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Probing the Telomere Damage Response

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Abstract

Telomere dysfunctions, rendered through replicative attrition of telomeric DNA or due to the inhibition of shelterin components, are recognized as DNA double stranded breaks (DSBs) by the DNA damage repair (DDR) pathway. This leads to the activation of DNA damage checkpoint sensors including the Mre11-Rad50-Nbs1 (MRN) complex, γ -H2AX and 53BP1, the ATM and ATR signal transducing kinases and downstream effectors including Chk1, Chk2 and p53. Robust DNA damage response signals at dysfunctional telomeres, achieved by complete deletion of TRF2 or by expressing dominant negative mutant TPP1 ^{Δ RD}, can be detected by their association with γ -H2AX and 53BP1 forming “telomere dysfunction induced foci (TIFs)”. Induction of TIFs at telomeres provides an opportunity to quantify the extent of telomere dysfunction and monitor the signaling pathways.

Keywords

DNA damage; Telomere dysfunction; Telomere induced foci; Telomere-FISH

1. Introduction

Telomeres are nucleoprotein complexes at the chromosome ends; consist of TTAGGG repetitive sequences, a 3' single strand G-overhang, and telomere binding proteins. In mammals, telomere binding proteins form a highly organized functional unit known as telosome or the shelterin complex formed by six telomeric core proteins: telomeric-repeat-binding factor 1 (TRF1) and 2 (TRF2), TRF1-interacting protein 2 (TIN2), transcriptional repressor/activator protein RAP1, protection of telomeres 1 (POT1) and POT1- and TIN2-organizing protein (TPP1) (1). The telosome/shelterin complex contributes to the formation of protective telomere loop (t-loop) structures; where the single-stranded 3'-overhang invades the telomeric duplex in order to prevent the chromosome ends to be recognized as DSBs by DDR (2). Failure of the protective features of telomeres due to replicative attrition or due to genetic alteration of shelterin components leads to 'dysfunction' or 'uncapped' telomeres (3). Uncapped telomeres sensed by MRN complex (Mre11/Rad50/Nbs1) activates ATM/ATR mediated DNA damage signaling cascade leading to the recruitment of DNA damage response factors such as 53BP1, γ -H2AX, MDC1, and initiation of inappropriate DNA repair pathways (Non Homologous End Joining and Homologous Recombination) at the chromosome ends. The recruitment of DNA damage response factors colocalized with telosome or telomeres are referred as telomere induced foci (4, 5). TIFs can be efficiently visualized in the interphase nuclei using telomere FISH (Fluorescent *In Situ* Hybridization).

2. Materials

2.1 . Cell Culture

1. Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone SH30022.01) supplemented with 10% fetal bovine serum (FBS, Sigma F-6178), OPTI-MEM (Gibco 31985)
2. 293T (ATCC) and Mouse Embryonic Fibroblast (MEFs) Cells.
3. 0.25% Trypsin (Gibco 25200).
4. PBS (Hyclone SH30028.02)

2.2 . Retroviral Production and Infection

1. pCL Eco, Empty pBabe, p-Babe shTRF2, Empty pQCXIP, pQCXIP TPP1^{ΔRD}.
2. Polybrene Stock 6 mg/ml. Filter through a 0.2 μm filter, aliquot, and store at −20°C.
3. Lipofectamine Plus Reagent (Invitrogen), Fugene 6 (Roche)
4. 0.45μm syringe filters (Millipore)

