ORTA DOĞU TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ MÜDÜRLÜĞÜ

TEZ ŞABLONU ONAY FORMU
THESIS TEMPLATE CONFIRMATION FORM

1. Şablonda verilen yerleşim ve boşluklar değiştirilmemelidir.
2. **Jüri tarihi** Başlık Sayfası, İmza Sayfası, Abstract ve Öz'de ilgili yerlere yazılmalıdır.
3. İmza sayfasında jüri üyelerinin unvanları doğru olarak yazılmalıdır. Tüm imzalar **mavi pilot kalemle** atılmalıdır.
4. **Disiplinlerarası** programlarda görevlendirilen öğretim üyeleri için jüri üyeleri kısmında tam zamanlı olarak çalıştıkları anabilim dalı başkanlığının ismi yazılmalıdır. Örneğin: bir öğretim üyesi Biyoteknoloji programında görev yapıyor ve biyoloji bölümünde tam zamanlı çalışıyorsa, İmza sayfasına biyoloji bölümü yazılmalıdır. İstisnai olarak, disiplinler arası program başkanı ve tez danışmanı için disiplinlerarası program adı yazılmalıdır.
5. Tezin **son sayfasının sayfa** numarası Abstract ve Öz'de ilgili yerlere yazılmalıdır.
6. Bütün chapterlar, referanslar, ekler ve CV sağ sayfada başlamalıdır. Bunun için **kesmeler** kullanılmıştır. **Kesmelerin kayması** fazladan boş sayfaların oluşmasına sebep olabilir. Bu gibi durumlarda paragraf (¶) işaretine tıklayarak kesmeleri görünür hale getirin ve yerlerini **kontrol edin**.
7. Figürler ve tablolar kenar boşluklarına taşmamalıdır.
8. Şablonda yorum olarak eklenen uyarılar dikkatle okunmalı ve uygulanmalıdır.
9. Tez yazdırılmadan önce PDF olarak kaydedilmelidir. Şablonda yorum olarak eklenen uyarılar PDF dokümanında yer almamalıdır.
10. Tez taslaklarının kontrol işlemleri tamamlandığında, bu durum öğrencilere METU uzantılı öğrenci e-posta adresleri aracılığıyla duyurulacaktır.
11. Tez yazım süreci ile ilgili herhangi bir sıkıntı yaşarsanız, Sıkça Sorulan Sorular (SSS) sayfamızı ziyaret ederek yaşadığınız sıkıntıyla ilgili bir çözüm bulabilirsiniz.

1. Do not change the spacing and placement in the template.
2. Write **defense date** to the related places given on Title page, Approval page, Abstract and Öz.
3. Write the titles of the examining committee members correctly on Approval Page. **Blue ink** must be used for all signatures.
4. For faculty members working in **interdisciplinary programs**, the name of the department that they work full-time should be written on the Approval page. For example, if a faculty member staffs in the biotechnology program and works full-time in the biology department, the department of biology should be written on the approval page. Exceptionally, for the interdisciplinary program chair and your thesis supervisor, the interdisciplinary program name should be written.
5. Write **the page number of the last page** in the related places given on Abstract and Öz pages.
6. All chapters, references, appendices and CV must be started on the right page. **Section Breaks** were used for this. **Change in the placement** of section breaks can result in extra blank pages. In such cases, make the section breaks visible by clicking paragraph (¶) mark and **check their position.**
7. All figures and tables must be given inside the page. Nothing must appear in the margins.
8. All the warnings given on the comments section through the thesis template must be read and applied.
9. Save your thesis as pdf and Disable all the comments before taking the printout.
10. This will be announced to the students via their METU students e-mail addresses when the control of the thesis drafts has been completed.
11. If you have any problems with the thesis writing process, you may visit our Frequently Asked Questions (FAQ) page and find a solution to your problem.

☒ **Yukarıda bulunan tüm maddeleri okudum, anladım ve kabul ediyorum. / I have read, understand and accept all of the items above.**

Name : Ayça
Surname : Çırçır
E-Mail : ayca.circir@gmail.com
Date :
Signature : _____

SNX3 LOSS ENHANCES EGFR OVEREXPRESSION IN A MOUSE TRIPLE NEGATIVE BREAST CANCER MODEL

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

AYÇA ÇIRÇIR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOLOGY

JANUARY 2023

Approval of the thesis:

SNX3 LOSS ENHANCES EGFR OVEREXPRESSION IN A MOUSE TRIPLE NEGATIVE BREAST CANCER MODEL

submitted by **AYÇA ÇIRÇIR** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** i**n Biology, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Ayşe Gül Gözen
Head of the Department, **Biology** _____

Prof. Dr. Ayşe Elif Erson Bensan
Supervisor, **Biology, METU** _____

Examining Committee Members:

Prof. Dr. Mesut Muyan
Biology, METU _____

Prof. Dr. Ayşe Elif Erson Bensan
Biology, METU _____

Prof. Dr. Sreeparna Banerjee
Biology, METU _____

Assoc. Prof. Dr. Özlen Konu Karakayalı
Molecular Biology and Genetics, Bilkent Uni. _____

Asst. Prof. Dr. Onur Çizmecioğlu
Molecular Biology and Genetics, Bilkent Uni. _____

Date: 23.01.2023

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Last name : Ayça Çırçır

Signature :

ABSTRACT

SNX3 LOSS ENHANCES EGFR OVEREXPRESSION IN A MOUSE TRIPLE NEGATIVE BREAST CANCER MODEL

Çırçır, Ayça
Doctor of Philosophy, Biology
Supervisor : Prof. Dr. A.Elif Erson-Bensan

January 2023, 62 pages

Sorting nexin 3 (SNX3) is one of the sorting nexin family proteins and recruits the retromer complex in endosomal trafficking. SNX3 binds specifically to Phosphotidylinositol3Phosphate (PtdIns(3)P) via its Phox-domain (PX) domain but can also interact with cargo proteins which are mostly membrane receptors. Internalized receptors can be recycled back to the plasma membrane or the trans-Golgi network or degraded in lysosomes or proteosomes. In addition to the role of SNX3 in Wntless (Wls) and Transferrin receptor (Tfrc) turnover, previous work and ours demonstrated the epidermal growth factor receptor (EGFR) as a recycling cargo of SNX3. Considering the critical role of EGFR in cell survival, proliferation, invasion/migration and clonogenicity of EGFR positive triple negative breast cancers (TNBCs), we hypothesized that SNX3 might modulate EGFR levels in TNBCs and deregulation of SNX3 might result in the alteration of clinical phenotypes. To experiment with our hypothesis, we generated Snx3 knockdown mouse mammary cell line models and evaluated the alterations in the neoplastic phenotypes of the cells. Early response to Snx3 downregulation decreased the Egfr protein, whereas extended silencing of Snx3 resulted in both Egfr protein and Egfr

mRNA upregulation. Upregulation of the Egfr protein enhanced the phenotype of 4T1 cells to a more aggressive state. In summary, Snx3 levels are critical for Egfr protein levels. Our results suggest that Snx3 loss may explain Egfr overexpression cases in triple negative breast cancer patients who do not have genomic amplification.

Keywords: Sorting nexin 3, Epidermal growth factor receptor, Triple negative breast cancer, Phenotypic assay

ÖZ

SNX3 KAYBININ FARE ÜÇLÜ NEGATİF MEME KANSERİ MODELLERİNDE EGFR AŞIRI İFADESİNİ ARTIRMASI

Çırçır, Ayça
Doktora, Biyoloji
Tez Yöneticisi: Prof. Dr. A.Elif Erson-Bensan

Ocak 2023, 62 sayfa

Sorting nexin 3 (SNX3) sorting neksin ailesine ait ve retromer kompleksinin endozomda görev alımını yönlendiren bir proteindir. SNX3, PX domaini sayesinde speisifik olarak fosfotidilinositol-3-fosfata (PtdIns(3)P) bağlanır ve aynı zamanda çoğunluğunu membrane proteinlerin oluşturduğu kargo proteinlerine de bağlanır. Hücre içine alınan reseptörler, tekrar hücre membranınına dönebilir ya da trans-Golgi ağına gidebilir veya lizozom ya da proteozomda yıkıma uğrayabilir. SNX3'ün, Wntless (Wls) ve Transferin reseptörünün (Tfrc) geri dönüşümünde rol almasına ek olarak, yeni bulgular epidermal büyüme factörü reseptörünün (EGFR) de SNX3 tarafından geri dönüştürüldüğünü göstermektedir. EGFR'ın üçlü negative meme kanseri (TNBC) hücrelerinde, hücre sağkalımı, çoğalması, migrasyon/invazyonu ve coloni oluşturma kapasitesi üzerindeki kritik rolünü düşünerek, SNX3'ün üçlü negative meme kanserlerinde EGFR seviyesini düzenleyebileceğini hipotez ettik. Hipotezimizi test etmek için Snx3 susturulmuş fare meme hücre hattı oluşturduk ve fenotipik değişimleri değerlendirdik. Snx3 baskılanmasına karşı ilk yanıt olarak Egfr protein seviyesi azalırken, baskılama süresinin uzaması hem Egfr protein hem de Egfr mRNA seviyesinin artışı ile sonuçlandı. Egfr seviyesindeki düzensizliğin hücre fenotipini daha agresif olma yönünde değiştirdiği bu durum; düşük Snx3, yüksek

Egfr mRNA seviyesi olan hastaların düşük oranda nükssüz sağkalıma sahip olmalarıyla tutarlıdır. Sonuç olarak, SNX3, üçlü negative meme kanseri hücrelerinde, EGFR proteini düzenlenmesi açısından kritik bir proteindir ve EGFR tarafından kontrol edilen kanser tipleri için de önemli bir aday olabilir.

Anahtar Kelimeler: Sorting Neksin 3, Epidermal büyüme faktörü reseptörü, Üçlü negative meme kanseri, Fenotipik analiz

To all the hardships I've been through

ACKNOWLEDGMENTS

I want to express my warmest thanks to my supervisor Prof. Dr. Ayşe Elif Erson Bensan, who made this work possible. Her invaluable guidance, endless support, and academic stimulus encouraged me throughout this project's challenging stages.

I want to thank all my thesis committee members, Assoc.Prof. Dr. Özlen Konu Karakayalılar, Asst. Prof. Dr. Onur Çizmecioğlu, Dr. Begüm Akman Tuncer and especially Prof. Dr. Mesut Muyan. I will never forget his encouragement when I hesitated to move forward in my academic and personal life.

I want to thank Muyan Lab, Banerjee Lab, Terzi Lab, and Gürsel Lab for sharing their resources.

I am incredibly grateful to Prof. Dr. Özgür Şahin and Asst. Prof. Dr. Özge Akbulut Çalışkan for *in vivo* syngenic model mouse experiments.

Many thanks go to Dr. Murat Erdem for his sincere friendship, patience with me, support, and fun inside and outside the lab.

An exceptional thank is to Dr. Merve Öyken. She has always been with me since the first day I came to the lab and in very early mornings and long nights. Not only physically, but with her support, patience, lengthy discussions on problems, and all the fun, she was always nearby me.

I want to thank all previous and current members of Erson Lab for their friendships. Ela Almeriç, İrem Eroğlu, and Utku Cem Yılmaz, I especially thank you for making my most challenging last year unforgettable with your joy.

Last but not least, I would like to express my endless gratitude to my family; my mother, Lefagat Çırçır, my sister Ayşegül Çırçır and my late father, Zeki Çırçır, who are always by my side throughout my life.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

ACTB	Actin Beta
AMS	Aspirin-induced membrane compartment
BAR	Bin, amphiphysin, Rvs
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
ERBB	Epidermal growth factor receptor family
ESE	Early sorting endosome
FERM	Protein 4.1/ezrin/radixin/moesin
FHA	Forkhead associated domain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
HER	Human epidermal growth factor receptor
IP	Immunoprecipitation
KM	Kinesin motor domain
MIT	Microtubule interacting and trafficking domain
NT	Non-targeting
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDZ	Postsynaptic density 95/discs large/zonula occludens domain
PM	Plasma membrane
PtdIns(3)P or PI3P	Phosphatidyl-inositol-3-phosphate
PR	Progesterone receptor
PX	Phox (phagocyte oxidase)-homolgy

RB	Rab5-binding domain
RGS	Regulator of G-protein signaling domain
RPLP0	Ribosomal protein lateral stalk subunit P0
RTK	Receptor tyrosine kinase
shRNA	Short-hairpin RNA
siRNA	Small-interfering RNA
SNX	Sorting nexin
TetR	Tetracycline repressor
TfRC	Transferrin receptor
TGN	Trans-Golgi Network
TNBC	Triple negative breast cancer
VPS	Vacuolar protein sorting
WCL	Whole-cell lysate
WLS	Wntless
WT	Wild-type

CHAPTER 1

INTRODUCTION

1.1 Basic properties of Sorting Nexins and SNX3

The SNX (Sorting Nexin) family is made up of a wide variety of cytoplasmic and membrane-associated proteins that are involved in numerous processes of cargo endocytosis and endosome trafficking (Gallon & Cullen, 2015; Hanley & Cooper, 2020). The evolutionarily conventional 100–130 amino acid phagocyte oxidase (PHOX) homology domain (PX) is present in all members of the SNX family (SEET & HONG, 2006; Yang et al., 2019). The PX domain facilitates SNXs interaction with phosphatidylinositols, most often phosphatidylinositol 3-phosphate (PI3P) (Chandra et al., 2019). Thus, PI3P-bearing early endosomes are linked to the majority of SNXs (Gallon & Cullen, 2015). The SNX family is divided into five subfamilies based on their domains, including SNX-BAR (Bin/Amphiphysin/Rvs), SNX-PX, SNX-FERM (protein 4.1/ezrin/radixin/moesin), SNX-PXA-RGS-PXC, and additional distinct SNX subfamilies (Amatya et al., 2021; Yong et al., 2020; H. Zhang et al., 2018). Some SNXs contain these domains as well as other unique structures. Some members of subfamilies exhibit distinctive characteristics, such as the PDZ domain in the FERM subfamily (Bauler et al., 2008; Ghai et al., 2014), which is only present on SNX27 and the N-terminal transmembrane domain (PXC) predicted on SNX13/SNX14/SNX19 but not on SNX25 in the PXA-RGS subfamily, as well as the absence of the RGS domain in SXN19 (Mas et al., 2014; Paul et al., 2022; Teasdale & Collins, 2012; Vieira et al., 2021; Yang et al., 2019).

