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Snail transcription factor NLS and importin β1 regulate the subcellular localization of Cathepsin L and Cux1

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ABSTRACT

Several recent studies have highlighted an additional unexpected localization and site of action for Cathepsin L (Cat L) protease within the nucleus in breast, colon and prostate cancer, however, its role in the nucleus was unclear. It was proposed to mediate proteolytic processing of the transcription factor CCAAT-displacement protein/cut homeobox transcription factor (Cux1) from the full-length p200 isoform to generate the p110 and p90 isoforms, of which the p110 isoform was shown to act as a cell cycle regulator to accelerate entry into the S phase. The p110 isoform has also been shown to bind to the promoter regions of Snail and E-cadherin to activate Snail and inactivate E-cadherin transcription, thus promoting epithelial mesenchymal transition (EMT). Mechanistic studies on what drives Cat L nuclear localization have not been reported. Our hypothesis is that Snail shuttles into the nucleus with Cat L through binding to importin-β. Snail knockdown with siRNA in MDA-MB-468 breast cancer cells led to nuclear to cytoplasmic shuttling of Cat L and decreased levels of Cux1, while overexpression of Snail in MCF-7 breast cancer cells or HEK-293 human embryonic kidney cells led to increased nuclear expression of both Cat L and Cux1. Additionally, transient transfection of Snail NLS mutants not only abrogated Snail nuclear localization but also nuclear localization of Cat L and Cux1. Interestingly, importin β1 knockdown with siRNA decreased Snail and Cux1 levels, as well as nuclear localization of Cat L. Therefore, we show for the first time that the nuclear localization of Cat L and its substrate Cux1can be positively regulated by Snail NLS and importin β1, suggesting that Snail, Cat L and Cux1 all utilize importin β1 for nuclear import.
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1. Introduction

Many proteins targeted to the nucleus contain a classical nuclear localization signal (NLS) that is recognized by a heterodimeric import receptor comprised of importin α and importin β [1]. Through the NLS(s), the cargo protein binds to the nuclear import receptor proteins called importins (importin β1 in most cases and importin α/β1 complex in other cases). Importin β1 then binds to the nucleoporins of the nuclear pore complexes that subsequently translocate the cargo protein to the nucleus. Each protein that localizes to the nucleus must possess a functional NLS or is required to bind to cargo proteins which possess a NLS(s) [1]. Research studies have shown that importin β1 mediates the nuclear import of Snail through direct binding with its zinc finger domains [2]. More specifically, basic residues within the three zinc fingers have been shown to be required for nuclear localization of Snail [3].

Nuclear Cat L protein expression has been observed in malignant prostate, breast and colorectal cancers [4–6]. The studies were mainly reported as observations, and failed to investigate the mechanisms of Cat L localization. So far, only few substrates of nuclear Cat L have been identified, namely Cux1, the histones H1 or H3, and 53BP1 [7]. Previous reports show that the p110 isoform of Cux1 works in cooperation with Snail gene to repress the transcriptional activation of E-cadherin, while also increasing Snail transcription [8]. Our recent findings show that a positive feedback loop exists, whereby Snail transcription factor can promote Cat L expression and activity, nuclear localization of Cat L, and subsequent Cux1 cleavage, which then further promotes Snail transcription and EMT [9]. In this study, Snail overexpression led to nuclear localization of Cat L, increased degradation of its Cux1 substrate into the p110 and p90 isoforms, and increased EMT associated with increased migration and invasion [9]. However, we never addressed the mechanism of nuclear localization of Cat L. It is

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unclear how Snail would drive nuclear localization of Cat L. In this report we show that Snail NLS is required for nuclear translocation of Cat L, and interestingly, also for nuclear localization of Cux1. We further show that importin β1 knockdown prevents nuclear localization of Cat L and decreases Cux1 expression, suggesting that the same importin β1 that is well known to transport Snail, is also responsible for the transport of Cat L.

2. Materials and methods

2.1. Cell culture, reagents and antibodies

The human breast cancer cells lines, MCF-7, and MDA-MB-468, and human embryonic kidney cells, HEK-293, were obtained from ATCC, Manassas, VA. The MCF-7 cells stably transfected with empty Neo vector (MCF-7 Neo) and or constitutively active Snail (MCF-7 Snail) used for most of these studies and were generated previously [10]. Cells were grown in RPMI medium (VWR Int., West Chester, PA) supplemented with 10% fetal bovine serum (FBS, Hyclone, South Logan, UT) and 1% penicillin/streptomycin (VWR Int., West Chester, PA), at 37 °C with 5% CO_2 in a humidified incubator. Charcoal/dextran treated FBS (DCC-FBS) was from Hyclone, South Logan, UT. Anti-mouse α-tubulin antibody was from Sigma—Aldrich, St Louis, MO. Rat monoclonal anti-Snail, horseradish peroxidase (HRP)-conjugated goat anti-rat and anti-rabbit, and importin β1 antibodies were from Cell Signaling Technology, Danvers, MA. Goat monoclonal anti-Cat L antibody was purchased from R&D Systems (Minneapolis, MN). The HRP-conjugated donkey anti-goat secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated sheep anti-rabbit secondary antibody was purchased from Amersham Biosciences, Buckinghamshire, UK. Luminata Forte HRP chemiluminescence detection reagent was purchased from EMD Millipore (Billerica, MA). The protease inhibitor cocktail was from Roche Molecular Biochemicals, Indianapolis, IN.