2.3 . Immunofluorescence Telomere FISH

1. Nunc Lab-Tek 8 well slide chambers (Nalgene Nunc, 12-565-1), Microscope cover slips 18×18-1 (Fisher-12-548-A), Microscope Slides (Fisher 12-544-3).
2. Phosphate buffer saline (PBS): Phosphate buffered saline (PBS): Prepare 10X stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄ (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by dilution of one part with nine parts water.
3. Fix Solution: 2% paraformaldehyde (USB 199431) / 2% sucrose (Sigma S-1888).
4. Blocking Solution PBG: 0.2% fish gelatin (Sigma G-7765), 0.5% BSA (Sigma A-2153). Aliquot PBG and store at −20°C.
5. Permeabilization Buffer : 0.5% (v/v) Nonidet-P40 (USB 19628) in PBS.
6. TBST: 1XPBS with 0.1% Triton (Sigma T9284).
7. Primary antibodies: 53BP1 and γ-H2AX antibodies (Upstate 05-636).
8. Secondary antibodies: Alexa Fluor 568 goat anti mouse IgG {H+L} A-11004).
9. 20XSSC (Invitrogen 15557-036).
10. Formamide (Fisher BP227-500).
11. TelC-TAMRA, TAMRA-OO-(CCCTAA)₃ PNA Probe (Pan agene-F2002). Stock solution 1μg/μl in H₂O.
12. Yeast tRNA (Invitrogen 15401-011). Stock solution 1mg/ml in H₂O.
13. Wash Solution I: (70 % formamide, 0.1% Tween 20 Sigma BP337 500, 0.1% BSA, mM Tris-HCl, pH 7.5).
14. Wash Solution II: (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% BSA, 0.1% Tween-20).
15. Mounting Medium with Antifate: DAPI (Vectashield H-1200).

3. Methods

Robust TIFs are monitored by disrupting the protective function of telomere through inhibition of TRF2 or through expression of dominant negative mutant TPP1^{ΔRD}, which efficiently remove endogenous TPP1-POT1a complex from telomeres (6, 7). Eventually, TIFs can also be visualized with a dominant negative allele TRF2^{ΔBΔM} (3) and siRNA mediated inhibition of TRF2 or TPP1 in Osteosarcoma U2OS or Hela cells. To accomplish efficient induction of TIFs in MEFs (Mouse Embryonic Fibroblasts), it is recommended to perform retroviral infections for complete removal of TRF2 using shRNA against TRF2 or expression of TPP1^{ΔRD}. For infecting MEFs, use pCL-ECO, and for infecting human cells, use VSV-G/Gag-Pol packaging constructs for generating high titer shTRF2 or TPP1^{ΔRD} retroviral particles. To achieve high titer retroviral particles, use early passage 293T cells and high quality DNA preparations. Digital images can be acquired and analyzed using a Nikon Eclipse 800 microscope, X63 apo-plan objectives and a CCD camera with a 5 megapixel, 9×9 micron chip. Cells 53BP1 or γ-H2AX signals co localized with telomere signals considered as TIFs positive cells. Score at least 100-200 TIFs positive cells to reach the statistical significance.

3.1. Retrovirus Production in 293T cells

1. **Day 0:** On the day before transfection, plate $0.5-1 \times 10^6$ 293T cells into 6cm dish in 4.0 ml DMEM supplemented 10%FBS. (*see Note 1*). The cells are ready for transfection after 18-20 hours, or when they are about 60-70% confluent. Plate slightly more cells when making VSV-G pseudotyped viruses.
2. **Day 1:** Aspirate the medium and replace the cells with 2.0ml DMEM without FBS and any antibiotic. Transfect 293T cells with 2.0μg pCL-ECO and 4.0μg of transfer vectors (p-Babe shTRF2, PQXCIP TPP1^{ΔRD}) using Lipofectamine Plus Reagent following the manufacturer's protocol. For making VSV-G retroviruses by triple transfection use 0.9μg Gag/Pol expression vector, 0.1μg VSV-G expression vector, and 1.0μg transfer vector. For making VSV-G pseudotype viruses use Eugene 6 following the manufactures protocol.
3. Change the medium 5-7 hours after transfection with 4.0 ml of fresh medium (*see Note 2*)
4. **Day 3:** Harvest the viral supernatants at 48 hours post-transfection. Filter the viral supernatant with 0.45μm syringe filter. Add 10ml of fresh DMEM medium supplemented with 10% FBS to the cells.
5. **Day 4:** Harvest the viral supernatant at 72 hours of post infection as above.