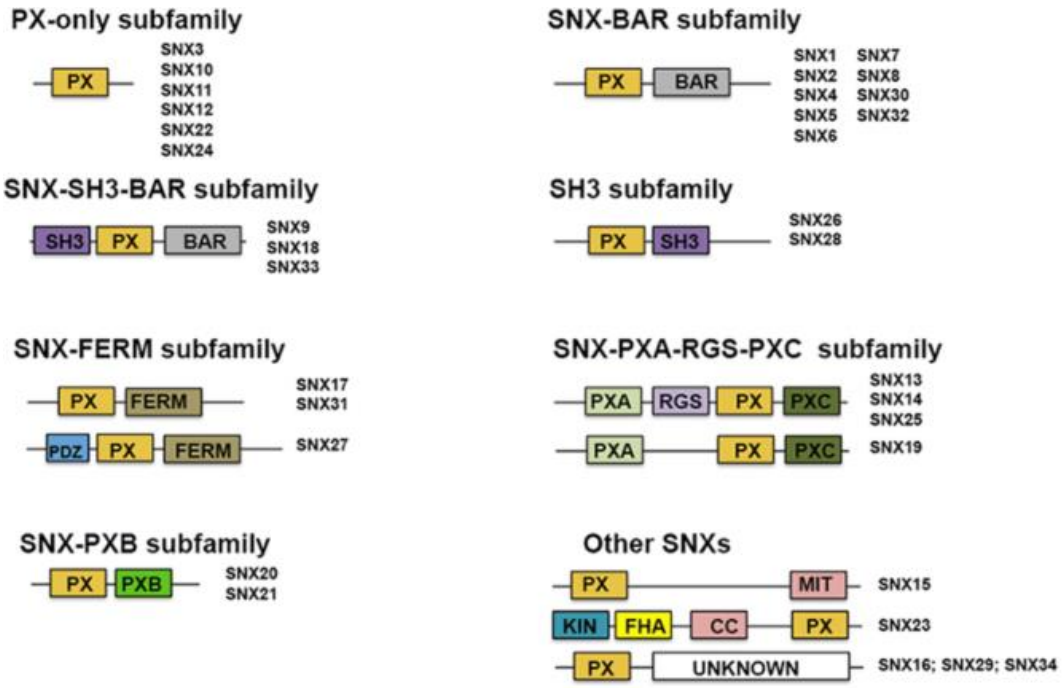


Figure 1.1. The organization of the SNX family is shown schematically based on conserved domains. All annotated members of the family contain the PX domain. The picture includes representations of additional domain abbreviations. BAR, Bin/Amphiphysin/Rvs domain; FERM, protein 4.1/ezrin/radixin/moesin domain; FHA, forkhead associated domain; KM, kinesin motor domain; MIT, microtubule interacting and trafficking domain; PDZ, postsynaptic density 95/discs large/zonula occludens domain; PX, phagocyte oxidase (phox) homology domain; PXA, PX-associated domain A; PXC, PX-associated domain C; RB, Rab5-binding domain; RGS, the regulator of G-protein signaling domain; SNX, sorting nexin (Vieira et al., 2021).

SNX proteins participate in endocytic trafficking in several aspects (Worby & Dixon, 2002). First, the most crucial physiological function of SNXs is recycling (Vieira et al., 2021). SNXs are a part of the retromer, a multi-subunit protein complex that mediates the recycling or retrograde trafficking of transmembrane proteins from endosomes to the trans-Golgi network (TGN) or back to the plasma membrane (Haft et al., 1998; Steinberg et al., 2013; Tu et al., 2020). Recycled proteins, also known

as cargo proteins, are transmembrane proteins, G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), transporters for enzymes and the Wnt sorting receptor, glucose , and metal ion transporters, and polarity proteins (Atwell et al., 2022; González-Mancha et al., 2022; Hanley & Cooper, 2020; McGough et al., 2018; Steinberg et al., 2013; Vieira et al., 2021).

The retromer complex comprises two subunits: the vacuolar protein sorting associated protein (VPS26-VPS29-VPS35) trimeric subcomplex, which interacts with and concentrates the cargo, and a membrane-associated SNX dimer, which binds to endosomal membranes and leads to coordination of cargo proteins to their destination (K.-E. Chen et al., 2021; Lucas et al., 2016).

The mammalian retromer complex has three unique forms: SNX-BAR-retromer, SNX3-retromer, and SNX27-retromer (Kvainickas et al., 2017; Yong et al., 2021). Some SNXs, including SNX1/2/5/6 of the SNX-BAR subfamily, SNX3 of the SNX-PX subfamily, and SNX27 of the SNX-FERM subfamily, have been the subject of attention due to their cargo proteins (Steinberg et al., 2013).

For instance, heterodimers of the SNX-BAR proteins, SNX1, SNX2, SNX5, and SNX6, are necessary for the retrograde transport of the cation-independent mannose 6-phosphate receptor from the endosomes to the Trans-Golgi network (TGN) (Kvainickas et al., 2017; Seaman, 2007). The Wnt-binding protein Wntless is transported from the endosomes to the TGN by the SNX3-retromer (Brown et al., 2020; Harterink et al., 2011; P. Zhang et al., 2011) and the SNX27-retromer, on the other hand, facilitates direct endosome-to-plasma membrane trafficking, bypassing the TGN, for recycling of glucose transporter type 1 (Shinde & Maddika, 2017; Steinberg et al., 2013). Without being a part of the retromer complex, SNX9 has a role in clathrin-mediated endocytosis and interacts with dynamins in clathrin-coated pits or through protein-protein interactions (Lundmark & Carlsson, 2003; Soulet et al., 2005). SNX11 facilitates the movement of TRPV3 from the plasma membrane to the lysosomes for destruction. The roles of SNX proteins are not restricted to a single direction (Li et al., 2016; Xu et al., 2020). For example, while SNX17 hinders

the lysosomal degradation of β1 integrins by attaching to its tail (Böttcher et al., 2012; Steinberg et al., 2012), it also prevents the entrance of P-selectin to the lysosome for its degradation (Knauth et al., 2005; Williams et al., 2004).

1.2 Role of SNX3 in endosomal trafficking and its targets

SNX3 plays a role in endosomal trafficking by recruiting VPS26-VPS35-VPS29 retromer trimer to early endosomes (Bean et al., 2017; Harrison et al., 2014a; Lucas et al., 2016). Early endosome is enriched with Phosphatidyl-inositol-3-phosphate (PtdIns(3)P or PI3P), and binding of SNX3 to PI3P is crucial in attracting the retromer complex to early endosome (Gu et al., 2001).

PI3P is present and enriched on the early endosomal membranes and recognized by several proteins. SNX3 and PI3P interaction is facilitated by the bilayer insertion of a proximal loop of the SNX3 PX domain. This interaction can be regulated by the phosporylation od specific serine residues on SNX3(Lenoir et al., 2018). In case of SNX3 binding to early endosmes, the retromer complex is recruited, however, dual recognition by SNX3 and RAB7 is necessary for this reqruitment. C-terminal part of VPS35 binds to SNX3 and GTP-bound RAB7 to form a binding pocket together for the other VPS proteins for cargo protein (Harrison et al., 2014b).

Among this complex of proteins, SNX3 emerges as the specificty factor for the fate of the retromer-dependent transport of Wntless, a membrane protein necessary for Wnt secretion, in worms and flies. For Wnt secretion and the development of morphogenic gradients, Wntless delivers Wnt ligands to the cell surface. The endosome-to-Golgi transport route needs SNX3-retromer to recycle endocytosed Wntless. Therefore, after internalization of surface receptor Wntless, early endosomes start sorting the cargo to TGN instead of the degradative axis if SNX3-retromer is recruited. Otherwise, in the absence of SNX3, the internalized cargo enters the degradation route in which early endosomes mature into late endosomes

and merge with a lysosome at the end (Brown et al., 2020; Harterink et al., 2011; P. Zhang et al., 2011).

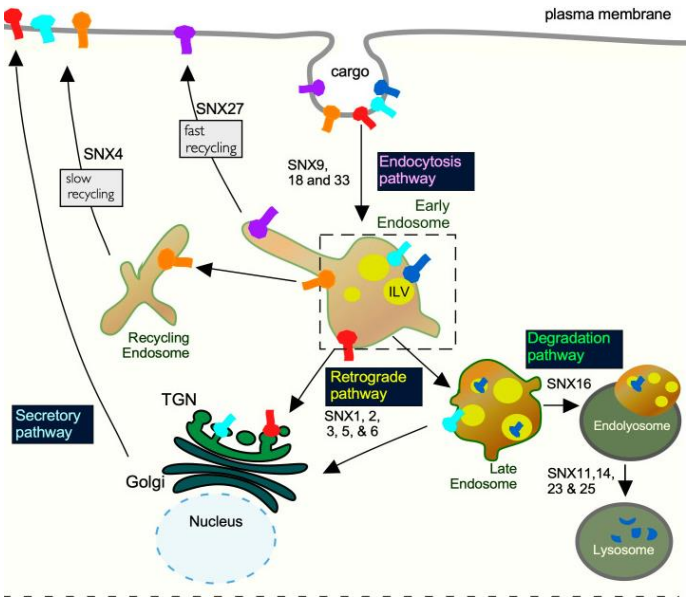


Figure 1.2. The function of sorting nexin proteins in the endocytic pathway. Internalized transmembrane proteins are transported from the plasma membrane to early endosomes. Membrane proteins must be transported retrogradely in order to be recycled back to the plasma membrane. This route requires the involvement of recycling endosomes and the trans-Golgi network. Plasma membrane proteins to be degraded are sent to late endosomes and lysosomes to generate the endolysosomes (Hanley & Cooper, 2020).

The role of SNX3 is known for the Transferrin receptor (Tfrc) turnover in which silencing of SNX3 resulted in anemia and hemoglobin defects because transferrin receptor-mediated iron absorption was hindered. The interaction of Snx3 and Vps35 seems critical for the recruitment of Tfrc to the recycling endosomes (C. Chen et al., 2013).

1.3 Epidermal Growth Factor Receptor (EGFR) and SNX3

EGFR belongs to the protein kinase superfamily and is a transmembrane glycoprotein receptor. The classic description of transmembrane receptor activity involves initial ligand binding, EGF, and other epidermal growth factor family members, followed by intracellular signaling and internalization (Oda et al., 2005; Sabbah et al., 2020). At this time, EGFR is either degraded or recycled back to the plasma membrane (Baldys & Raymond, 2009; Baumdick et al., 2015; Tomas et al., 2014). Tyrosine autophosphorylation at the cytoplasmic tail of receptor and homo or hetero dimerizations with other ERBB family receptors (ERBB2, ERBB3, ERBB4) are brought about by the protein's binding to a ligand, which activates downstream signaling pathways (Abe et al., 2006; Hajdu et al., 2020; Liu et al., 2012; Needham et al., 2016).

The SNX family member SNX1 regulates the lysosomal targeting motif in the EGFR molecule, and its overexpression promotes EGFR breakdown (Chin et al., 2001; Kurten et al., 1996; Nishimura & Itoh, 2019). In contrast, SNX3 delays EGFR degradation in the late endosomes and lysosomes. Chiow and colleagues showed that overexpressing SNX3 in A-431 cells caused endocytosed-EGFR to accumulate in the early endosomes and delay its destruction in the lysosomes (Chiow et al., 2012). In contrast, suppressing SNX3 by siRNA transfection increased the degradation of EGFR in both A-431 cells and HeLa cells (Chiow et al., 2012; Pons et al., 2008).

Hence when a cargo protein is transported from the ESE to the late endosomes and lysosomes for destruction, SNX3 seems to be crucial in negatively regulating this process by enhancing recycling.

1.4 EGFR in triple-negative breast cancers

Triple-negative breast cancer (TNBC) is characterized by the absence of expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) (Gluz et al., 2009; Yin et al., 2020). TNBC subtype is 15–20% of all breast cancer patients (Ahn et al., 2016; Santonja et al., 2018). TNBC is typically distinguished from other breast cancer subtypes by an aggressive clinical course and a poor prognosis (Ovcaricek et al., 2011; Reyes et al., 2017; Zagami & Carey, 2022). Moreover, chemotherapy remains the primary option for systemic treatment due to the absence of druggable targets, such as ER and HER2. TNBC exhibits EGFR overexpression more commonly than other breast cancer subtypes, and EGFR expression is linked to a poor prognosis for TNBCs (Masuda et al., 2012a; Nakai et al., 2016; Y. Tang et al., 2012; Zakaria et al., 2019). Due to EGFR gene amplification, protein overexpression, mutations, or in-frame deletions, EGFR signaling is commonly changed in various human malignancies (Roskoski, 2014; Uribe et al., 2021). EGFR amplification or overexpression causes a rise in EGFR density at the plasma membrane (PM), which stimulates receptor homo- and heterodimerization and activates kinases (Sigismund et al., 2018).

Last but not least, it's critical to emphasize that, in addition to oncogenic modifications, EGFR over-activation in cancer might result from impaired receptor endocytosis and trafficking (Khan & Steeg, 2021; Sigismund et al., 2018; Tomas et al., 2014) There are two ways to accomplish this: through changed endocytic/trafficking genes to increase the signal's duration and amplitude or mutant RTKs (receptor tyrosine kinase) hijacking the endocytic apparatus, fostering their signaling qualities (Sigismund et al., 2018). In fact, numerous aggressive tumors have changes in the equilibrium between receptor recycling and degradation (T. Tang et al., 2020).

1.5 Aim of the Study

Current literature and results from our laboratory showed that SNX3 facilitates the recycling of transmembrane receptors, including EGFR. However, the role of SNX3 in breast cancers in relation to EGFR is not known. In this study, we hypothesized that SNX3 might be a critical cancer-related gene, and we tested this hypothesis in a TNBC model.

CHAPTER 2

MATERIAL AND METHODS

2.1 Cell culture

4T1 (mouse breast cancer cell line) cells were cultured in DMEM high glucose (01-052-1A, Biological Industries) media with 10% fetal bovine serum (cat#181T-500, Biowest), 1% Penicillin/Streptomycin (cat#03-031-1), 1% sodium pyruvate (03-042-1B, BI) and 1% non-essential amino acids (cat#01-340-1B, BI). Cells were incubated at 37°C in a 95% humidified environment with 5% CO_2. 4T1 cells were a kind gift from Prof. Dr. Ozgur Sahin.

2.2 Plasmids

pSUPER retro.neo-GFP (a kind gift from Prof. Dr. Uygar Tazebay) was used to clone Mouse Snx3 short-hairpin RNA (shRNA)(5'GATCCCCAACTTCCTCGAGATCGACGTTCAAGAGACGTCGATCTCGAGGAAGTTTTTTTA-3') and non-targeting (NT) shRNA (5'-CGTACGCGGAATACTTCGATT-3'). The same shRNA sequences were cloned into pSUPERIOR retro.neo-GFP for inducible transfection. pCDNA6/TR vector was used to generate TetR (Tet repressor) expressing stable 4T1 cells. Top10 E.coli strain was used to amplify the vectors. Vectors were isolated using the Thermo GeneJET Plasmid Mini-Prep Kit according to the manufacturer's instructions. Maestrogen Nano was used to determine vector concentrations.

2.3 Transfections

2.3.1 Transient and stable cell line generation

4T1 cells were plated onto 6-well plates. The next day, for each well of the 6-well plate, 2.5 μg of pSUPER-Snx3sh or pSUPER-Ntsh vectors were transfected by Lipofectamine LTX and PLUS Reagent kit. 7.5 μl LTX and 2.5 μl Plus Reagent were used according to the manufacturer's instructions. Transiently transfected cells were collected at indicated times. A day after transfection, stably transfected cells' medium was replaced with a selection medium containing 350 μg/ml G418 (cat#04727878001, Merck) as the final concentration. Cells were grown with the antibiotic until untransfected cells died. Monoclonal transfectants were selected, and they were grown in a maintenance medium containing 175 μg/ml G418 as the final concentration.

