Published in final edited form as:

Biochem Biophys Res Commun. 2017 September 09; 491(1): 59–64. doi:10.1016/j.bbrc.2017.07.039.

Snail transcription factor NLS and importin β 1 regulate the subcellular localization of Cathepsin L and Cux1

Liza J. Burton, Veronica Henderson, Latiffa Liburd, and Valerie A. Odero-Marah

Center for Cancer Research and Therapeutic Development, Department of Biological Sciences, Clark Atlanta University, Atlanta, Georgia, USA

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 β 1 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 Cux1 can be positively regulated by Snail NLS and importin β 1, suggesting that Snail, Cat L and Cux1 all utilize importin β 1 for nuclear import.

Keywords

Snail; Cat L; Cux1; Importin β 1

Address correspondence to Valerie Odero-Marah, The Department of Biological Sciences, Clark Atlanta University, 223 James P Brawley Dr SW, Atlanta, GA 30314, Phone:(404)-880-6764, Fax:(404)-880-8065, voderomarah@cau.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest

The authors declare no conflict of interest.

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 β 1 in most cases and importin α/β 1 complex in other cases). Importin β 1 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 β 1 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 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 β 1 knockdown prevents nuclear localization of Cat L and decreases Cux1 expression, suggesting that the same importin β 1 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% CO₂ 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 β 1 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' - A C T G C A A A T A C T G C A A C G A G G A A T A C C T C A G C C T G - 3' Reverse 3' - T G A C G T T T A T G A C G T T G C T C C T T A T G G A G T C G G A C - 5') and K161E (Forward 5' C G T G C C T T C G C T G A C G A G T C C A A C C T G C G G G C C - 3' Reverse 3' - G C A C G G A A G C G A C T G C T C A G G T T G G A C G C C C G G - 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

5×10³ 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 volume 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 5×10³ MDA-MB-468 or MCF-Snail treated with importin β1 siRNA for 72 h, as described previously, and probed with importin β1, Snail, Cat L and Cux1 antibodies.

Fluorescence microscopy was performed using Zeiss microscope and Axiovision Rel 4.8 software.

3 Results

3.1 Snail regulates the expression and subcellular localization of Cat L and its substrate Cux1

We previously published that Snail overexpression increased nuclear localization of Cat L which was associated with increased Cux1 degradation from the p200 form to the p110 and p90 isoforms, and further increase in Snail transcription in both breast and prostate cancer [9]. We sought to investigate further Snail regulation of nuclear Cat L expression/localization. Using breast cancer cells as a model, we knocked down endogenous Snail

utilizing Snail siRNA in MDA-MB-468 cells and compared to control siRNA knockdown. Western blot analysis of whole cell lysates showed that Snail knockdown led to a decrease in Cat L and Cux1 expression (Fig. 1A). Subcellular fractionation showed that Snail knockdown in MDA-MB-468 cells led to decreased levels of Snail and Cat L in the nucleus, and higher levels within the cytoplasm, concomitant with decreased levels of nuclear Cux1 (Fig. 1B). This shows that upon Snail knockdown, the decreased levels of Cat L is now predominantly cytoplasmic, suggesting that Snail can regulate not only Cat L expression, but also its nuclear localization. Additionally, Snail appears to also regulate Cux1 expression.

3.2 Snail NLS is required for nuclear localization of Cat L and Cux1

Studies have shown that Snail transcription factor contains NLS sequences within its zinc fingers which is necessary for its nuclear localization [2, 3]. Since our observations above suggest that Snail expression may regulate Cat L nuclear localization and Cux1 expression, we examined whether Snail NLS plays a role. We mutated the lysine to glutamate in residue 161 and arginine to glutamate in residue 220 to create K161E and R220E within the NLS of Snail's zinc finger 1 and 3, respectively. Transient transfection of wild-type (WT) Snail in MCF-7 breast cancer cells led to increased nuclear/cytoplasmic Snail expression associated with increased nuclear/cytoplasmic expression of Cat L and Cux1, as observed by immunofluorescence; conversely, Cat L levels were lower and predominantly cytoplasmic, while Snail and Cux1 were undetectable in untreated (Control) MCF-7 cells (Fig. 2). Transient transfection of mutant Snail (K161E or R220E) in MCF-7 cells resulted in exclusive localization of Snail, Cat L and Cux1 within the cytoplasm (Fig. 2). Similar results were obtained when the experiment was performed in HEK-293 cells (Fig. 2). Therefore, Snail NLS is required for nuclear localization of Cat L and Cux1.