3.2. Retroviral Infection

1. **Day 2:** Grow 20% confluent target cells either on coverslips in 6 well plates or in 8 well slide chambers (*see Note 3*)
2. **Day 3:** Infect the cells with 48 hours harvested retroviral particles. Add 2.0 ml viral particles (1:1 diluted in DMEM/10%FBS) in 6 well plate and 500μl in 8 well chambers to infect MEFs/Human cells. Add 6μg/ml polybrene for infecting MEFs and 4μg/ml polybrene for infecting Hela or U2OS cells (*see Note 4*).

¹It is very important to have single cells suspensions (trypsinize well) and evenly distributed cells.

²293T cells detach easily, be careful with all medium changes.

³Cover slips must be autoclave or sterilized using flame with 95% alcohol. Place the cover slips in 6 well plates to cool down.

⁴Polybrene enhances the rate of infection.

3. **Day 4:** After 24 hours of first infection, reinfect the cells with 72 hours harvested retrovirus particles.
4. **Day 5:** Replace the cells with fresh medium (*see* Note 5).

3.3. Immunofluorescence-Telomere FISH

1. **Day 6:** Aspirate the medium and wash the cells twice for 5 min each with 1XPBS at RT.
2. Fix the cells with 2% paraformaldehyde / 2% sucrose for 10 minutes at RT.
3. Wash the fixed cells twice for 5 min each with 1XPBS.
4. Permeabilize the cells with 0.5% Nonidet-P40 for 10 minutes at RT.
5. Wash the cells thrice for 5min with 1XPBS.
6. Incubate the cells with PBG for 1 hour to block the non-specific binding.
7. Incubate the cells with the primary antibody diluted in PBG (1:2000 53BP1 or γ -H2AX) for overnight at 4°C in humidifier chamber (*see* Note 6). Add 60 μ l antibody for 8 well chambers, 30-50 μ l for cells on coverslips, place cells face down on antibody drop over paraffin film).
8. **Day 7:** Wash the cells thrice for 5 min each with PBST followed by 5 minutes blocking with PBG.
9. Incubate the cells with appropriate secondary antibody diluted in PBG (1:2000) for 1hour at RT. Incubation should be performed in dark.
10. Wash the cells thrice for 5 min each with 1XPBST.
11. Post-fix the secondary antibody to primary antibody by incubating the cells in 4% paraformaldehyde for 10 minutes at room temperature (*see* Note 7).
12. Wash the cells twice for 5 min each with PBS, RT
13. Add freshly prepared PNA-FISH hybridization mix: (30 μ l/coverslip, face down on paraffinized slides; 60 μ l per 8 chamber slide).
14. Denature the slide at 85°C on a hot plate for 3 minutes; place the slide in the dark for overnight in a humidified chamber (*see* Note 8).
15. **Day 8:** Wash twice for 15 min each in Wash Solution I. For slides use coplin jar, for coverslips use 6-well plate.
16. Wash thrice for 5 min each in Wash Solution II.
17. Ethanol dehydrate the slides for 2 minutes each with 70%, 85%, and 95% Ethanol.
18. Counterstain with DAPI and seal with nail vanish. Cover slip should be carefully inverted to a drop of mounting medium on a microscope slides. For 8 well chambers, carefully remove the gasket and place few drops of mounting medium

⁵Visualize at any GFP control plates under the fluorescent microscope to be sure the cells are expressing GFP. If they are, you can assume the cells have taken in the DNA and are producing virus.

⁶Make sure cells are covered and will not dry out.

⁷It is critical to post-fix the secondary antibody to primary antibody using paraformaldehyde in order to retain the primary antibody signals.

⁸Make absolutely sure that the temperature is exactly 85°C using a thermometer.

and cover with a coverslips (*see* Note 9). The slides can be viewed immediately when the varnish is dried or be stored in the dark at 4°C for up to a month.

19. Image on a fluorescent microscope.

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⁹Air bubbles are undesirable in the mounting medium.

H2O	2.85μl
2% BSA	1.5μl
100μg/ml tRNA	0.15μl
0.6XSSC	3.0μl
100% Formamide	21.0μl
10ng/μl PNA Probe	1.5μl
Total	30.0μl