2.3.2 Inducible cell line generation

4T1 cells were transfected with pcDNA6/TR Tet repressor vector, and monoclones were selected with 4 μg/ml Blasicidine S hydrochloride (cat#15205, Sigma). TetR expression of cells was controlled by RT-PCR. TetR primer pairs (F: 5'-AACAACCCGTAAACTCGCCC-3' and R: 5'-TCTCAATGGCTAAGGCGTCG-3', 101 bp product, annealing temperature 56°C) were used with cDNAs from TetR transfected cells. Next, confirmed monoclones were re-transfected with pSUPERIOR constructs, and selected clones were maintained in Tet-free FBS containing growth medium supplied with 175 μg/ml G418 and 4 μg/ml Blasticidine as the final concentration.

2.4 DNase Treatment

10 μg of isolated total RNA mixed with 10 μL 10X DNase incubation buffer, 5 μl DNase (cat#047167280-01, Merck) and reaction volume was completed to 100μL with RNase-DNase free water (cat#BI01-866-1A, BI) and incubated at 37°C for an hour in a water bath. After incubation, samples were mixed with 100μL of phenol-chloroform-isoamyl alcohol (25:24:1) and vortexed for 30 seconds, followed by a 10-minute incubation on ice. Then tubes were centrifuged at 14000 rpm for 20 minutes at 4°C. 80 μL of the upper phase of the solution was mixed with 240 μL EtOH (100%) and 8 μL of 3 M sodium acetate. After overnight incubation of samples at -20°C, they were centrifuged again under the same conditions for 30 minutes. The supernatant was discarded carefully, the RNA pellet was washed with 70% EtOH, and samples were spun at 13000 rpm for 15 minutes at 4°C. After discarding the supernatant, pellets were left to air-dry and suspended in DNase-RNase-free water. Dissolved RNAs were analyzed in terms of quality and quantity by using Maestrogen Nano. RNAs were stored at -80°C. To control whether DNase treatment completely degraded DNAs in RNA samples, Rplp0 (Ribosomal Protein Lateral Stalk Subunit P0) specific primers were used in PCR where the template was isolated RNAs. The primer couple is Rplp0_F: 5'-GAGGCCACACTGCTGAACAT-3', Rplp0_R: 5'-ATGCTGCCGTTGTCAAACAC-3' with a product size of 83 base pairs. PCR reaction conditions were as follows; 94°C for 10 min initial denaturation, 94°C for 30 sec denaturation, 60°C for 30-sec annealing, and 72°C for a 20-sec extension. The last three steps were repeated along 35 cycles, and the reaction was finalized with 72°C for a 10-minute final extension. As a positive control, gDNA was used in PCR.

2.5 cDNA Synthesis

1 μg of RNA was converted to cDNA by using RevertAid First Strand cDNA Synthesis Kit (cat#LSG-K1622, Thermo) according to the manufacturer's instructions. cDNA products were kept at -20°C.

2.6 RT-qPCR

SYBR® Green Mastermix (cat#1725121, BioRad) was used for all RT-qPCR reactions. 10 μL of total reaction volume was prepared with 300 nM of gene-specific primers. Mouse Egfr primer pairs sequences were F: 5'-GGACTGTGTCTCCTGCCAGAAT-3' and R: 5'-GGCAGACATTCTGGATGGCACT-3' with 129 bp product size. CFX Connect (BioRad) PCR cycler was used, and normalization of Egfr expression was done against Rplp0, whose primer sequences are given above. RT-qPCR reaction conditions were 95°C for 10 min initial denaturation, 40 cycles of 94°C for 15-sec denaturation, 60°C and 67°C annealing temperatures for Rplp0 and Egfr, respectively, and 72°C for a 30-sec extension. Additional steps for melt curve analysis were 95°C for 10 sec and 50°C to 99°C with 1°C increments before the plate read. According to the o ΔΔCq equation, relative quantification was calculated by following the MIQE Guidelines. Statistical analysis tests were performed by using GraphPad Prism.

2.7 Protein isolations

2.7.1 Protein isolation from tumor samples

50-100 mg tumors were taken into an Eppendorf tube and washed with 1X PBS by vortexing. Then samples were centrifuged at 13000 rpm for a minute, and PBS was discarded. 150 µl of RIPA buffer (20 mM sodium phosphate buffer, 150 mM NaCl, five mM EDTA, 1% Triton X-100, and 0.5% sodium deoxycholate added with one complete tablet of protease inhibitor (cat#1187350001, Roche) and 1 mM sodium orthovanadate (pH 7.4)) was added to each sample, and they were sonicated for 3 minutes on ice when pulser was set to 10 sec ON and 10 sec OFF. Then centrifugation was applied at 13000 rpm for 40 minutes at 4°C. Finally, the supernatant was transferred to a new tube and kept at -80°C until use. From cell lines: M-PER Mammalian Protein Extraction Reagent (cat#78501, Thermo) was used according to the manufacturer's protocol. M-PER was added with 1X PhosSTOP (cat#04906837001, Roche) and 1X Protease inhibitor (cat#1187350001, Roche) before mixing with samples. After isolation, Pierce BCA Protein Assay Kit (cat#23227, Thermo) was used to measure protein concentrations by basing on the kit manual both for cell and tumor isolates.

2.7.2 Protein isolation from cell lines

Total protein was isolated from cell lines using M-PER Mammalian Protein Extraction Reagent (78501, Thermo Scientific). Trypsinized cells were collected and washed with 1X PBS with 2000 rpm 5 min centrifugation. Pellet was suspended in M-PER, containing 1X phosSTOP (05056489001, Roche Life Science) and 1X protease inhibitor cocktail (4906845001, Roche Life Science) as final concentrations by adjusting the volume of the reagent according to pellet amount. Suspended samples were incubated on ice for 30 minutes and vortexed after every 10 minutes.

Then, samples were centrifuged at 13000 rpm for 20 minutes at 4°C. Collected supernatants were taken into clean Eppendorf tubes and stored at -80 C until use.

2.8 Protein Quantification

Pierce BCA Protein Assay Kit (23227, Thermo Fisher Scientific) was used for protein concentration determination. The kit protocol was modified as 200 µl Reagent A, and 4 µl Reagent B were mixed for each protein sample, and 5 µl of protein and 20 µl of nuclease-free water were added to the mixture in a 96-well plate. A blank sample was prepared with 25 µl of nuclease-free water without protein. Then, the plate was incubated in 37°C water bath for 30 minutes. Then the absorbance was measured at 562 nm with Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific)

2.9 Western blotting

6X Laemmli buffer (12% SDS, 30% 2-mercaptoethanol, 60% Glycerol, 0.012% Bromophenol blue, 0.375M Tris) was added to 100 µg protein isolates as 1X final concentration, and samples were boiled at 95°C for 5 minutes. Boiled samples were spun down after cooling and run onto 10% SDS-polyacrylamide gel. Then proteins were blotted onto the PVDF membrane (cat#03010040001, Roche). Depending on the primary antibody used, skim-milk (cat#170-6404, BioRad) or Bovine serum albumin (BSA-cat#11934.01, Serva) in TBS-T (20mM Tris, 137 mM NaCl, 0.1% Tween 20, pH: 7.6) were used for the blocking step. After overnight primary antibody incubation at 4°C, membranes were washed with 1% TBS-T three times for 10 minutes and incubated in appropriate HRP-conjugated secondary antibody for an hour at RT. After washing, the membranes were incubated with WesternBright ECL (cat#K12045-D50, Advansta) for 5 minutes and visualized using Chemidoc MP Imaging System (BioRad).

2.10 Proliferation and Viability Assays

2.10.1 xCELLigence Real-Time Cell Analyzer Proliferation Assay

pSUPER-NTshRNA and pSUPER-Snx3shRNA stably transfected 4T1 cells were counted with (Countess Automated Cell Counter, Thermo) and seeded at a density of 2×10^4 cells/well on E-plates placed to the RTCA plate station (xCELLigence Real-Time Cell Analyzer, ACEA Biosciences). Impedance was recorded live for 48 h with 15-min intervals.

2.10.2 MTT Metabolic Activity and Viability Assay

Counted cells were seeded at a density of 2×10^3 cells/well in three 96-well plates. After overnight growth, cells were treated with MTT (cat#M5655, Sigma) solution (0.5% in 1X PBS) as 10 μl in 100 μl growth medium. 4 hours later, 100 μl SDS-HCl solution was added (10%SDS in 0.01M HCl) and left overnight incubation. The next day, OD measurement was taken at 570 nm with a plate reader (Multiscan GO Microplate Spectrophotometer, Thermo). MTT was added to the time 0 plate right after the cell seeding to be used as seeding density normalization control.

For AG1478 (cat#1276, Tocris) treatment, cells were treated with AG1478 (15 μM) or vehicle control on the next day of plating. After 72 hours of incubation, the MTT protocol was performed.

2.11 Transwell Migration and Invasion Assays

70% confluent pSUPER-NTshRNA and pSUPER-Snx3shRNA stably transfected 4T1 cells were trypsinized and collected with 10% FBS growth medium. After centrifugation at 2000 rpm for 5 minutes, the supernatant was aspirated, and cells were washed with PBS twice and suspended in a 1% FBS-containing growth medium. Counted cells were seeded at a density of $5x10^4$ cells onto the upper

chamber of transwell inserts (cat#662638, Greiner) in a 100 µl 1% FBS growth medium. The bottom of the transwell inserts residing in the 24-well plate was filled with 600 µl 10% FBS growth medium, and cells were allowed to migrate for 8 hours at 37°C.

For invasion assay, transwell chambers were coated with 100 µl Matrigel (cat#356231, Corning) as a final concentration of 300 µg/ml and were dried for 4 hours at 37°C. Later, 8×10^4 cells were seeded per well to be incubated for 12 hours. Cell count and other plate preparation steps are the same as the migration assay except for Matrigel coating. At the end of migration and invasion durations, transwells were removed from the plate, and the inner surface of the transwell was cleaned with cotton sticks carefully not to scratch the membrane. 24-well plate wells were filled with 1mL methanol, and transwells were immersed for 10 minutes. After that, inserts were taken into Giemsa-filled wells and kept for 2 minutes. After staining, inserts were transferred to dH_2O-filled wells for washing. The inner parts of the destained transwell inserts were cleaned with a cotton stick, and the membrane was cut from the insert with a razor blade without touching the bottom surface. The cut membrane was placed onto a glass slide as the bottom surface up position. The membrane surface was covered with a tiny drop of immersion oil and covered with a coverslip by sealing the edges with colorless nail polish. Stained migrated/invaded cells were photographed under the light microscope at 10X magnification to be counted.

2.12 Colony formation assay

Stably transfected 4T1 cells were harvested and seeded on a 6-well plate as 500 colonies per well and allowed to grow for 7 days. For the inhibitor treatment experiment, seeded cells were treated with AG1478 (IC50=15 µM) or DMSO vehicle control during the same time. Cells were washed with 1X PBS and fixed with pure methanol for 20 minutes. Then, colonies were stained with crystal violet and

photographed to be counted with countPHICS (Plot HIstograms of Colony Size) software (Brzozowska et al., 2019).

CHAPTER 3

RESULTS AND DISCUSSION

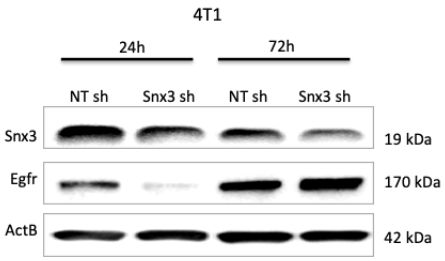
Earlier, we described 3'UTR shortening of *SNX3* upon EGF treatment in EGFR-positive triple-negative breast cancer cells. In support of the role of SNX3 in EGFR biology, Chiow et al. suggested the involvement of SNX3 in EGFR recycling in an epidermoid carcinoma cell line A-431. Our lab also generated data to show SNX3localization in early endosomes and with EGFR. In this thesis, I investigated whether SNX3 has cancer-related functions considering its link to EGFR recycling. To this end, I generated an Snx3 knock-down cell line model using the mouse 4T1 cells representing human TNBCs (Pulaski, 2001). 4T1 cells are highly tumorigenic and metastatic in mice.

3.1 Effect of Transient Silencing of Snx3 on Egfr Expression

To test the efficiency of silencing, I first performed transient transfection of an shRNA targeting Snx3 in 4T1 cells. 24- and 72-hours post-transfection, lysates were collected for western blotting. I used a non-targeting shRNA vector as a control. Figure 3.1 shows the result of the western blot and silencing of Snx3 in Snx3sh transfected cells compared to NT shRNA transfected cells.

I also checked Egfr levels as decreased Snx3 levels would cause less recycling of Egfr and hence decreased total protein levels. Egfr protein level was initially decreased when Snx3 protein level was downregulated after 24 hours of Snx3 shRNA transfection; however, at 72 hours, Egfr protein levels were higher upon Snx3 silencing. To better profile the effect of Snx3 silencing on Egfr protein levels, we moved on to an inducible system.

A.



B.

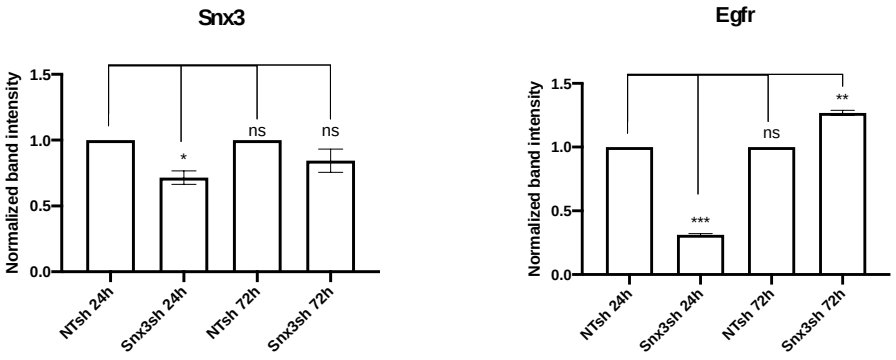


Figure 3.1. 4T1 cells were transfected transiently with pSUPER- NTshRNA and pSUPER- Snx3shRN. A. Western blot image of Snx3 and Egr protein levels. Cells were collected as indicated, and 50 μg protein was used in SDS-PAGE. ActB was used as loading control. B. Densitometry analysis of Snx3 and Egfr. t-test was performed for statistical analysis (*<0.05, **<0.01, ***< 0.001) (n=2)

3.2 Inducible Silencing of Snx3 in 4T1 Cells

For inducible silencing, 4T1 cells were first transfected with pcDNA6/TR, tetracycline repressor (TetR) expressing vector. Transfected and untransfected cells were treated with 8 μg/mL Blasticidine. I selected monoclones that grew in the presence of Blasticidin. Surviving monoclones were collected, and cDNA was synthesized after total RNA isolation. TetR specific primers were used in a colony PCR. Colonies with high expression of TetR were selected for the following experiments (Figure 3.2). Then I transfected these monoclones with pSUPERIOR-retro-neo-GFP vector with either NT or *Snx3* shRNA.

