleus.
   a. FociLab 2.0 recognizes the nucleus with Otsu algorithm.
   b. For identification of foci, FociLab 2.0 firstly calculates the intensity gradient of each pixel by comparing it with the around 8 pixels, defines the position of foci with the distribution curve of intensity gradient of each pixels and the parameters (Std and minimum cutoff) defined by user, output the information of intensity and area of each foci.
   c. For analyzing the co-localization, FociLab 2.0 calculates the distance between each foci from FITC channel (telomere) and 594 channel (BLM or EdU) in the same nucleus, and output the information of co-localized foci if their distance is <= the overlap setting score (it can be 2 or 3).

⚠ CRITICAL: For setting the parameters of foci identification (Std and minimum cutoff) and co-localization analysis (overlap setting score), it's important to test different scores with a representative sample image as standard, and check which score can generate output matching the manual counting.

Note: FociLab 2.0 is specially designed for analyzing foci in images generated from biological studies including DNA repair and telomere maintenance. FociLab 2.0 can find the nucleus, quantify the foci of up to 3 channels in each nucleus, and analyze the co-localization of foci

between any 2 channels. For the image analysis, software Cellprofiler can also be used as alternatives.

2. GraphPad Prism 7 was used for statistical analysis. unpaired Student's t test was used for statistical analysis, and standard deviations (SD) were determined.

LIMITATIONS

Although the ABA-dependent tethering of PML-IV to telomeres can induce APB formation and telomere clustering in both ALT^+ and ALT^- cancer cells, it only stimulates telomere DNA synthesis in ALT^+ cells (Zhang et al., 2021). Thus, this system can be used to study APB formation and telomere clustering in both ALT^+ and ALT^- cells, but its effects on telomere DNA synthesis can only be studied in ALT^+ cells.

In addition, this assay may not recapitulate some of the early steps of ALT. Since PML-IV is directly tethered to telomeres after ABA treatment in this system, the natural ALT events that recruit PML-IV to telomeres may be bypassed. For example, BLM is required for the formation of APBs and telomere clustering in normal ALT^+ cells, but the depletion of BLM doesn't affect the ABA-induced formation of APBs and telomere clustering in cells expressing PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV (Zhang et al., 2021).

Finally, although the dramatic stimulation of ALT activity by ABA makes it easier to analyze the ALT pathway, it may bypass some regulatory mechanisms. The combination of this inducible system with other assays monitoring ALT activity, such as the assay for ALT telomere synthesis in APBs (ATSA), may help us obtain a better understanding of the process of ALT (Zhang et al., 2019).

TROUBLESHOOTING

Problem 1

The Immunofluorescence signal is weak.

Potential solution

- Good fixation is important for IF. Make sure the quality of PFA is good. (step 5).
- Make sure the quality of Triton X-100 used for permeabilization is good and the concentration of Triton X-100 in permeabilization buffer is correct. (step 6).
- For EdU detection by Click-iT, make sure the Click-IT reaction mixture is prepared freshly, and used immediately. (steps 7 and 8).
- Increase the antibody concentration, and incubation time. (step 10).
- Dehydration and denaturation steps during FISH can abolish the IF signal. Make sure coverslips are fixed again by PFA before processing for FISH. (step 15).

Problem 2

The background of immunofluorescence signal is high.

Potential solution

- Use coverslips with low fluorescent background. (before you begin step 7).
- Try different blocking solutions (3% BSA in PBST, 5% milk in PBST, 3% BSA 5% milk in PBST). (step 9).
- Incubation with the antibody solution at 4°C can help decrease the background signal. (step 10).

Problem 3

The FISH signals of telomeres are weak.

Potential solution

- Make sure that the hybridization mixture is prepared correctly, and that dextran sulfate used for FISH is thawed freshly. The stock of dextran sulfate should be kept at −20°C. (step 18).
- Seal the coverslip with rubber cement quickly. It can help increase the hybridization efficiency. (step 22).
- Increase the incubation time at 20°C–22°C from 2 h to 12–24 h. (step 23).

Problem 4

The background of FISH signal is high.

Potential solution

- Always use coverslips with low fluorescent background for FISH. (before you begin step 7).
- Make sure to preheat the hybridization solution and coverslips. (step 20).
- Increase the number of washing steps can decrease background. (steps 25 and 26).
- Avoid the coverslips dry when changing the wash solution during washing steps. (steps 25 and 26).