3.3 Importin β 1 regulates the subcellular localization/expression of Cat L and Cux1

Next we wanted to examine if importin β 1 which has been shown to regulate the nuclear import of Snail [2] could also mediate nuclear import of Cat L and/or Cux1. We knocked down importin β 1 in MDA-MB-468 cells and performed western blot analysis following subcellular fractionation, as well as immunofluorescence. We confirmed knockdown of importin β 1 (Fig. 3A) and observed that Snail and Cux1 protein expression decreased, while Cat L localization changed from nuclear to predominantly cytoplasmic (Fig. 3B, C) in MDA-MB-468 cells. We repeated importin β 1 knockdown in MCF-7 cells stably overexpressing Snail; interestingly, Snail overexpression increased the expression importin β 1 which could be decreased by importin β 1 siRNA (Fig. 4A). Furthermore, importin β 1 knockdown led to decreased expression of Snail, Cat L and Cux1 (Fig. 4B, C). Thus, importin β 1 may mediate not only Snail, but also Cat L and Cux1 nuclear import and expression.

4 Discussion

Snail, a zinc finger transcription factor, is a key regulator of EMT and represses transcription of the cell adhesion molecule E-cadherin while inducing transcription of mesenchymal genes. The zinc finger domain of Snail serves as its nuclear localization sequence and authorizes its movement into the nucleus [2, 3]. Importin β 1 mediates the nuclear import of

Snail through direct binding with its zinc finger domains [2]. Importins bind to exposed basic residues and in the case of Snail, have been reported to recognize a NLS within zinc fingers 1–3, constituted by pairs of basic residues K161/K170, K187/R191, and R220/R224 [3]. Mingot et al. showed that any of these residues can be mutated individually without impairing nuclear localization of Snail [3]. Our findings differ from this research by showing that single point mutations in K161 and R220 actually prevented nuclear localization of Snail in MCF-7; since this diverged from the previous study that showed double mutations were required, we sought to confirm it in another cell type, and HEK-293 cells showed similar results. We also noted that in the previous study, only a few cells were shown but our study shows a larger field of cells.

Our previously published reports show that there is a positive feedback loop between Snail-Cat L-Cux1 whereby the increase in Snail expression leads to an increase in Cat L expression within the nucleus and increased activity associated with the cleavage of Cux1 and further EMT [9]. However, the mechanism by which Cat L localizes to the nucleus has not been reported. Nuclear localization of Cat L has been associated with decreased survival in breast and colorectal cancer, and is frequently upregulated in triple negative breast cancer patients and patients with either a germline or somatic mutation in BRCA1 tumor suppressor gene [5]. This has been shown to allow resistance to radiation and genotoxic chemotherapeutics such as cisplatin, PARP inhibitors and mitomycin C [5]. By identifying transport pathways required for nuclear localization of Cat L, our project may reveal additional targets for therapeutic development to hinder cancer metastasis and improve patient survival. We show for the first time that by knocking down Snail or importin β 1, or mutating Snail NLS, we are able to relocalize Cat L from the nucleus and into the cytoplasm. This suggests that Cat L utilizes importin β 1 for import into the nucleus, which relies also on Snail NLS. Interestingly, Cux1 nuclear import also depends on Snail NLS; it is plausible that Snail, Cat L and Cux1 are all co-imported together by importin β . Indeed co-import of proteins into the nucleus has been previously described [12].

Snail overexpression increases Cux1 nuclear expression in MCF-7 cells, while Snail knockdown decreases Cux1 knockdown in MDA-MB-468 cells. It is unclear why Snail knockdown would decrease the levels of Cux1, but it is possible that Snail may indirectly increase Cux1 transcription and/or translation; it has been published that similar to Snail, Cux1 is a transcriptional target of TGF- β [13]. Another possibility is that in the absence of Snail to regulate nuclear import of Cux1, cytoplasmic Cux1 is unstable and is rapidly degraded. However, in MDA-MB-468 and MCF-7 Snail cells, we observe both nuclear and cytoplasmic Cux1, suggesting stability of Cux1 in the cytoplasm, which is lost by either Snail knockdown or importin β 1 knockdown (which also leads to decreased Snail expression), therefore the presence of Snail seems to be a stronger determinant of Cux1 expression. It is possible that when importin β 1 is knocked down preventing Snail nuclear localization, then the cytoplasmic Snail is subject to the well-known proteosomal degradation pathway mediated by GSK3 β [14]; this may subsequently lead to loss of Cux1. All these hypotheses warrant further investigation. Of note, importin β 1 knockdown led to relocalization of Cat L from the nucleus to the cytoplasm in MDA-MB-468 cells, while it led to decreased Cat L expression in MCF-7 Snail cells, which highlights cell line differences but does not detract from the overall theme. Another noteworthy observation was

that Snail overexpression increased importin $\beta 1$ expression; this supports a report that more aggressive breast cancer cells overexpress importin $\beta 1$, where it was further shown that importin $\beta 1$ silencing was more potent in malignant compared to non-transformed breast cancer cells [15]. It has also been shown that importin $\beta 1$ is overexpressed in cervical cancer and inhibition with siRNA inhibited tumor cell proliferation without affecting normal cervical epithelium [16]. More recently, a small molecule inhibitor to importin $\beta 1$ was shown to induce cell cycle arrest and apoptosis in mitotic cells without affecting microtubule polymerization [17].