In this system, in the absence of tetracycline, shRNA expression is suppressed via TetR expression. When tetracycline is given to the cells, it blocks the repression via binding to TetR. TetR-expressing monoclones were confirmed with RT-PCR (Figure 3.2).

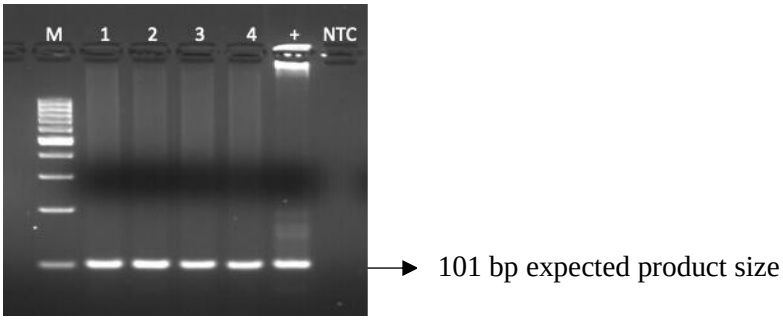


Figure 3.2. TetR expression of monoclones with RT-PCR. cDNA synthesized from isolated total RNA was used as a template in the PCR reaction. 100 bp Plus DNA ladder was used (#SM0321, Thermo) for size control, and gDNA and NTC (no template control) was used as positive and negative PCR controls, respectively.

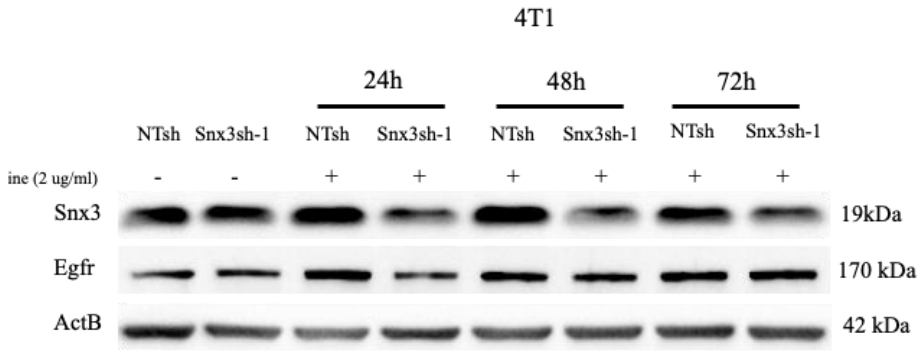
While this PCR was not quantitative, all monoclones seemed to express TetR. I chose a colony (lane 2) and transfected these TetR+ monoclonal cells with pSUPERIOR-retro-neo-GFP-NTshRNA and pSUPERIOR-retro-neo-GFP-*Snx3* shRNA inducible

shRNA vectors. 350 μg/mL G418 was used to select TetR+/shRNA+ monoclones, and these clones were maintained in 175 μg/mL G418 and 4 μg/mL Blasticidine-containing medium. Inducible monoclones were first confirmed with PCR using pSUPERIOR-retro-neo-GFP specific primers and isolated gDNA as a template.

Cells were grown in Tetracycline free FBS containing medium and induced with 2 μg/mL Tetracycline every 24 hours till 72 h. Cells were collected at each time point and used to isolate total protein. The extent of Snx3 silencing was verified by changes in protein level via Western Blot analysis (Figure 3.3, A)

In agreement with the transient transfection result, inducible knockdown of Snx3 in 4T1 cells resulted in the Egfr downregulation first, yet it was upregulated once the Snx3 silencing was extended to 72h (Figure 3.3, A and B)

A.



B.

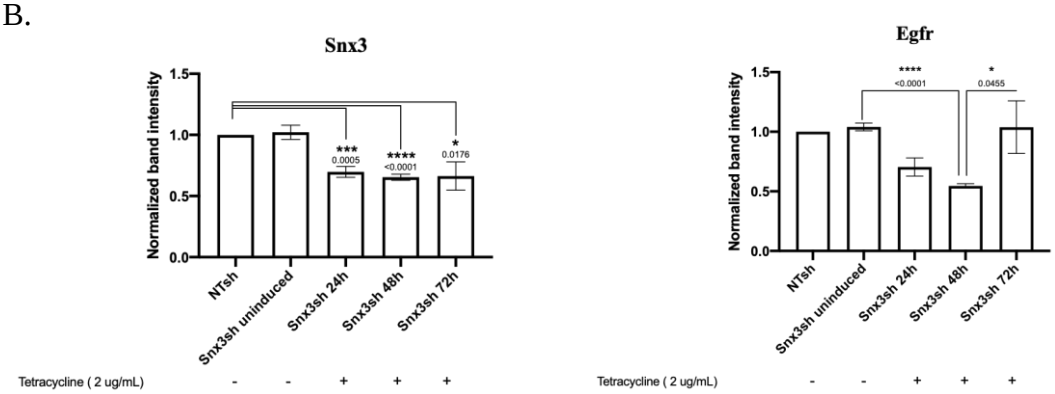


Figure 3.3. Snx3 and Egfr protein levels in an inducible silencing model. A. pSUPERIOR-retro-neo-GFP-NTshRNA and pSUPERIOR-retro-neo-GFP-Snx3 shRNA transfected 4T1 cells were induced with 2 μg/mL Tetracycline till 72 h. Cells were collected at each indicated time point, and 50 μg of total protein was loaded onto 10% SDS gel. ActB was used as a loading control. B. Densitometry analysis of a) Snx3 and b) Egfr in Western blot. t-test was applied for significance analysis (*: $p<0.05$, ***: $p<0.001$, ****: $p<0.0001$). (n=3)

3.3 Stable Silencing of Snx3 in 4T1 Cells

Next, to see the long-term effects of Snx3 silencing, I also generated a stable silencing model cell line using 4T1 cells. For this, cells were transfected with pSUPER-NTshRNA and pSUPER-Snx3shRNA vector systems. Resistant monoclones were selected with 350 μg/mL G418 both for NTshRNA and Snx3shRNA transfected cells. The extent of Snx3 silencing was verified by Western Blot analysis (Figure 3.4, A)

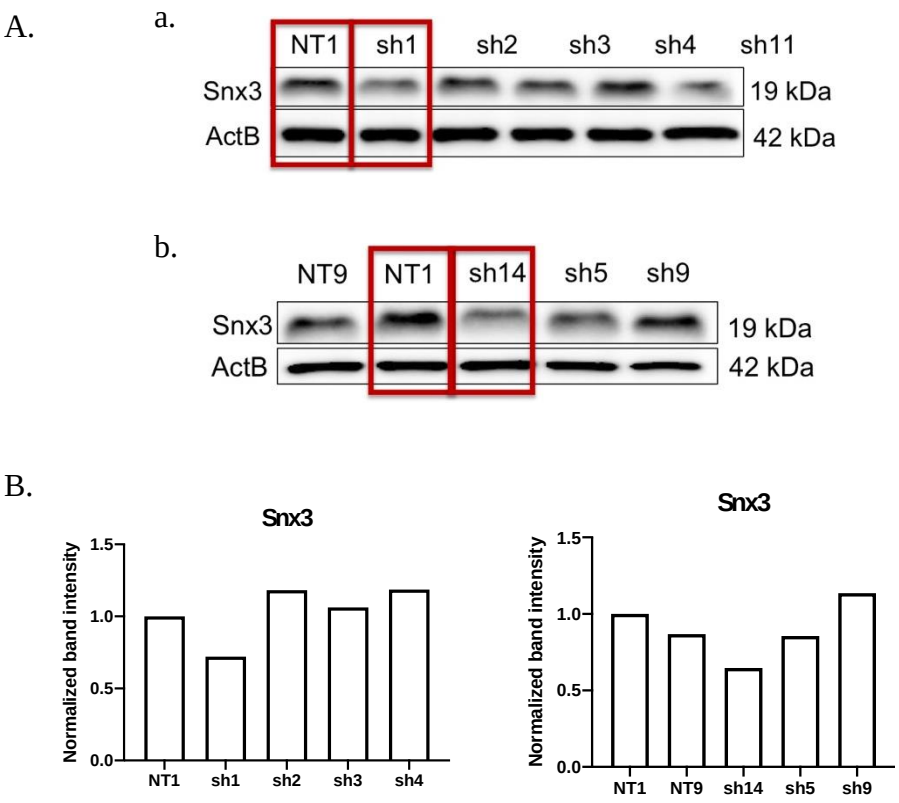
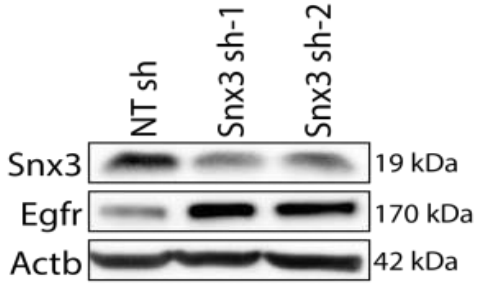


Figure 3.4. Selection of monoclones. A. 50 μg of total protein isolated from selected monoclones was used in western blot analysis. ActB was used as a protein loading control. B. Densitometry analysis of western blots. (n=1).

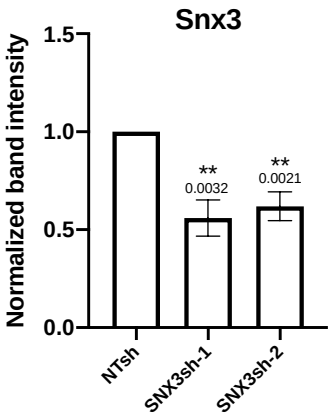
Comparison of NT1 (non-target monoclone) and sh1, sh14 (Snx3sh monoclones) showed silencing of Snx3. Thus, we continued with these monoclones in the following experiments.

In previous experiments, we observed an increased protein level of Egfr with extended Snx3 silencing. Therefore, we wanted to see how the Egfr protein level changed in response to stable Snx3 downregulation.

A.



B.



C.

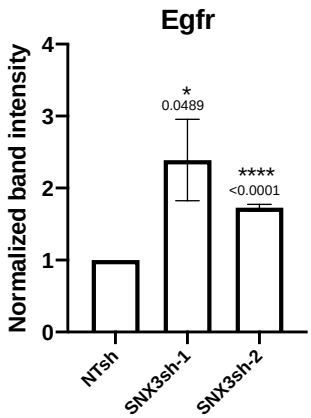


Figure 3.5. Egfr upregulation with Snx3 stable silencing. A. Total protein was isolated from 4T1 stable cells, and 50 μg of protein was loaded onto SDS-gel. ActB was used as a protein loading control. B. Snx3 and C. Egfr are densitometry analysis. The significance of the results was tested with an unpaired t-test, *:p<0.05, **:p < 0.01, ****<0.0001 (n = 3).

In agreement with the previous results, Snx3 silenced 4T1 cells had upregulation of Egfr protein level with Snx3 knockdown (Figure 3.5). Our data so far suggested that Snx3 silencing initially correlated with downregulated Egfr protein levels, but Egfr protein levels were upregulated when Snx3 silencing was extended for a longer period.

3.4 *Egfr* mRNA Level in Stably Snx3 Silenced 4T1 Cells

Next, we wanted to investigate how EGFR proteins were upregulated in Snx3 silenced cells. I looked into transcriptional regulation. I isolated RNA, synthesized cDNA, and performed RT-qPCR with stable shRNA expressing 4T1 cells.

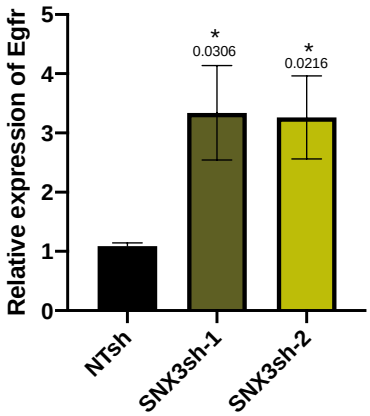


Figure 3.6. *Egfr* mRNA levels in NTsh and Snx3sh monoclonal populations, detected by RT-qPCR. The significance of the results was analyzed with an unpaired t-test,*:p < 0.05 (n = 3).

While the short-term silencing of Snx3 resulted in reduced Egfr protein levels, possibly due to decreased recycling, prolonged silencing of Snx3 led to the recovery of Egfr protein levels. These data suggested that transcriptional upregulation of *Egfr* mRNA in Snx3 silenced cells may explain the upregulation of Egfr protein levels.

3.5 Functional Assays in Stable Snx3 Silenced 4T1 models

The next step was to test the phenotypic changes in 4T1 cells with Egfr overexpression and Snx3 silencing.

3.5.1 Effect of Snx3 Silencing on 4T1 Proliferation

First, I looked into the proliferative behavior of 4T1 cells. I used both end-point and real-time analysis methods. Both the MTT Viability assay (Figure 3.7, A) and real-time monitoring with the XCelligence Proliferation assay (Figure 3.7, B) results showed an increase in cell proliferation in Egfr upregulated Snx3sh stable 4T1 cells.

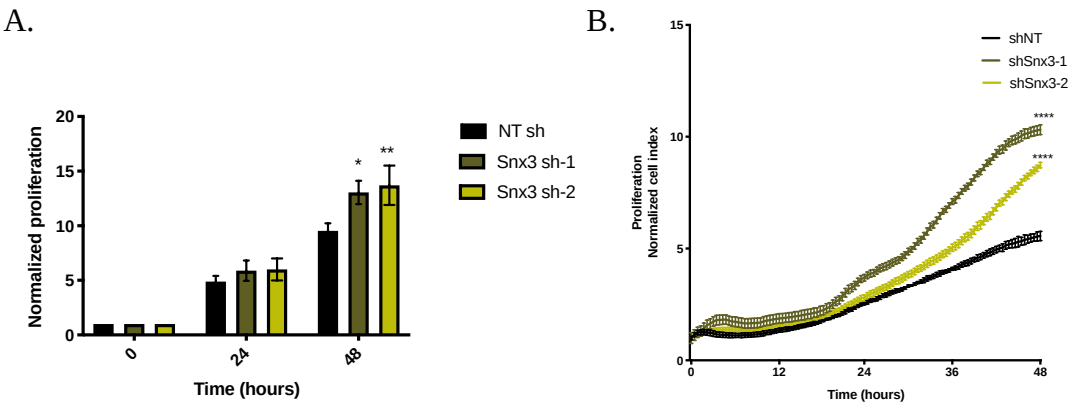


Figure 3.7. Proliferation assays.

A. pSUPER-NTshRNA and pSUPER-Snx3shRNA stably transfected 4T1 cells were seeded at a density of 2x103 in 96-well flat-bottom plates, and cell viability was analyzed for 48 hours (n=3). B. For real-time proliferation analysis, 2x104 cells/well

were plated on E-plates placed in the RTCA plate station. Impedance was monitored live for 48 hours at 15-minute intervals (n=2). Two-way ANOVA was used for significance analysis, *:p<0.05, **:p < 0.01, ****<0.0001 (n = 3).