Problem 5

Cell loss during the experiment.

Potential solution

- When pipetting ABA or EdU stock into the medium, shake the plates immediately. Since ABA and EdU are all prepared with DMSO, local high concentration of DMSO will kill the cells. (step 2).
- When adding the solutions into the well, let the liquid flow down the side of well, unless the cells may be easily washed away. (steps 3–5).

Problem 6

The RFP and GFP signals of PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV persist after FISH.

Potential solution

Dehydration and hybridization steps during FISH can largely destroy TagRFP and GFP signal from PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV (Figures 3A and 3B). So after FISH, the fluorescence of GFP and RFP will be quenched, and not affect the normal observation of telomere (FITC) and BLM or EdU (594).

- Make sure the incubation time of steps 16 and 22 is not shorter than required.
- After the FISH procedure, the RFP signal of PYL1-TagRFP-TRF1 is largely abolished, but there is still a very weak residual GFP foci signal of ABI-EGFP-PML-IV. If other proteins with very weak signal are detected, It's better to observe it with 594. (step 28).
- If Telomere FISH signal is not required for the experiments, FISH procedures can still be done without telomere probes in order to quench the signal of GFP and RFP.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lee Zou (zou.lee@mgh.harvard.edu).

Materials availability

All unique reagents generated in this study will be made available upon request with appropriate institutional review and approval. We may require a completed Materials Transfer Agreement and a payment if the reagents are used for commercial applications.

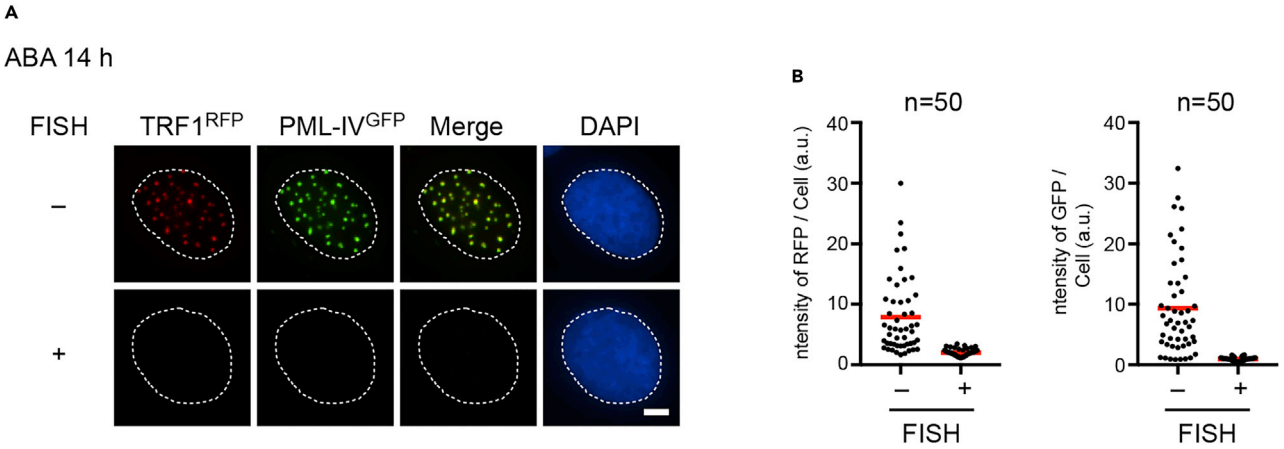

Figure 3. The RFP and GFP signals of PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV are largely abolished after procedures of FISH

(A) U2OS cells expressing $TRF1^{RFP}$ and PML-IV^{GFP} were treated with ABA for 14 h, and then fixed by PFA for 15 min but without primary and secondary antibody incubation. The sample (FISH+) is treated with dehydration and Hybridization (steps 16–22) but without the teloC probe. The sample (FISH–) is not treated with dehydration and Hybridization (steps 16–22). GFP and RFP signal were observed directly under fluorescence microscope. Scale bar: 5 μm.

(B) The RFP and GFP signals of PYL1-TagRFP-TRF1 and ABI-EGFP-PML-IV in each cell (n=50) with and without FISH treatment are quantified. The red lines represent the mean intensity of fluorescence per cell.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

J.-M.Z. performed the experiments and data analysis. J.-M.Z. and L.Z. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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