Biochemical and Biophysical Research Communications 498 (2018) 72–78

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ZSCAN4 is negatively regulated by the ubiquitin-proteasome system and the E3 ubiquitin ligase RNF20

Benjamin A. Portney [a, 1], Raju Khatri [a, 1], W. Alex Meltzer [a], Jennifer M. Mariano [a], Michal Zalzman [a, b, c, d, *]

[a] Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[b] Department of Otorhinolaryngology-Head and Neck Surgery, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[c] Marlene and Stewart Greenbaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[d] The Center for Stem Cell Biology and Regenerative Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA

ARTICLE INFO

Article history:
Received 9 February 2018
Accepted 18 February 2018
Available online 2 March 2018

Keywords:
ZSCAN4
RNF20
Proteasome
Lysine-48 ubiquitination
Cancer
Stem cells
Telomeres

ABSTRACT

Zscan4 is an early embryonic gene cluster expressed in mouse embryonic stem and induced pluripotent stem cells where it plays critical roles in genomic stability, telomere maintenance, and pluripotency. *Zscan4* expression is transient, and characterized by infrequent high expression peaks that are quickly down-regulated, suggesting its expression is tightly controlled. However, little is known about the protein degradation pathway responsible for regulating the human ZSCAN4 protein levels. In this study we determine for the first time the ZSCAN4 protein half-life and degradation pathway, including key factors involved in the process, responsible for the regulation of ZSCAN4 stability. We demonstrate lysine 48 specific polyubiquitination and subsequent proteasome dependent degradation of ZSCAN4, which may explain how this key factor is efficiently cleared from the cells. Importantly, our data indicate an interaction between ZSCAN4 and the E3 ubiquitin ligase RNF20. Moreover, our results show that RNF20 depletion by gene knockdown does not affect ZSCAN4 transcription levels, but instead results in increased ZSCAN4 protein levels. Further, RNF20 depletion stabilizes the ZSCAN4 protein half-life, suggesting that RNF20 negatively regulates ZSCAN4 stability. Due to the significant cellular functions of ZSCAN4, our results have important implications in telomere regulation, stem cell biology, and cancer.

1. Introduction

The embryonic gene *Zscan4* (Zinc finger and SCAN domain containing 4) promotes telomere and genomic stability in mouse embryonic stem (ES) cells [1]. Knockdown of *Zscan4* in mouse ES cells results in telomere shortening and karyotype abnormalities, slowing cell proliferation until reaching culture crisis. *Zscan4* is highly but transiently expressed [1], with protein expression bursts that facilitate chromatin remodeling [2,3] and transcriptional reprogramming during the generation of induced pluripotent stem (iPS) cells [4–6]. A short expression burst of *Zscan4* was further demonstrated to replace *Myc* and enhance the efficiency of mouse iPS cell formation through activation of early embryonic genes [4]. The human ZSCAN4 has been shown to interact with factors important for telomere maintenance [7,8], and has been suggested to play a role in cancer [1,8]. Given the important role of ZSCAN4 and its transitory nature in the cell [1,9], maintaining the delicate balance between its protein synthesis and degradation is critical for stem cell and potentially cancer cell function.

Concentrations and spatial gradients of specific proteins must be able to rapidly change in response to extracellular cues and according to current cell state [10]. Small protein imbalances can drastically impact such important cellular processes. Therefore, intracellular protein degradation and turnover play a significant role in cell life cycle [11]. Two major pathways responsible for the degradation of proteins in cells are autophagy and the ubiquitin

Abbreviations: UPS, ubiquitin-proteasome system; ES cells, embryonic stem cells; iPS cells, induced pluripotent stem cells; Dox, doxycycline; CHX, cycloheximide; co-IP, co-immunoprecipitaiton; kDA, kilo Dalton; K48Ub, lysine-48 Ubiquitination; DAPI, diamidino-2-phenylindole; Cyto, cytoplasmic cell fraction; Nuc, nuclear cell fraction.

* Corresponding author. Department of Biochemistry & Molecular Biology, Department of Otorhinolaryngology Head and Neck Surgery, University of Maryland School of Medicine, 108 N. Greene Street, Baltimore, MD 21201, USA.
E-mail address: MZalzman@som.umaryland.edu (M. Zalzman).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bbrc.2018.02.155

proteasome system (UPS). Autophagy is the process responsible for degradation of longer lived, structural proteins and organelles. This process depends on the formation of a double membrane autophagosome, which takes up its cargo and subsequently fuses with lysosomes, leading to degradation [12,13]. The canonical UPS is an ATP-dependent degradation pathway [14]. Proteins are marked for proteasomal degradation by ubiquitin, a small 8.5 kDa regulatory protein, which is added to lysine residues of the target protein. Polyubiquitination, or formation of an ubiquitin side chain, specifically on lysine residue 48 of the ubiquitin moieties, targets proteins to the 26S proteasome for degradation. The ubiquitination process involves three steps: activation of the ubiquitin molecule by E1 ubiquitin enzymes, conjugation of ubiquitin to an E2 ubiquitin ligase, and ligation of the ubiquitin molecule to substrate. E3 ubiquitin ligases play a particularly important role as they connote substrate specificity and facilitate the ligation of the ubiquitin molecule to the target protein [15].