2.2. Western blot analysis

Cells were lysed in a modified RIPA buffer as described previously (10). Supernatants were collected and quantified using a micro BCA assay (Promega, Madison, WI). 30 μg of cell lysate was resolved using 10% sodium dodecyl sulfate—polyacrylamide gel electrophoresis, followed by *trans*-blotting onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with appropriate primary and secondary antibody, followed by visualization using Luminata Forte ECL reagent. The membranes were stripped using Restore western blot stripping buffer (Pierce Biotechnology, Rockford, IL) prior to reprobing with a different antibody.

2.3. Short interfering RNA (siRNA) transfection

Transient transfections were performed with 25 nM of non-silencing ON-TARGET plus SMARTpool control siRNA (Catalog #D-001810-10), importin β1 siRNA (Catalog #L-005841-00) or Snail siRNA (Thermo Scientific - Dharmacon, Lafayette, CO) as per the manufacturer's instructions. The SMARTpool siRNA we use combines four highly potent siRNAs that target four different mRNA regions at once, and also use a dual-strand modification pattern to guarantee knockdown while reducing off-target effects. Pooling also reduces the concentration of each individual siRNA, a widely accepted strategy for reducing off-target effects; the company claims their pool siRNA reduces off-target effects by up to 90%. Briefly, MDA-MB-468, MCF-7 Neo or MCF-7-Snail cells were seeded overnight in 6-well dishes then incubated with either non-silencing control, importin β1 or Snail siRNA (25 nM) in phenol-free RPMI without FBS or antibiotics for 5 h; subsequently the media were replaced with 5% DCC phenol-free RPMI for an additional 72 h. Lysates from whole cell, nuclear and cytoplasmic extracts were harvested and quantitated for respective experiments.

2.4. Subcellular fractionation

Subcellular fractionations were performed per the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). Briefly, cells at 80–90% confluence were lysed in a series of buffers containing protease inhibitors (25X) with CERI (250 μl), CERII (11 μl), or NER (100 μl). Centrifugation steps were performed to obtain a non-nuclear fraction and an intact nuclear pellet, followed by further lysing to isolate the nuclear fraction. 30 μg of non-nuclear and nuclear fractions were utilized for Western blot analysis. Mouse *anti*-topoisomerase I (Santa Cruz Biotechnology Santa Cruz, CA) and rabbit *anti*-GAPDH antibodies (Cell Signaling Technology, Inc., Danvers, MA) were used to ensure the integrity of nuclear and cytoplasmic fractions, respectively. Rabbit *anti*-Calnexin antibody (Santa Cruz Biotechnology Santa Cruz, CA) was utilized as an added control to ensure that the nuclear fraction was pure and not contaminated with endoplasmic reticulum.

2.5. Site-directed mutagenesis

We obtained pGEX-2T Snail-GFP construct from Dr. Yoneda at Osaka University in Japan who has published with this construct [11]. K161E and R220E point mutations within the Snail NLS, were generated using the Quik Change XL Site-Directed Mutagenesis Kit (Stratagene). K161E and R220E are point mutations within the zinc finger 1 and zinc finger 3 domains which in combination with other mutations within these domains have been published to prevent nuclear localization of Snail in MCF-7 breast cancer cells [3]. The forward and reverse primers utilized were: R220E (Forward 5′-ACTGCAAATACTGCAACGAGGAATACCTCAGCCTG-3′ Reverse 3′-TGACGTTTATGACGTTGCTCCTTATGGAGTCGGAC-5′) and K161E (Forward 5′CGTGCCTTCGCTGACGAGTCCAACCTGCGGGCC-3′ Reverse3′-GCACGGAAGCGACTGCTCAGGTTGGACGCCCGG-5′). Accuracy of the mutations was confirmed by DNA sequencing on an ABI 3130 xl Gene Analyzer Sequencer at Morehouse School of Medicine, Atlanta, GA.

2.6. Immunofluorescence

5×10^3 MCF-7 or HEK-293 cells were plated overnight into 16 well chamber slides (Bio-Tek, Nunc, Winooski, VT). Subsequently, cells were subjected to transient transfection of wildtype Snail cDNA or mutated plasmids (R220E or K161E) for 48 h using lipofectamine 2000 according to manufacturer instructions. Cells were then fixed with methanol/ethanol 1:1 vol for 5 min, followed by washes with 1 × PBS and blocking with protein blocking solution without serum (Dako, Camarillo, CA) for 10 min at room temp. Subsequently, slides were incubated with 1:50 dilution of Snail, Cat L or Cux1 primary antibody in Dako antibody diluent solution for 1 h at room temp. Slides were washed with 1 × TBS-T (Dako, Camarillo, CA), then incubated with secondary antibody in the dark for 1 h at room temp. Secondary antibodies used were anti-rabbit Oregon green 488 or anti-goat Texas red (Vector Laboratories, Burlingame, CA). Slides were washed with 1 × TBS-T and double deionized water, prior to counterstaining with DAPI (1 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA). Slides were mounted using Fluorogel mounting medium (Electron Microscopy Sciences, Hatfield, PA). Immunofluorescence was also performed on 5×10^3

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