3.5.2 Effect of Snx3 Silencing on Colony Formation Ability of 4T1 Cells

Later, I continued with the colony formation assay, which is based on the expansion capability of a single cell. The technique shows the ability of a single cell to grow into a colony, which in turn may correlate with the tumor initiation capacity of cancer cells (Franken, 2006; Gruber, 2019). 70% confluent cells were harvested and suspended in a minimum of 2 mL of medium to obtain the 500000 cells/mL, and serial dilution was applied to reach the 500 cells/mL. Then 1 mL of diluted cell suspension was added to each well. Cells were allowed to grow and form colonies for seven days. Then I fixed and dyed colonies and counted them (Figure 3.8).

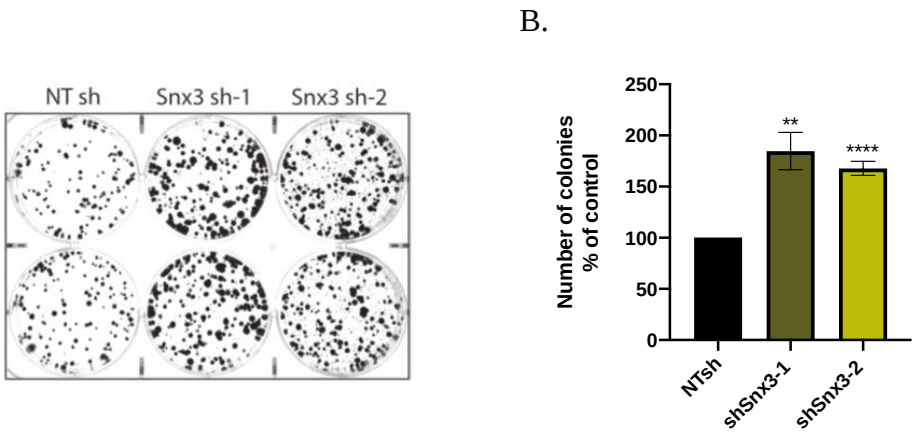


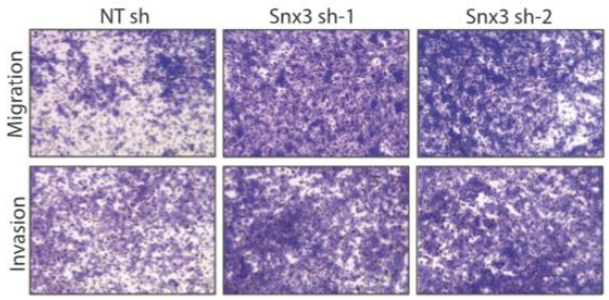
Figure 3.8. Colony formation assay. Stably transfected 4T1 cells (500 cells/well) were grown in 6-well plates for seven days. Following methanol fixation, colonies were stained with 0.5 % crystal violet. A. Representative image of colonies on a 6-well plate. B. Normalized graph of % count of colonies. The size cut-off for counted colonies is ≥ 1000. The significance of the results was analyzed with the unpaired t-test, **:p < 0.01, ****<0.0001 (n = 3).

With downregulated Snx3 and increased Egfr protein levels, we observed that the colony forming potential of shSnx3 cells increased by more than 60%. (Figure 3.8 B)

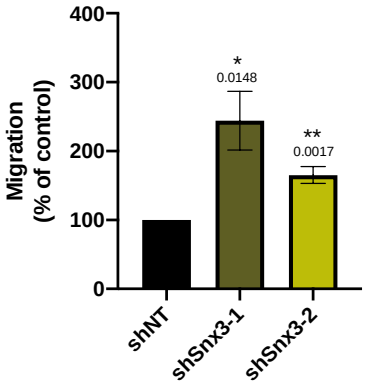
3.5.3 Effect of Snx3 Silencing on Migratory and Invasive Property of 4T1 Cells

Later, we wanted to test the migratory and invasive properties of Snx3 silenced cells. 50000 cells/well were seeded at the upper chamber of transwell plates for migration assay, and cells were allowed to migrate from low FBS to high FBS-containing medium through the chamber. For the invasion assay, chambers were coated with Matrigel to mimic the extracellular matrix, and 80000 cells per well were seeded to invade towards the high FBS side. Five random pictures were taken at 4X magnification, and cells on the membrane were counted.

A.



B.



C.

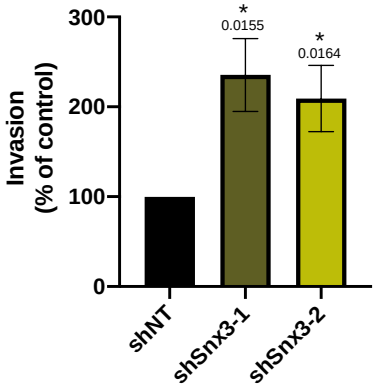


Figure 3.9. Transwell migration and invasion of 4T1 Snx3sh cells. Cells were allowed to migrate for 8 h. For invasion assay, cells were allowed to invade Matrigel-coated chambers for 12 h. A. Representative pictures were taken at 4X magnification. B. Migration and C. Invasion graphs show cell counts normalized to percent of control. Three independent experiments were analyzed with unpaired t-tests (*$p < 0.05$,**$p < 0.01$). daha cok resmin varsa koyabilirsin

Hence, stable silencing of Snx3 and overexpression of Egfr resulted in increased migration and invasion in 4T1 cells. Both shSNx3-1 and shSnx3-2 showed an almost 2-fold change in the invasion, and we observed 2-fold and 1.5-fold changes in migration for shSNx3-1 and shSnx3-2, respectively.

Overall, these data showed that the Snx3 downregulation and Egfr overexpression in 4T1 cells led to increased proliferation, colony-forming capability, and transwell migration and invasion.

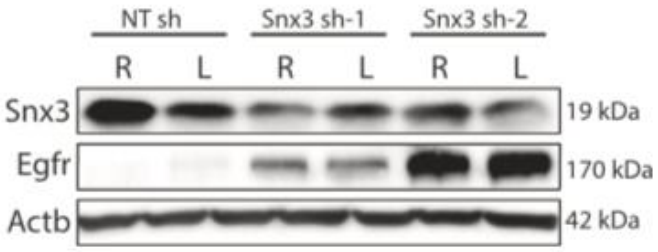
3.6 Effect of Snx3 Silencing on Tumorigenic Properties of 4T1 *in vivo*

Next, we moved on to further test whether Snx3 knockdown and elevated levels of Egfr have a role in tumor progression and metastasis *in vivo*. *In vivo* experiments were conducted by Özge Akbulut in Prof. Özgür Şahin's lab at Bilkent University. Snx3sh and NTsh 4T1 cells were injected subcutaneously into mammary fat pads (both right and left fat pads) to generate an orthotropic mouse model (BALB/c mouse).

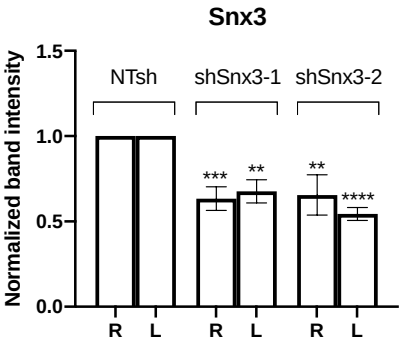
Outcomes of in vivo experiments were larger tumor formation and gross metastasis to lungs in mice with Snx3sh tumors compared to the control group of mice (Cicek E, Circir A, Oyken M, et al. 2022). Isolated tumors from sacrificed mice were taken from Dr. Sahin's lab, and I isolated proteins from the primary tumors.

I performed a western blot to test whether Snx3 silencing and Egfr overexpression status were maintained in primary tumors.

A.



B.



C.

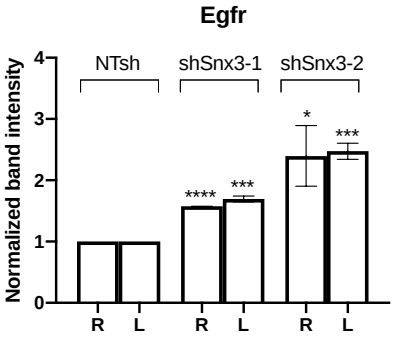


Figure 3.10. Snx3 and Egfr protein levels in collected primary tumors. A. Primary tumors were collected, and isolated proteins were used in western blot analysis. ActB was used as a sample loading control. Densitometry analysis graphs are shown in A. Snx3, B. Egfr. An unpaired t-test was used for significance analysis. *p < 0.05, **p < 0.01, ***<0.001***p < 0.0001, unpaired t-test) (R: right side tumor, L: left side tumor).

Indeed, Snx3 silencing and Egfr overexpression was maintained in tumors. Despite the fact that Snx3 silencing was less than 50%, Egfr was signficantly overexpressed.

3.7 Effect of Snx3 Silencing on Tfrc Protein Level

The next question was whether enhanced tumorigenic properties of shSNx3 cells were due to upregulated Egfr. Snx3 is a general sorting protein that may be involved in the recycling of other proteins, such as Wntless (Wls) (Harterink, 2010) or transferrin receptor (Tfrc) (Chen, 2013).

To understand if other Snx3-regulated receptor targets were affected in the same way as Egfr, we checked the transferrin receptor protein levels in both Snx3sh cell lysates and tumor lysates.

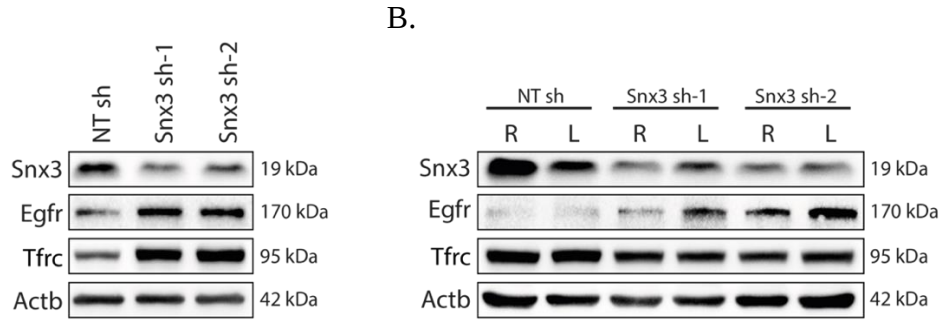


Figure 3.11. Western blot of Transferrin receptor. Total lysates of A. Snx3sh and NTsh 4T1 cells and lysates of primary tumors. The same blot was stripped and reused with all Egfr (cat#: sc-514312, SantaCruz), Tfrc (cat#: 13-6890, Thermo), and Snx3 (cat# 10772,-I-AP, Proteintech) antibodies ActB was used as a loading control (R: right side tumor, L: left side tumor)

Data showed that Snx3 silencing also caused an upregulation of Trfc in 4T1 cells. However, this upregulation was not maintained in the primary tumors. This result suggested that Trfc upregulation in the cell line model was not contributing to tumor formation in animal models.

3.8 EGFR Inhibition Impact on Cell Proliferation

Next, to further test whether the Egfr overexpression was indeed responsible for the observed phenotypes in functional assays, I used a small molecule inhibitor, AG1478, to block Egfr action. AG1478 is a specific inhibitor of EGFR, which competitively binds to the ATP binding site of EGFR and prevents tyrosine kinase activity by hindering its phosphorylation (Wang, 2018).

For proliferation and viability and colony formation assays, Snx3sh and control NTsh 4T1 cells were treated with AG1478 (IC50=15μM) for 72 hours or vehicle control DMSO.

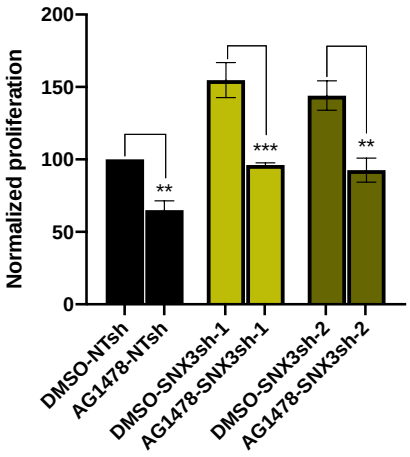


Figure 3.12. EGFR inhibition downregulates proliferation in Snx3 silenced cells. MTT proliferation assay of AG1478 treated Snx3sh cells for 72h. Three independent experiments were conducted. Results were analyzed with an unpaired t-test (**: $p<0.01$, ***$p<0.001$).

The proliferation rate of AG1478-treated SNx3sh-1 and SNx3sh-2 cells decreased approximately 0.4 fold ($p=0.0006$ and $p=0.0023$, respectively) compared to their DMSO controls.

3.9 Effect of EGFR Inhibition on Colony Formation

Consistent with the MTT result, when we performed the colony formation assay with AG1478 treatment, the elevated colony-forming potential of SNX3sh cells was decreased. Additionally, we noted a decrease in the size of the colonies (Figure 3.13).

A. B.

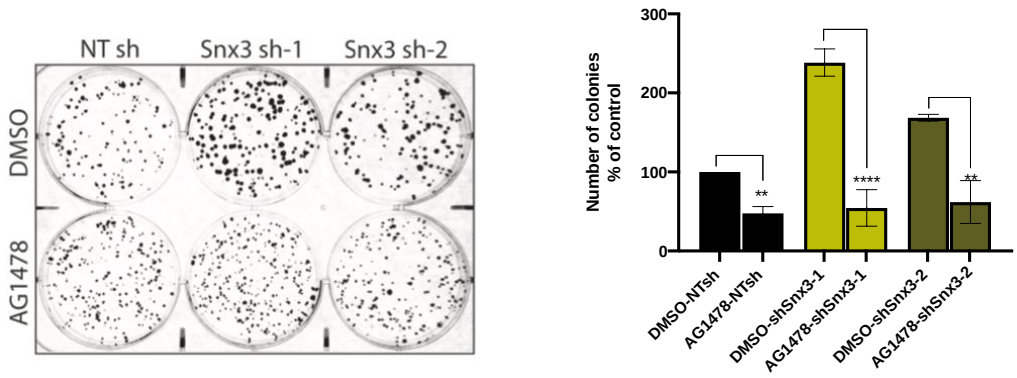


Figure 3.13. Colony formation assay with AG1478 treatment. NTsh and Snx3sh cells were treated with AG1478 or DMSO for seven days. Bars show the number of large colonies (≥1000 pixels) counted. (n = 3 independent experiments, significance was tested by unpaired t-test, (**p < 0.01, ****p < 0.0001).

The colony formation capacity of AG1478-treated SNx3sh-1 and SNx3sh-2 cells were decreased by approximately 0.8 ($p<0.0001$) and 0.6 ($p<0.0027$) fold, respectively when compared to their DMSO controls.

To conclude, blocking the EGFR kinase activity with AG1478 in SNx3sh cells decreased the proliferation rate and colony-forming potential of 4T1 cells.

Overall, these results implied that overexpression of Egfr because Snx3 knockdown triggered 4T1 cells to become more aggressive.

CHAPTER 4

CONCLUSION

The retromer complex and SNXs are critical endosomal trafficking regulators, and they primarily carry out their specialized activities by interacting with and sorting transmembrane proteins (Zhang, 2018). SNX3 is one of the nexin proteins having a role in the recycling retromer complex. Chiow et al. proposed the involvement of SNX3 in EGFR recycling in an epidermoid carcinoma cell line, suggesting a function for SNX3 in EGFR biology.