Overall, we have compelling novel data indicating a role for importin $\beta 1$ in nuclear import of both Cat L and Cux1, which also requires Snail NLS suggesting possible co-import of 3 molecules (Snail, Cat L and Cux1) that are involved in the same signaling pathway that promotes EMT. Therefore, targeting either Snail or importin $\beta 1$ may target multiple proteins to prevent EMT and metastasis in future.

Acknowledgments

This work was supported by grants from the National Institutes of Health; 5F31CA200362-02-(to L.J.B), 8 G12 MD007590 and 2 P20 MD002285 (to V.O.M).

References

1. Lange A, Mills R, Lange C, Stewart M, et al. Classical nuclear localization signals: definition, function, and interaction with importin alpha. *J Biol Chem.* 2007; 282:5101–5105. [PubMed: 17170104]
2. Yamasaki H, Sekimoto T, Ohkubo T, Douchi T, Nagata Y. Zinc Finger domain of Snail Functions as a Nuclear Localization for importin-B mediated nuclear import pathway. *Genes Cell.* 2005; 10:455–464.
3. Mingot JM, Vega S, Maestro B, Sanz JM, Nieto MA. Characterization of Snail nuclear import pathways as representatives of C2H2 zinc finger transcription factors. *Journal of cell science.* 2009; 122:1452–1460. [PubMed: 19386897]
4. Burton L, Smith B, Smith B, Loyd Q, Nagappan P, McKeithen D, Wilder C, Platt M, Hudson TS, Otero-Marah V. Muscadine grape skin extract can antagonize Snail-cathepsin L-mediated invasion, migration and osteoclastogenesis in prostate and breast cancer cells. *Carcinogenesis.* 2015; 36:1019–1027. [PubMed: 26069256]
5. Gotsky DE. BRCA1 loss activates cathepsin L-mediated degradation of 53BP1 in breast cancer cells. *J Cell Bio.* 2013; 200:187–202. [PubMed: 23337117]
6. Sullivan S, Tosetto M, Devans D, Coss A, Wang L, et al. Localization of nuclear cathepsin L and its association with disease progression and poor outcome in colorectal cancer. *Int J cancer.* 2009; 125:54–61. [PubMed: 19291794]
7. Tamhane T, Illukkumbura R, Lu S, Maelandsmo G, Haugen M, Brix K. Nuclear cathepsin L activity is required for cell cycle progression of colorectal carcinoma cells. *Biochimie.* 2015
8. Keding V, Sansregret L, Harada R, et al. p110 CUX1 Homeodomain Protein Stimulates Cell Migration and Invasion in Part through a Regulatory Cascade Culminating in the Repression of E-cadherin and Occludin. *J Biol Chem.* 2009; 284:27701–27711. [PubMed: 19635798]
9. Burton LJ, Dougan J, Jones J, Smith B, Randle D, Henderson V, Otero-Marah V. Targeting the Nuclear Cathepsin L CCAAT Displacement Protein/Cut Homeobox Transcription Factor-Epithelial Mesenchymal Transition Pathway in Prostate and Breast Cancer Cells with the Z-FY-CHO Inhibitor. *Mol Cell Biol.* 2017; 37
10. Smith BN, Burton LJ, Henderson V, Randle DD, Morton DJ, Smith BA, Taliaferro-Smith L, Nagappan P, Yates C, Zayzafoon M, Chung LW, Otero-Marah VA. Snail promotes epithelial

mesenchymal transition in breast cancer cells in part via activation of nuclear ERK2. PloS one. 2014; 9:e104987. [PubMed: 25122124]

