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ZSCAN4 and TRF1: A functionally indirect interaction in cancer cells independent of telomerase activity

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ABSTRACT

Purpose: Recently, the newly identified embryonic stem cell marker, Zinc finger and SCAN domain containing 4 gene (ZSCAN4), which plays a key role in genomic stability by regulating telomere elongation, was shown to co-localize with TRF1 foci. This suggests that the interaction of ZSCAN4 with TRF1 functions in regulation of telomere elongation in ESC. Based on these studies, we hypothesized that ZSCAN4 binds to TRF1 in cancer cells to function in regulating telomere length. The purpose of this study was to determine whether this interaction occurred across different cell lineage-derived cancers and whether telomerase status impacted this relationship. To that end, telomerase positive cervical cancer cells (HeLa) and breast cancer cells (MCF7), and telomerase negative osteosarcoma cells (SaOS2), were analyzed for ZSCAN4 and TRF1 interactions.

Results: Immunocytochemistry demonstrated co-localization of ZSCAN4 and TRF1 to the nucleus. This functional relationship was confirmed using BiFC imaging analysis based on distance *in situ*. Co-immunoprecipitation and pull-down assay results demonstrated that ZSCAN4 binds with TRF1 *in vitro* indirectly. All three cell types showed similar results.

Conclusions: In this study, we revealed, for the *first time*, that ZSCAN4 indirectly interacts with TRF1 (functional association protein) in cancer cells. Furthermore, we show that ZSCAN4 plays an important role independent of telomere maintenance pathways (telomerase positive and ALT) or cell lineage.

1. Introduction

A hallmark of cancer cells is their capacity for unlimited proliferation in contrast to human somatic cells, which are limited to approximately 50–70 cell divisions before entry into a senescent state [1]. Telomere length maintenance is the limiting factor [2]. While the literature on telomeres in extensive, the mechanisms governing telomere replication in humans still remain elusive [3]. Telomere repeat binding factors and their associated proteins, located in the shelterin complex, control telomere length [4,5]. Although each shelterin protein has its own function, TRF1 has the primary role in telomere length regulation [6–8]. Overexpression of TRF1 results in the progressive shortening of telomere length, while dominant-negative inhibition of TRF1 leads to telomere elongation [7–9]. Even though the shelterin complex has been identified as key in regulating telomere elongation, the mechanism of control behind telomere length regulation is not fully understood. Studies have shown that telomere-associated proteins possess diverse functions such as DNA repair/damage checkpoint proteins, nucleotide excision repair/base excision repair, etc., [2,10]. However, another aspect to consider is that the telomere protein complex may associate with other proteins with different biological processes at the telomere [3]. Since the mechanism behind telomere length maintenance also regulates transcription of mammalian telomeres [11,12], we investigated the potential interaction of the embryonic stem cell (ESC) marker, ZSCAN4 with TRF1 in controlling telomere length regulation in cancer cells.

ZSCAN4 is expressed exclusively in late 2-cell embryos and ESC

Abbreviations: ALT, alternative lengthening of telomeres; BIFC, bimolecular fluorescence complementation; Co-IP, co-immunoprecipitation; ESC, embryonic stem cells; GST, glutathione S-transferase; PinX1, PIN2/TERF1 interacting, telomerase inhibitor 1; POT1, protection of telomeres 1; RAP1, their associated proteins repressor/activator protein 1; TIN2, TRF1-interacting nuclear protein 1; TPP1, tripeptidyl peptidase 1; TRF1, telomeric repeat binding factor 1; TRF2, telomeric repeat binding factor 2; T-SCE, telomere sister chromatid exchange; ZSCAN4, zinc finger and SCAN domain containing 4.

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and is a novel regulator of ESC pluripotency that includes six paralogous genes (ZSCAN4a-f) [13–15]. Previous studies demonstrate overexpression of ZSCAN4 in ESC rescues cell proliferation, causes rapid telomere extension and also co-localizes with TRF1 foci [15]. Another function of ZSCAN4 is control of T-SCE (strongly related to the ALT pathway) [15]. Therefore, ZSCAN4 may potentially participate in telomere length extension through interaction with telomeric proteins of the shelterin complex or proteins associated with the ALT pathway in human cancer cells. The objective of this study was to address whether ZSCAN4 interacted with TRF1 in human cancer cells, demonstrating similar functions with respect to telomere regulation, as those identified in ESC, and determine whether this interaction was linked to telomerase activity.

2. Methods

2.1. Cell culture

The following human cancer cell lines: HeLa (cervical), MCF7 (breast), and SaOS2 (osteosarcoma) were purchased from the American Tissue and Culture Collection (ATCC), Manassas, VA, USA. HeLa, and MCF7 cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 IU/ml and 100 μg/ml, respectively). SaOS2 cells were cultured in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine, and 0.01% penicillin/streptomycin. All cell lines were grown under humidity in 5% CO_2 at 37 °C.