Earlier in our lab, we detected the 3'UTR shortening of *SNX3* upon EGF treatment in EGFR-positive triple-negative breast cancer cells (Akman et al., 2014). In other thesis work performed in our laboratory, we showed EGF induced upregulation of SNX3 and the close proximity of SNX3 and EGFR in early endosomes. Here, I investigated the role of Snx3 in both Egfr positive triple negative mouse breast cancer cells

Firstly, we showed that the short and long-term knockdown of Snx3 differentially affected Egfr protein levels. In Egfr-positive mouse mammary cells, the transient knockdown of Snx3 resulted in decreased levels of Egfr as an early response. However, with the prolonged silencing of Snx3, the level of Egfr protein increased. Consistent with the differential effect of Snx3 downregulation on Egfr, inducible silencing of Snx3 led to the same down and upregulation of Egfr protein level with extended Snx3 silencing. Additionally, stably silenced 4T1 cells had increased Egfr protein levels. Hence multiple lines of evidence showed that Egfr levels were sensitive to Snx3 protein levels. These findings were interesting specifically in a TNBC model as EGFR overexpression is commonly detected in this agressive subtype of breast cancers.

Oncogenic activity of EGFR is linked to poor prognosis in breast and other cancer types (Lurje & Lenz, 2009; Masuda et al., 2012b; Rakha et al., 2007). However, *EGFR* gene amplifications only partly explain EGFR overexpression in tumors. In addition to genomic copy number increases, gain of function mutations are also resposible for EGFR overactivity in certain cancer types such as non-small cell lung cancer, glioblastoma, colon adenocarcinoma and lung adenocarcinoma. (Thomas & Weihua, 2019). In breast cancers, despite the low frequency of DNA level alterations (Kim et al., 2017), EGFR overexpression is seen in approximately 50% of the triple negative breast cancer cases (Masuda et al., 2012b). Hence it is of interest to explain how EGFR protein becomes overexpressed in tumors by DNA-independent mechanisms.

As we have observed EGFR protein overexpression to develop very quickly after SNX3 down regulation,we focused on understanding why Egfr protein levels were upregulated in our models. The first mechanism we turned to was transcriptional regulation. Indeed I showed transcriptional upregulation of *Egfr* mRNA in 4T1 cells when Snx3 expression is downregulated. This transcriptional upregulation could be part of the compensatory mechanism as a response to the loss of Egfr in the short-term silencing of Snx3, potentially due to decreased recycling of Egfr. Currently it is not clear what kind of compensatory mechanisms are causing this transcriptional activation, however there is evidence for similar types of receptor overexpression cases in other contexts. For example, studies on trafficking of nicotinic acetylcholine receptors (nAChRs) show that denervation or exposure to sustained nicotine exposure increases the number of surface nAChRs in skeletal muscles. Alternatively, inhibition of proteasome activity increases the number of nAChRs on cell surface, due to increased assembly of nAChR subunits in the ER (reviewed in St john, 2009).

For EGFR there are also previous findings suggesting existence of feedback or compensatory mechanisms. For example, the impairment of entrance into lysosomal degradation pathway resulted with the increased Egfr sorting to plasma membrane (Gui et al., 2012)

Overall, it is not clear yet how and which compensatory signals rewire the transcriptional regulation of Egfr in our model, yet, there could be a feedback mechanisms in which downregulation of recycling to activate the transcriptional upregulation of *Egfr* mRNA.

The EGFR signaling pathway is critical for many cellular mechanisms. Downstream EGFR signaling regulates cell proliferation, survival, migration, invasion, and tumorigenesis (Uribe et al., 2021; Wee & Wang, 2017). Indeed our functional assays showed loss of Snx3 to enhance the oncogenic phenotype of cells by promoting cell proliferation, migration, invasion, and colony formation. these enhanced oncogenic phenotypes were reversible with the use of use of an Egfr specific inhibitor, emphasizing the role of increased Egfr protein levels. This experiment was important because Snx3 loss could have other consequences. Indeed, EGFR is not the only target for SNX3. It has a role in the turnover of varying plasma membrane receptor proteins such as Transferrin receptor. We observed that the transferrin receptor protein level was increased in Snx3 silenced 4T1 cells as well but not in primary tumor samples. Primary tumors developed in mice clearly indicated no upregulation of transferrin receptor but a signficant upregulation of Egfr protein levels. These results suggested that cells did not depend on the transferrin receptor during tumorigenesis. Moreover, AG1478 treatment in 4T1 cells was effective in inhibiting the neoplastic phenotypes. But for future work, the subsellular localization of overexpressed Egfr and the downstream signalling cascades should be characterized in Snx3 downregulated systems.

In conlusion, our results indicate a dynamic relationship between Egfr protein levels and Snx3. We were able to identify this observation through transient, inducable and long term silencing experiments. This approach clearly showed that early and late term effects of gene silencing may differ and must be monitored to get a better perspective on the dynamic regulatory events. In closing, the relationship we describe between Snx3 loss and Egfr protein upregulation may have implications for the EGFR overexpressing tumors.

REFERENCES

Abe, M., Kuroda, Y., Hirose, M., Watanabe, Y., Nakano, M., & Handa, T. (2006). Inhibition of autophosphorylation of epidermal growth factor receptor by small peptides *in vitro*. *British Journal of Pharmacology*, *147*(4), 402–411. https://doi.org/10.1038/sj.bjp.0706634

Ahn, S. G., Kim, S. J., Kim, C., & Jeong, J. (2016). Molecular Classification of Triple-Negative Breast Cancer. *Journal of Breast Cancer*, *19*(3), 223. https://doi.org/10.4048/jbc.2016.19.3.223

Akman, H., Erson-Bensan, A., B. Akman, H., & E. Erson-Bensan, A. (2014). Alternative Polyadenylation and Its Impact on Cellular Processes. *MicroRNA*, *3*(1), 2–9. https://doi.org/10.2174/2211536602666131210001152

Amatya, B., Lee, H., Asico, L. D., Konkalmatt, P., Armando, I., Felder, R. A., & Jose, P. A. (2021). SNX-PXA-RGS-PXC Subfamily of SNXs in the Regulation of Receptor-Mediated Signaling and Membrane Trafficking. *International Journal of Molecular Sciences*, *22*(5), 2319. https://doi.org/10.3390/ijms22052319

Atwell, B., Chen, C.-Y., Christofferson, M., Montfort, W. R., & Schroeder, J. (2022). Sorting nexin-dependent therapeutic targeting of oncogenic epidermal growth factor receptor. *Cancer Gene Therapy*. https://doi.org/10.1038/s41417-022-00541-7

Baldys, A., & Raymond, J. R. (2009). Critical Role of ESCRT Machinery in EGFR Recycling. *Biochemistry*, *48*(40), 9321–9323. https://doi.org/10.1021/bi900865u

Bauler, T. J., Hendriks, W. J. A. J., & King, P. D. (2008). The FERM and PDZ Domain-Containing Protein Tyrosine Phosphatases, PTPN4 and PTPN3, Are

Both Dispensable for T Cell Receptor Signal Transduction. *PLoS ONE, 3*(12), e4014. https://doi.org/10.1371/journal.pone.0004014

Baumdick, M., Brüggemann, Y., Schmick, M., Xouri, G., Sabet, O., Davis, L., Chin, J. W., & Bastiaens, P. I. H. (2015). EGF-dependent re-routing of vesicular recycling switches spontaneous phosphorylation suppression to EGFR signaling. *ELife, 4*. https://doi.org/10.7554/eLife.12223

Bean, B. D. M., Davey, M., & Conibear, E. (2017). Cargo selectivity of yeast sorting nexins. *Traffic*, *18*(2), 110–122. https://doi.org/10.1111/tra.12459

Böttcher, R. T., Stremmel, C., Meves, A., Meyer, H., Widmaier, M., Tseng, H.-Y., & Fässler, R. (2012). Sorting nexin 17 prevents lysosomal degradation of β1 integrins by binding to the β1-integrin tail. *Nature Cell Biology*, *14*(6), 584–592. https://doi.org/10.1038/ncb2501

Brown, H. M., Murray, S. A., Northrup, H., Au, K. S., & Niswander, L. A. (2020). *Snx3* is important for mammalian neural tube closure via its role in canonical and non-canonical WNT signaling. *Development, 147*(22). https://doi.org/10.1242/dev.192518

Brzozowska, B., Gałecki, M., Tartas, A., Ginter, J., Kaźmierczak, U., & Lundholm, L. (2019). Freeware tool for analysing numbers and sizes of cell colonies. *Radiation and Environmental Biophysics*, *58*(1), 109–117. https://doi.org/10.1007/s00411-018-00772-z

Chandra, M., Chin, Y. K.-Y., Mas, C., Feathers, J. R., Paul, B., Datta, S., Chen, K.-E., Jia, X., Yang, Z., Norwood, S. J., Mohanty, B., Bugarcic, A., Teasdale, R. D., Henne, W. M., Mobli, M., & Collins, B. M. (2019). Classification of the human phox homology (PX) domains based on their phosphoinositide binding specificities. *Nature Communications*, *10*(1), 1528. https://doi.org/10.1038/s41467-019-09355-y

Chen, C., Garcia-Santos, D., Ishikawa, Y., Seguin, A., Li, L., Fegan, K. H., Hildick-Smith, G. J., Shah, D. I., Cooney, J. D., Chen, W., King, M. J., Yien,

Y. Y., Schultz, I. J., Anderson, H., Dalton, A. J., Freedman, M. L., Kingsley, P. D., Palis, J., Hattangadi, S. M., … Paw, B. H. (2013). Snx3 Regulates Recycling of the Transferrin Receptor and Iron Assimilation. *Cell Metabolism*, *17*(3), 343–352. https://doi.org/10.1016/j.cmet.2013.01.013

Chen, K.-E., Guo, Q., Hill, T. A., Cui, Y., Kendall, A. K., Yang, Z., Hall, R. J., Healy, M. D., Sacharz, J., Norwood, S. J., Fonseka, S., Xie, B., Reid, R. C., Leneva, N., Parton, R. G., Ghai, R., Stroud, D. A., Fairlie, D. P., Suga, H., … Collins, B. M. (2021). De novo macrocyclic peptides for inhibiting, stabilizing, and probing the function of the retromer endosomal trafficking complex. *Science Advances*, *7*(49). https://doi.org/10.1126/sciadv.abg4007

Chin, L.-S., Raynor, M. C., Wei, X., Chen, H.-Q., & Li, L. (2001). Hrs Interacts with Sorting Nexin 1 and Regulates Degradation of Epidermal Growth Factor Receptor. *Journal of Biological Chemistry*, *276*(10), 7069–7078. https://doi.org/10.1074/jbc.M004129200

Chiow, K. H., Tan, Y., Chua, R. Y., Huang, D., Ng, M. L. M., Torta, F., Wenk, M. R., & Wong, S. H. (2012). SNX3-dependent regulation of epidermal growth factor receptor (EGFR) trafficking and degradation by aspirin in epidermoid carcinoma (A-431) cells. *Cellular and Molecular Life Sciences*, *69*(9), 1505–1521. https://doi.org/10.1007/s00018-011-0887-z

Gallon, M., & Cullen, P. J. (2015). Retromer and sorting nexins in endosomal sorting. *Biochemical Society Transactions*, *43*(1), 33–47. https://doi.org/10.1042/BST20140290

Ghai, R., Tello-Lafoz, M., Norwood, S. J., Yang, Z., Clairfeuille, T., Teasdale, R. D., Mérida, I., & Collins, B. M. (2014). Phosphoinositide binding by the SNX27 FERM domain regulates localisation at the immune synapse of activated T-cells. *Journal of Cell Science*. https://doi.org/10.1242/jcs.158204

Gluz, O., Liedtke, C., Gottschalk, N., Pusztai, L., Nitz, U., & Harbeck, N. (2009). Triple-negative breast cancer—current status and future directions. *Annals of Oncology*, *20*(12), 1913–1927. https://doi.org/10.1093/annonc/mdp492

González-Mancha, N., Rodríguez-Rodríguez, C., Alcover, A., & Merida, I. (2022). Sorting Nexin 27 Enables MTOC and Secretory Machinery Translocation to the Immune Synapse. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.814570

Gu, F., Crump, C. M., & Thomas, G. (2001). Trans-Golgi network sorting. *Cellular and Molecular Life Sciences*, *58*(8), 1067–1084. https://doi.org/10.1007/PL00000922

Gui, A., Kobayashi, A., Motoyama, H., Kitazawa, M., Takeoka, M., & Miyagawa, S. (2012). Impaired degradation followed by enhanced recycling of epidermal growth factor receptor caused by hypo-phosphorylation of tyrosine 1045 in RBE cells. *BMC Cancer*, *12*(1), 179. https://doi.org/10.1186/1471-2407-12-179

Haft, C. R., de la Luz Sierra, M., Barr, V. A., Haft, D. H., & Taylor, S. I. (1998). Identification of a Family of Sorting Nexin Molecules and Characterization of Their Association with Receptors. *Molecular and Cellular Biology*, *18*(12), 7278–7287. https://doi.org/10.1128/MCB.18.12.7278

Hajdu, T., Váradi, T., Rebenku, I., Kovács, T., Szöllösi, J., & Nagy, P. (2020). Comprehensive Model for Epidermal Growth Factor Receptor Ligand Binding Involving Conformational States of the Extracellular and the Kinase Domains. *Frontiers in Cell and Developmental Biology*, *8*. https://doi.org/10.3389/fcell.2020.00776

Hanley, S. E., & Cooper, K. F. (2020). Sorting Nexins in Protein Homeostasis. *Cells*, *10*(1), 17. https://doi.org/10.3390/cells10010017

Harrison, M. S., Hung, C.-S., Liu, T., Christiano, R., Walther, T. C., & Burd, C. G. (2014a). A mechanism for retromer endosomal coat complex assembly with

cargo. *Proceedings of the National Academy of Sciences*, *111*(1), 267–272. https://doi.org/10.1073/pnas.1316482111

Harrison, M. S., Hung, C.-S., Liu, T., Christiano, R., Walther, T. C., & Burd, C. G. (2014b). A mechanism for retromer endosomal coat complex assembly with cargo. *Proceedings of the National Academy of Sciences*, *111*(1), 267–272. https://doi.org/10.1073/pnas.1316482111

Harterink, M., Port, F., Lorenowicz, M. J., McGough, I. J., Silhankova, M., Betist, M. C., van Weering, J. R. T., van Heesbeen, R. G. H. P., Middelkoop, T. C., Basler, K., Cullen, P. J., & Korswagen, H. C. (2011). A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion. *Nature Cell Biology*, *13*(8), 914–923. https://doi.org/10.1038/ncb2281

Khan, I., & Steeg, P. S. (2021). Endocytosis: a pivotal pathway for regulating metastasis. *British Journal of Cancer*, *124*(1), 66–75. https://doi.org/10.1038/s41416-020-01179-8