11. Yamasaki H, Sekimoto T, Ohkubo T, Douchi T, Nagata Y, Ozawa M, Yoneda Y. Zinc finger domain of Snail functions as a nuclear localization signal for importin beta-mediated nuclear import pathway. Genes Cells. 2005; 10:455–464. [PubMed: 15836774]
12. Bange G, Murat G, Sinning I, Hurt E, Kressler D. New twist to nuclear import: When two travel together. Communicative & integrative biology. 2013; 6:e24792. [PubMed: 23940825]
13. Michl P, Ramjaun AR, Pardo OE, Warne PH, Wagner M, Poulsom R, D'Arrigo C, Ryder K, Menke A, Gress T, Downward J. CUTL1 is a target of TGF(beta) signaling that enhances cancer cell motility and invasiveness. Cancer cell. 2005; 7:521–532. [PubMed: 15950902]
14. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nature cell biology. 2004; 6:931–940. [PubMed: 15448698]
15. Kuusisto HV, Jans DA. Hyper-dependence of breast cancer cell types on the nuclear transporter Importin beta1. Biochimica et biophysica acta. 2015; 1853:1870–1878. [PubMed: 25960398]
16. van der Watt PJ, Maske CP, Hendricks DT, Parker MI, Denny L, Govender D, Birrer MJ, Leaner VD. The Karyopherin proteins, Crm1 and Karyopherin beta1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. Int J Cancer. 2009; 124:1829–1840. [PubMed: 19117056]
17. Ha S, Choi J, Min NY, Lee KH, Ham SW. Inhibition of Importin beta1 With a 2-Aminothiazole Derivative Resulted in G2/M Cell-cycle Arrest and Apoptosis. Anticancer research. 2017; 37:2373–2379. [PubMed: 28476803]

Highlights

- Snail expression is associated with increased nuclear expression of Cathepsin L (Cat L) and Cux1.
- Snail NLS is required for nuclear localization of Cat L and Cux1.
- Importin β 1 is required for Snail, Cat L and Cux1 expression/nuclear localization.

Fig. 1. Snail regulates the expression and subcellular localization of Cat L and its substrate Cux1
MDA-MB-468 cells were treated with Control or Snail siRNA for 72 hrs followed by probing for Snail, Cat L and Cux1 by western blot analysis of (A) whole cell lysates or (B) cytoplasmic and nuclear fractions. Alpha tubulin (α -Tubulin) was utilized as a loading control. GAPDH and Topoisomerase I (TOPI) were used as controls for integrity of cytoplasmic and nuclear fractions, respectively. Calnexin is used as a control to ensure that the nuclear fraction is not contaminated with endoplasmic reticulum. Data are representative of at least 3 independent experiments.

Fig. 2. Snail NLS is required for nuclear localization of Cat L and Cux1

MCF7 or HEK-293 cells were transiently transfected with wild-type or mutant (K161E or R220E) Snail cDNA for 48 hrs. The cells were subsequently fixed and probed by immunofluorescence using Snail, Cat L, and Cux1 antibodies. Nuclei were counterstained with DAPI (blue). Images were taken at 40× magnification using Axiovision software 4.8.2 with a Zeiss Axio Imager.z1 fluorescence microscope. Data are representative of at least 3 independent experiments.

Fig. 3. Importin β 1 regulates the subcellular localization/expression of Cat L and Cux1 in MDA-MB-468 cells

MDA-MB-468 cells were treated with control or importin β 1 siRNA for 72 hrs. (A) Western blot analysis was performed on whole cell lysate to confirm knockdown of importin β 1 (Imp. β 1). (B) Subcellular fractionation was performed followed by western blot analysis using Snail, Cat L and Cux1 antibodies. (C) Immunofluorescence was performed using Imp. β 1, Snail, Cat L and Cux1 antibodies. Alpha tubulin (α -Tubulin) was utilized as a loading control. GAPDH and Topoisomerase I (TOPI) were used as controls for integrity of cytoplasmic and nuclear fractions, respectively. Calnexin is used as a control to ensure that the nuclear fraction is not contaminated with endoplasmic reticulum. Data are representative of at least 3 independent experiments.

Fig. 4. Importin β 1 regulates the subcellular localization/expression of Cat L and Cux1 in MCF-7 cells overexpressing Snail

MCF-7 Neo (empty vector control) or MCF-7 cells overexpressing Snail (MCF-7 Snail) were treated with control or importin β 1 siRNA for 72 hrs. (A) Western blot analysis was performed on whole cell lysate to confirm knockdown of importin β 1 (Imp. β 1). (B) Subcellular fractionation was performed followed by western blot analysis using Snail, Cat L and Cux1 antibodies. (C) Immunofluorescence was performed using Imp. β 1, Snail, Cat L and Cux1 antibodies. Alpha tubulin (α -Tubulin) was utilized as a loading control. GAPDH and Topoisomerase I (TOPI) were used as controls for integrity of cytoplasmic and nuclear fractions, respectively. Calnexin is used as a control to ensure that the nuclear fraction is not contaminated with endoplasmic reticulum. Data are representative of at least 3 independent experiments.