2.2. Co-immunoprecipitation (Co-IP)

For immunoprecipitation, pre-cleared protein using 1 μg of normal mouse IgG from whole cell lysates has incubated with anti-TRF1 rabbit polyclonal antibody (Abcam) or anti-GFP rabbit polyclonal antibody already conjugated to pre-washed sepharose-conjugated protein A/G beads (20 μl) (Pierce) in the non-denaturing lysis buffer overnight at 4 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), visualized by Western blot with anti-ZSCAN4 antibody (LSBio, 1:200 rabbit polyclonal) and detected with X-ray film.

2.3. Cell-free protein expression (In vitro translation)

Cell-free protein synthesis was performed using the TNT® T7 Quick Coupled system (Promega). The pANT7-cGST-Rap1 (DNASU) was expressed using the TNT® T7 Quick Coupled system according to the manufacturer's instructions (Promega).

2.4. Recombinant fusion protein purification and quantitation

Recombinant pGEX-4T-1 vectors were transformed in *Escherichia coli* BL21 (DE3). Isopropyl-β-D-1-thiogalactopyranoside (IPTG)-induced cells were harvested and pellets were resuspended in pull-down binding buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.5% NP-40, 50 mM EDTA, 5 mM DTT and protease inhibitors. Cells were disrupted by sonication with a 3 mm probe in an Ultrasonic Processor (Cole Parmer). In order to obtain purified ZSCAN4 protein, the IPTG-induced-GST-ZSCAN4 cell supernatant was incubated with Thrombin (1 unit/mg protein) added in 2 volumes with the thrombin cleavage buffer (20 mM Tris–HCl pH 8.5, 100 mM NaCl, 0.3 mM $CaCl_2$, 1 mM DTT, and 0.1% Tween X-100). Thrombin was removed using a Thrombin Cleavage Capture kit according to manufacturer's instructions (Novagen, San Diego, USA).

2.5. GST-pull-down assay with total cell lysate

The bait protein (pGEX-4T-1-ZSCAN4) was expressed as described above. The GST-ZSCAN4 lysate was incubated with pre-washed glutathione agarose beads (Pierce), and then total cell lysate from the cancer cell lines was incubated with the bead-GST-ZSCAN4 complex. Immunoprecipitated protein complexes were collected and analyzed by Western with TRF1 antibody (Abcam) and detected on X-ray film.

2.6. GST-pull-down assay with cell free system

The bait protein (pANT7-cGST-RAP1) was generated using the cell-free protein expression procedure as described above. The purified ZSCAN4 and truncated ZSCAN4 were collected as described above. Immunoprecipitated protein complexes were collected for further immunoblot analysis against ZSCAN4 (LSBio).

2.7. Immunofluorescence microscopy

MCF7 and SaOS2 cancer cells were grown on 8-well chamber slides (Millipore). For initial immunostaining, the fixed cells were incubated in anti-ZSCAN4 antibody (LSBio, 1:200 rabbit polyclonal) for 24 h at 4 °C, washed three times in PBS, incubated in an Alexa Fluor 488 anti-rabbit IgG antibody (Molecular Probes, Eugene, USA) for 1 h at room temperature. A second immunostaining was performed as described above with anti-TRF1 antibody (Abcam, 1:200 mouse polyclonal) and Alexa Fluor 568 goat anti-mouse antibody (Invitrogen, 1:400). DNA was counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Imaging was performed using an Olympus IX71 inverted deconvolving epifluorescence microscope at 40X and analyzed using Simple PCI software (Compix).

2.8. Plasmid construction for BiFC

Plasmid pairs pBiFC-VN173 and pBiFC-VC155 were purchased from Addgene (Addgene, MA). The ZSCAN4 gene was amplified from ZSCAN4 (NM_152677) purified human protein (OriGene). The PCR product of ZSCAN4 amplification was cloned into a pBiFC-VC173 vector (Addgene). The PCR product was digested with *KpnI* and *EcoRI*, and then ligated into pBiFC-VN173 and pBiFC-VC155 (Addgene). The same method was used to construct the pBiFC–TRF1–VC155 with the TRF1 (BC029378) purchased pANT7-cGST-TRF1 (DNASU).

2.9. Transfections for BiFC analysis

Cancer cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Each cancer cell line was transfected with BiFC VN173-ZSCAN4 and BiFC VN155-TRF1. Post-transfection, the cells proteins cells were fixed, washed with PBS, and permeabilized. DNA was counterstained with DAPI containing 5% Phalloidin (Invitrogen). The cells were washed and visualized using an Olympus IX2-DSU (Disk Scanning Unit) confocal microscope (Hamamatsu Photonics, Japan) under an Olympus APON 60X TIRF NA 1.4. Objective. Slidebook 5.5 software by 3i (Intelligent Imaging Innovations, Denver, CO) was used for image capture and processing.

3. Results

3.1. Interactions between ZSCAN4 and TRF1 are observed in telomerase positive and negative cancer cell lines

It was hypothesized that ZSCAN4 interacts with one (or more) of