Kim, A., Jang, M. H., Lee, S. J., & Bae, Y. K. (2017). Mutations of the Epidermal Growth Factor Receptor Gene in Triple-Negative Breast Cancer. *Journal of Breast Cancer*, *20*(2), 150. https://doi.org/10.4048/jbc.2017.20.2.150

Knauth, P., Schlüter, T., Czubayko, M., Kirsch, C., Florian, V., Schreckenberger, S., Hahn, H., & Bohnensack, R. (2005). Functions of Sorting Nexin 17 Domains and Recognition Motif for P-selectin Trafficking. *Journal of Molecular Biology*, *347*(4), 813–825. https://doi.org/10.1016/j.jmb.2005.02.004

Kurten, R. C., Cadena, D. L., & Gill, G. N. (1996). Enhanced Degradation of EGF Receptors by a Sorting Nexin, SNX1. *Science*, *272*(5264), 1008–1010. https://doi.org/10.1126/science.272.5264.1008

Kvainickas, A., Jimenez-Orgaz, A., Nägele, H., Hu, Z., Dengjel, J., & Steinberg, F. (2017). Cargo-selective SNX-BAR proteins mediate retromer trimer

independent retrograde transport. *Journal of Cell Biology*, *216*(11), 3677–3693. https://doi.org/10.1083/jcb.201702137

Lenoir, M., Ustunel, C., Rajesh, S., Kaur, J., Moreau, D., Gruenberg, J., & Overduin, M. (2018). Phosphorylation of conserved phosphoinositide binding pocket regulates sorting nexin membrane targeting. *Nature Communications*, *9*(1), 993. https://doi.org/10.1038/s41467-018-03370-1

Li, C., Ma, W., Yin, S., Liang, X., Shu, X., Pei, D., Egan, T. M., Huang, J., Pan, A., & Li, Z. (2016). Sorting Nexin 11 Regulates Lysosomal Degradation of Plasma Membrane TRPV3. *Traffic*, *17*(5), 500–514. https://doi.org/10.1111/tra.12379

Liu, P., Cleveland, T. E., Bouyain, S., Byrne, P. O., Longo, P. A., & Leahy, D. J. (2012). A single ligand is sufficient to activate EGFR dimers. *Proceedings of the National Academy of Sciences*, *109*(27), 10861–10866. https://doi.org/10.1073/pnas.1201114109

Lucas, M., Gershlick, D. C., Vidaurrazaga, A., Rojas, A. L., Bonifacino, J. S., & Hierro, A. (2016). Structural Mechanism for Cargo Recognition by the Retromer Complex. *Cell*, *167*(6), 1623-1635.e14. https://doi.org/10.1016/j.cell.2016.10.056

Lundmark, R., & Carlsson, S. R. (2003). Sorting Nexin 9 Participates in Clathrin-mediated Endocytosis through Interactions with the Core Components. *Journal of Biological Chemistry*, *278*(47), 46772–46781. https://doi.org/10.1074/jbc.M307334200

Lurje, G., & Lenz, H.-J. (2009). EGFR Signaling and Drug Discovery. *Oncology*, *77*(6), 400–410. https://doi.org/10.1159/000279388

Mas, C., Norwood, S. J., Bugarcic, A., Kinna, G., Leneva, N., Kovtun, O., Ghai, R., Yanez, L. E. O., Davis, J. L., Teasdale, R. D., & Collins, B. M. (2014). Structural Basis for Different Phosphoinositide Specificities of the PX Domains of Sorting Nexins Regulating G-protein Signaling. *Journal of*

Biological Chemistry, *289*(41), 28554–28568. https://doi.org/10.1074/jbc.M114.595959

Masuda, H., Zhang, D., Bartholomeusz, C., Doihara, H., Hortobagyi, G. N., & Ueno, N. T. (2012a). Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Research and Treatment*, *136*(2), 331–345. https://doi.org/10.1007/s10549-012-2289-9

Masuda, H., Zhang, D., Bartholomeusz, C., Doihara, H., Hortobagyi, G. N., & Ueno, N. T. (2012b). Role of epidermal growth factor receptor in breast cancer. In *Breast Cancer Research and Treatment* (Vol. 136, Issue 2, pp. 331–345). https://doi.org/10.1007/s10549-012-2289-9

Masuda, H., Zhang, D., Bartholomeusz, C., Doihara, H., Hortobagyi, G. N., & Ueno, N. T. (2012c). Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Research and Treatment*, *136*(2), 331–345. https://doi.org/10.1007/s10549-012-2289-9

McGough, I. J., de Groot, R. E. A., Jellett, A. P., Betist, M. C., Varandas, K. C., Danson, C. M., Heesom, K. J., Korswagen, H. C., & Cullen, P. J. (2018). SNX3-retromer requires an evolutionary conserved MON2:DOPEY2:ATP9A complex to mediate Wntless sorting and Wnt secretion. *Nature Communications*, *9*(1), 3737. https://doi.org/10.1038/s41467-018-06114-3

Nakai, K., Hung, M.-C., & Yamaguchi, H. (2016). A perspective on anti-EGFR therapies targeting triple-negative breast cancer. *American Journal of Cancer Research*, *6*(8), 1609–1623.

Needham, S. R., Roberts, S. K., Arkhipov, A., Mysore, V. P., Tynan, C. J., Zanetti-Domingues, L. C., Kim, E. T., Losasso, V., Korovesis, D., Hirsch, M., Rolfe, D. J., Clarke, D. T., Winn, M. D., Lajevardipour, A., Clayton, A. H. A., Pike, L. J., Perani, M., Parker, P. J., Shan, Y., … Martin-Fernandez, M. L. (2016). EGFR oligomerization organizes kinase-active dimers into competent

signalling platforms. *Nature Communications*, *7*(1), 13307. https://doi.org/10.1038/ncomms13307

Nishimura, Y., & Itoh, K. (2019). Involvement of SNX1 in regulating EGFR endocytosis in a gefitinib-resistant NSCLC cell lines. *Cancer Drug Resistance*. https://doi.org/10.20517/cdr.2019.15

Oda, K., Matsuoka, Y., Funahashi, A., & Kitano, H. (2005). A comprehensive pathway map of epidermal growth factor receptor signaling. *Molecular Systems Biology*, *1*(1). https://doi.org/10.1038/msb4100014

Ovcaricek, T., Frkovic, S., Matos, E., Mozina, B., & Borstnar, S. (2011). Triple negative breast cancer - prognostic factors and survival. *Radiology and Oncology*, *45*(1). https://doi.org/10.2478/v10019-010-0054-4

Paul, B., Weeratunga, S., Tillu, V. A., Hariri, H., Henne, W. M., & Collins, B. M. (2022). Structural Predictions of the SNX-RGS Proteins Suggest They Belong to a New Class of Lipid Transfer Proteins. *Frontiers in Cell and Developmental Biology*, *10*. https://doi.org/10.3389/fcell.2022.826688

Pons, V., Luyet, P. P., Morel, E., Abrami, L., Van Der Goot, F. G., Parton, R. G., & Gruenberg, J. (2008). Hrs and SNX3 functions in sorting and membrane invagination within multivesicular bodies. *PLoS Biology*, *6*(9), 1942–1956. https://doi.org/10.1371/journal.pbio.0060214

Rakha, E. A., El-Sayed, M. E., Green, A. R., Lee, A. H. S., Robertson, J. F., & Ellis, I. O. (2007). Prognostic markers in triple-negative breast cancer. *Cancer*, *109*(1), 25–32. https://doi.org/10.1002/cncr.22381

Reyes, M. E., Fujii, T., Branstetter, D., Krishnamurthy, S., Masuda, H., Wang, X., Reuben, J. M., Woodward, W. A., Edwards, B. J., Hortobagyi, G. N., Tripathy, D., Dougall, W. C., Eckhardt, B. L., & Ueno, N. T. (2017). Poor prognosis of patients with triple-negative breast cancer can be stratified by RANK and RANKL dual expression. *Breast Cancer Research and Treatment*, *164*(1), 57–67. https://doi.org/10.1007/s10549-017-4233-5

Roskoski, R. (2014). The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacological Research, 79*, 34–74. https://doi.org/10.1016/j.phrs.2013.11.002

Sabbah, D. A., Hajjo, R., & Sweidan, K. (2020). Review on Epidermal Growth Factor Receptor (EGFR) Structure, Signaling Pathways, Interactions, and Recent Updates of EGFR Inhibitors. *Current Topics in Medicinal Chemistry*, *20*(10), 815–834. https://doi.org/10.2174/1568026620666200303123102

Santonja, A., Sánchez-Muñoz, A., Lluch, A., Chica-Parrado, M. R., Albanell, J., Chacón, J. I., Antolín, S., Jerez, J. M., de la Haba, J., de Luque, V., Sousa, C. E. F.-D., Vicioso, L., Plata, Y., Ramírez-Tortosa, C. L., Álvarez, M., Llácer, C., Zarcos-Pedrinaci, I., Carrasco, E., Caballero, R., … Alba, E. (2018). Triple negative breast cancer subtypes and pathologic complete response rate to neoadjuvant chemotherapy. *Oncotarget*, *9*(41), 26406–26416. https://doi.org/10.18632/oncotarget.25413

Seaman, M. N. J. (2007). Identification of a novel conserved sorting motif required for retromer-mediated endosome-to-TGN retrieval. *Journal of Cell Science*, *120*(14), 2378–2389. https://doi.org/10.1242/jcs.009654

SEET, L., & HONG, W. (2006). The Phox (PX) domain proteins and membrane traffic. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1761*(8), 878–896. https://doi.org/10.1016/j.bbalip.2006.04.011

Shinde, S. R., & Maddika, S. (2017). PTEN Regulates Glucose Transporter Recycling by Impairing SNX27 Retromer Assembly. *Cell Reports*, *21*(6), 1655–1666. https://doi.org/10.1016/j.celrep.2017.10.053

Sigismund, S., Avanzato, D., & Lanzetti, L. (2018). Emerging functions of the <scp>EGFR</scp> in cancer. *Molecular Oncology*, *12*(1), 3–20. https://doi.org/10.1002/1878-0261.12155

Soulet, F., Yarar, D., Leonard, M., & Schmid, S. L. (2005). SNX9 Regulates Dynamin Assembly and Is Required for Efficient Clathrin-mediated

Endocytosis. *Molecular Biology of the Cell*, *16*(4), 2058–2067. https://doi.org/10.1091/mbc.e04-11-1016

St john, P. A. (2009). Cellular trafficking of nicotinic acetylcholine receptors. *Acta Pharmacologica Sinica*, *30*(6), 656–662. https://doi.org/10.1038/aps.2009.76

Steinberg, F., Gallon, M., Winfield, M., Thomas, E. C., Bell, A. J., Heesom, K. J., Tavaré, J. M., & Cullen, P. J. (2013). A global analysis of SNX27–retromer assembly and cargo specificity reveals a function in glucose and metal ion transport. *Nature Cell Biology*, *15*(5), 461–471. https://doi.org/10.1038/ncb2721

Steinberg, F., Heesom, K. J., Bass, M. D., & Cullen, P. J. (2012). SNX17 protects integrins from degradation by sorting between lysosomal and recycling pathways. *Journal of Cell Biology*, *197*(2), 219–230. https://doi.org/10.1083/jcb.201111121

Tang, T., Yang, Z., Wang, D., Yang, X., Wang, J., Li, L., Wen, Q., Gao, L., Bian, X., & Yu, S. (2020). The role of lysosomes in cancer development and progression. *Cell & Bioscience*, *10*(1), 131. https://doi.org/10.1186/s13578-020-00489-x

Tang, Y., Zhu, L., Li, Y., Ji, J., Li, J., Yuan, F., Wang, D., Chen, W., Huang, O., Chen, X., Wu, J., Shen, K., Loo, W. T. Y., & Chow, L. W. C. (2012). Overexpression of epithelial growth factor receptor (EGFR) predicts better response to neo-adjuvant chemotherapy in patients with triple-negative breast cancer. *Journal of Translational Medicine*, *10*(S1), S4. https://doi.org/10.1186/1479-5876-10-S1-S4

Teasdale, R. D., & Collins, B. M. (2012). Insights into the PX (phox-homology) domain and SNX (sorting nexin) protein families: structures, functions and roles in disease. *Biochemical Journal*, *441*(1), 39–59. https://doi.org/10.1042/BJ20111226

Thomas, R., & Weihua, Z. (2019). Rethink of EGFR in Cancer With Its Kinase Independent Function on Board. *Frontiers in Oncology*, *9*. https://doi.org/10.3389/fonc.2019.00800

Tomas, A., Futter, C. E., & Eden, E. R. (2014). EGF receptor trafficking: consequences for signaling and cancer. *Trends in Cell Biology*, *24*(1), 26–34. https://doi.org/10.1016/j.tcb.2013.11.002

Tu, Y., Zhao, L., Billadeau, D. D., & Jia, D. (2020). Endosome-to-TGN Trafficking: Organelle-Vesicle and Organelle-Organelle Interactions. *Frontiers in Cell and Developmental Biology*, *8*. https://doi.org/10.3389/fcell.2020.00163

Uribe, M. L., Marrocco, I., & Yarden, Y. (2021). EGFR in Cancer: Signaling Mechanisms, Drugs, and Acquired Resistance. *Cancers*, *13*(11), 2748. https://doi.org/10.3390/cancers13112748

Vieira, N., Rito, T., Correia-Neves, M., & Sousa, N. (2021). Sorting Out Sorting Nexins Functions in the Nervous System in Health and Disease. *Molecular Neurobiology*, *58*(8), 4070–4106. https://doi.org/10.1007/s12035-021-02388-9

Wee, P., & Wang, Z. (2017). Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers*, *9*(5), 52. https://doi.org/10.3390/cancers9050052

Williams, R., Schlüter, T., Roberts, M. S., Knauth, P., Bohnensack, R., & Cutler, D. F. (2004). Sorting Nexin 17 Accelerates Internalization Yet Retards Degradation of P-selectin. *Molecular Biology of the Cell*, *15*(7), 3095–3105. https://doi.org/10.1091/mbc.e04-02-0143

Worby, C. A., & Dixon, J. E. (2002). Sorting out the cellular functions of sorting nexins. *Nature Reviews Molecular Cell Biology*, *3*(12), 919–931. https://doi.org/10.1038/nrm974

Xu, T., Gan, Q., Wu, B., Yin, M., Xu, J., Shu, X., & Liu, J. (2020). Molecular Basis for PI(3,5)P2 Recognition by SNX11, a Protein Involved in Lysosomal Degradation and Endosome Homeostasis Regulation. *Journal of Molecular Biology*, *432*(16), 4750–4761. https://doi.org/10.1016/j.jmb.2020.06.010

Yang, J., Villar, V. A. M., Rozyyev, S., Jose, P. A., & Zeng, C. (2019). The emerging role of sorting nexins in cardiovascular diseases. *Clinical Science*, *133*(5), 723–737. https://doi.org/10.1042/CS20190034

Yin, L., Duan, J.-J., Bian, X.-W., & Yu, S. (2020). Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Research*, *22*(1), 61. https://doi.org/10.1186/s13058-020-01296-5