Transient expression of high levels of *Zscan4* [1] leads to drastic changes in stem cell properties and potency [2,3]. Therefore, stringent regulation of the ZSCAN4 protein is required to effectively control its cellular functions. However, the regulation of human ZSCAN4 protein, and more specifically its turnover dynamics, remains obscure. As a growing body of evidence suggests a significant role for ZSCAN4 in stem cells and cancer, knowledge of its protein regulation is critical. In this study, we demonstrate for the first time that ZSCAN4 protein degradation is regulated by the ubiquitin-proteasome system. Further, we identify the E3 ubiquitin ligase RNF20 as an important negative regulator of ZSCAN4 protein stability.

2. Materials and methods

2.1. Cell culture

Tu167 cells were obtained from the University of Texas MD Anderson Cancer Center (Houston, TX, USA). All cell lines used in this study were grown in complete DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 2 mM GlutaMAX, penicillin (100 U/mL), streptomycin (100 μg/mL) and were tested free of mycoplasma.

2.2. RNF20 knockdown by siRNA

Wild type (WT) Tu167 cells were grown in monolayer to 70% confluence and transfected with 25 nM of either ON-TARGETplus individual siRNAs (targeting sequences GCUAAACAGUGGAGAUAAU and GUAUCAUCCUUAAACGUUA) or SMARTpool siRNA reagent targeting human RNF20 (Dharmacon). For non-targeting control conditions, Tu167 cells were transfected with 25 nM MISSION siRNA Fluorescent Universal Negative Control siRNA (Sigma). Cells were transfected using DharmaFECT reagent (Dharmacon) according to manufacturer's instructions. Knockdown was confirmed after 48 h incubation by immunoblot.

2.3. Determination of ZSCAN4 half-life

WT and doxycycline (Dox) inducible tet-ZSCAN4 Tu167 cells were treated with 1 μg/mL Dox (or kept untreated) for 24 h to induce ZSCAN4. For RNF20 knockdown, WT Tu167 cells were transfected for 48 h with either NTC-siRNA, pool RNF20 siRNA or RFN20 siRNA1-2 as described above. Cells were treated with 25 μg/ml CHX (Sigma), for the indicated time points. Total cell lysate in RIPA buffer was loaded on 10% SDS PAGE gel and immunoblotted with ZSCAN4 antibodies (1:1000; Origene) or controls, β-actin (1:1000; Millipore) and Lamin B antibodies (1:2000; Santa Cruz). Band intensities of ZSCAN4 were quantified using ImageJ software [16] and normalized to controls. The relative levels of ZSCAN4 in sample not treated with CHX was considered as initial level of ZSCAN4 and considered as 1 unit. The half-life of ZSCAN4 was determined using formula $t1/2 = \ln 2/k$ (k is the slope of the degradation curve).

2.4. Autophagy pathway assay

tet-ZSCAN4 Tu167 cells were treated for 24 h with Dox and then autophagy inhibitors: 5 nM of Bafilomycin A1 or 25 μM of Chloroquine (Sigma) were added for 24 h. Whole cell lysate (50 μg) in RIPA buffer was used on 8% SDS-PAGE analyzed by immunoblot to visualize the following antigens: anti-ZSCAN4 (1:1000; Origene), anti-p62 (1:5000; Sigma), anti-LC3 (1:1000; CellSignaling Technology), Anti-Beta Actin (1:10,000; Millipore). All data shown represent at least 3 independent experiments.

2.5. Immunoblot analysis

Nuclear proteins were fractionated using Nuclear Extraction Kit following manufacture's protocol (Active Motif). Total cell lysate was prepared in RIPA buffer and sonicated. For the detection of endogenous ZSCAN4 in Tu167 cells, cells were harvested by accutase (Millipore) and Cytoskeleton buffer (10 mM PIPES, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA and 0.5% Triton X100) was used to fractionate cytosolic proteins. Then, pellets were lysed in urea solution (8 M Urea in 0.01 Tris pH 8 + 0.1 M NaH_2PO_4) and sonicated. Nuclear proteins were electrophoresed in 8% polyacrylamide gels and transferred to a PVDF membrane. Immunoblot was performed using the following primary antibodies: ZSCAN4 (Origene; 1:1000), GAPDH (Santa Cruz; 1:5000), Actin (Sigma; 1:500), Lamin B (Santa Cruz; 1:2000) and with HRP (horseradish peroxidase) conjugated secondary antibodies (Millipore; 1:5000). Protein bands were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific). SuperSignal West Femto (Thermo Scientific) was used to detect endogenous ZSCAN4 in Tu167 cells. All immunoblots shown represent at least 3 independent experiments.

2.6. Ubiquitination assay

WT Tu167 cells were treated with indicated concentration of MG132 for 3–12 h. Cells treated with vehicle only were used as controls. Nuclear protein was isolated using urea extraction buffer. Then, 100 μg of nuclear lysate was diluted in IP-RIPA buffer with 0.1% SDS and denatured by heating to 90 °C for 10 min. Samples were taken for co-immunoprecipitation and immunoblot analyses.

2.7. Co-immunoprecipitation

WT Tu167 were lysed in RIPA buffer and sonicated. Protein A/G beads (Invitrogen) were incubated with 1 μg of anti-ZSCAN4 in 100 μL of RIPA buffer without SDS for 1 h at room temperature. Then, the beads were washed twice with RIPA buffer and crosslinked to the beads with 5 mM BS3 solution (ThermoFisher Scientific) according to manufacturer's protocols. Then the beads were precleared with 100 μg of nuclear lysate in 100 μL IP buffer without SDS (Cell Signaling Technology) overnight at 4 °C. Cell lysates were loaded and bound antigens were eluted with 25 μL RIPA with 0.1% SDS and 25 μL of 2x loading dye. Proteins were separated in 10% SDS PAGE and immunoblotted with corresponding antibodies.