Yong, X., Billadeau, D. D., & Jia, D. (2021). All ways lead to Rome: assembly of retromer on membranes with different sorting nexins. *Signal Transduction and Targeted Therapy*, *6*(1), 139. https://doi.org/10.1038/s41392-021-00561-z

Yong, X., Zhao, L., Deng, W., Sun, H., Zhou, X., Mao, L., Hu, W., Shen, X., Sun, Q., Billadeau, D. D., Xue, Y., & Jia, D. (2020). Mechanism of cargo recognition by retromer-linked SNX-BAR proteins. *PLOS Biology*, *18*(3), e3000631. https://doi.org/10.1371/journal.pbio.3000631

Zagami, P., & Carey, L. A. (2022). Triple negative breast cancer: Pitfalls and progress. *Npj Breast Cancer*, *8*(1), 95. https://doi.org/10.1038/s41523-022-00468-0

Zakaria, Z., Zulkifle, M. F., Hasan, W. A. N. W., Azhari, A. K., Raub, S. H. A., Eswaran, J., Soundararajan, M., & Husain, S. N. A. S. (2019). Epidermal growth factor receptor (EGFR) gene alteration and protein overexpression in Malaysian triple-negative breast cancer (TNBC) cohort . *OncoTargets and Therapy*, *Volume 12*, 7749–7756. https://doi.org/10.2147/OTT.S214611

Zhang, H., Huang, T., Hong, Y., Yang, W., Zhang, X., Luo, H., Xu, H., & Wang, X. (2018). The Retromer Complex and Sorting Nexins in Neurodegenerative Diseases. *Frontiers in Aging Neuroscience*, *10*. https://doi.org/10.3389/fnagi.2018.00079

Zhang, P., Wu, Y., Belenkaya, T. Y., & Lin, X. (2011). SNX3 controls Wingless/Wnt secretion through regulating retromer-dependent recycling of Wntless. *Cell Research*, *21*(12), 1677–1690. https://doi.org/10.1038/cr.2011.167

APPENDICES

A. Buffers and Solutions

10% Separating Gel Mix:

3.33 ml Acrylamide – Bisacrylamide (30%)

2.5 ml 1.5M Tris-HCl pH: 8.8

100 µl SDS (10%)

100 µl APS (10%)

4µl TEMED

3.96 ml dH_2O

5% Stacking Gel Mix:

1.36 ml Acrylamide – Bisacrylamide (30%)

1 ml 1M Tris-HCl pH: 6.8

80 µl SDS (10%)

80 µl APS (10%)

8 µl TEMED

5.44 ml dH2O

TBS-T:

20 mM Tris

137 mM NaCl

0.1% Tween 20

pH: 7.6

6X Laemmli Buffer:

12% SDS

30% 2-mercaptoethanol

60% Glycerol

0.012% bromophenol blue

0.375 M Tris

Mild Stripping Buffer:

15g Glycine

1 g SDS

10 ml Tween 20

Adjust the pH to 2.2

Complete to 1 L with dH2O

10X Running Buffer:

25 mM Tris base

190 mM Glycine

0.1% SDS

Dilute 1X with dH2O prior to use.

Transfer Buffer:

200 ml Methanol

100 ml 10X Blotting Buffer

700 ml dH2O

10 X Blotting Buffer:

30.3 g Trizma Base (0.25M)

144 g Glycine (1.92M)

pH: 8.3

10X PBS:

1.37 M NaCl

27 mM KCl

100 mM Na2HPO4

pH 7.4

Lysis buffer for proximity-based labelling assay:

0.8% NP-40

50 mM NaCl

50 mM Tris-HCl (pH 8.0)

5 mM MgCl2

Wash buffer for proximity-based labeling assay:

0.8% NP-40

150 mM NaCl

50 mM Tris-HCl (pH 8.0)

0.5% SDS

5 mM MgCl2

10X citrate saline buffer:

1.35 M KCl

0.15 M sodium citrate

Autoclaved

Diluted to 1X with Molecular Grade water

3X SDS sample loading buffer:

188 mM Tris-Cl (pH 6.8)

3% SDS

30% glycerol

0.01% bromophenol blue

20 mM DTT (freshly added)

2X SDS sample loading buffer:

Tris-Cl (100 mM, pH 6.8)

SDS (electrophoresis grade) (4%)

Bromophenol blue (0.2%)

Glycerol (20%)

10 mM DTT (freshly added)

B. Vector Maps

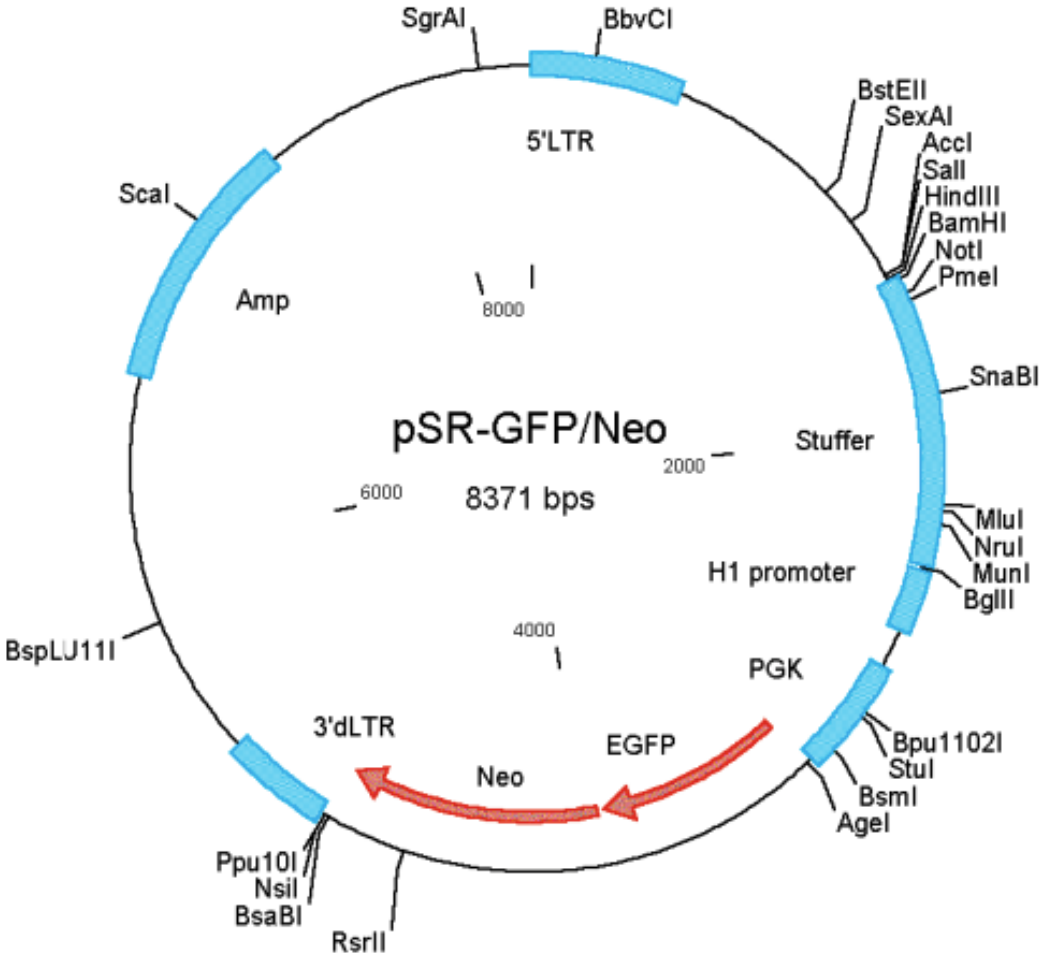


Figure B. 1 Vector map of pSUPER.retro.neo+GFP.

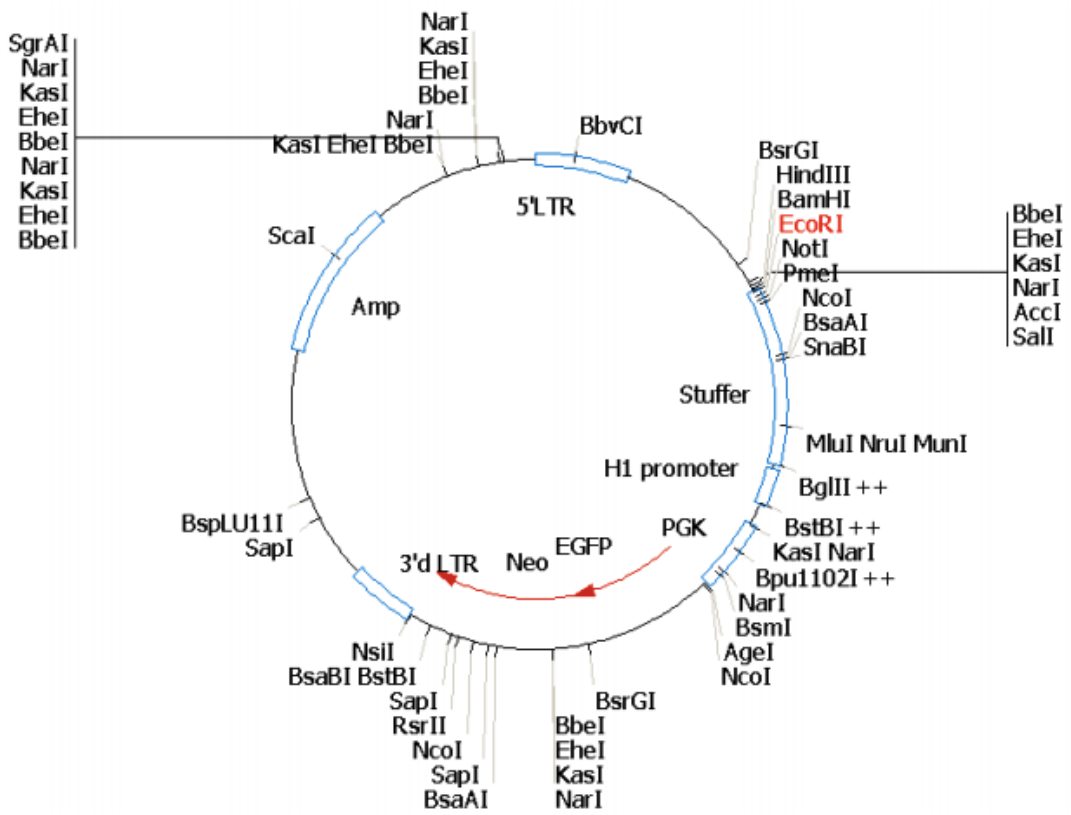


Figure B. 2 Vector map of pSUPERIOR.retro.neo+GFP

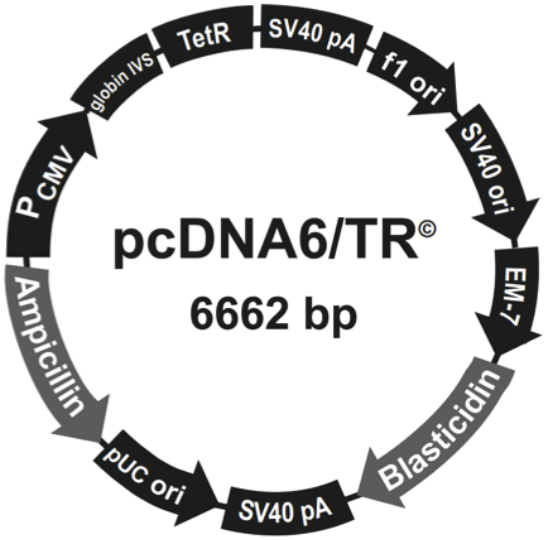


Figure B. 3 Vector map of pcDNA6/TR.

C. Markers

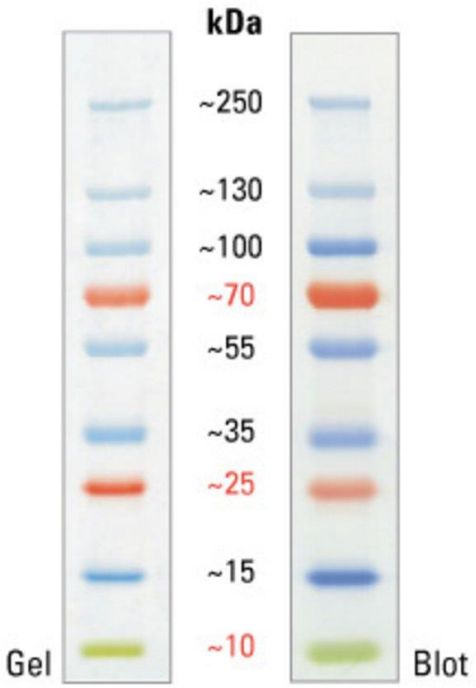


Figure C. 1 Protein ladder (Thermo PageRuler Plus Prestained, Cat #: 26619).

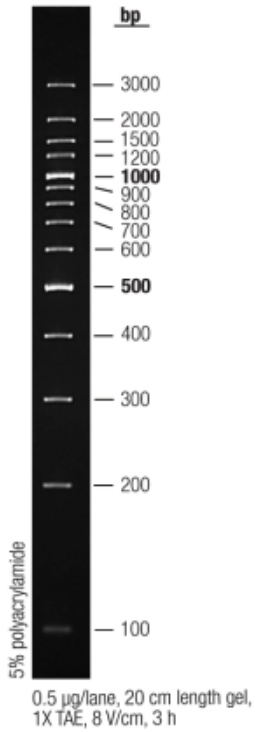


Figure C. 2 DNA Ladder (Thermo GeneRuler 100 bp Plus, Cat #: SM0321).

CURRICULUM VITAE

Surname, Name: Çırçır, Ayça

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biology	2014
BS	METU Biology	2011
High School	Gazi Anadolu High School, Ankara	2005

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

1. Circir, A. (2014). Development of Recombinant Vaccine Candidates Composed of LktA from Mannheimia haemolytica A1 and PlpeC-OmpH from Pasteurella multocida A:3 against Bovine Respiratory Disease. Master's Thesis, Middle East Technical University

2. Circir A*, Oyken M*, Cicek E*, Akbulut O, Dioken DN, Guntekin-Ergun S, Cetin-Atalay R, Sapmaz A, Ovaa H, Sahin O, Erson-Bensan AE, EGF-SNX3-EGFR axis drives tumor progression and metastasis in triple negative breast cancers. "Oncogene", 41(2), (2022 Jan), 220-232. Epub 2021 Oct 30.

*Equal Contribution

3. Circir A, Koksal Bicakci G, Savas B, et al. A C-term truncated EIF2Bγ protein encoded by an intronically polyadenylated isoform introduces unfavorable EIF2Bγ-EIF2γ interactions. Proteins. 2022 Mar;90(3):889-897. DOI: 10.1002/prot.26284. PMID: 34796993.

4. Circir Hatil, A., Cicek, E., Oyken, M., Erson Bensan, A. E., EGFR (Epidermal Growth Factor Receptor), Atlas of Genetics and Cytogenetics in Oncology and Haematology, 2019, DOI: 10.4267/2042/70782.

5.Cicek, E., Circir Hatil, A., Öyken, M., Cingoz, H., Erson Bensan, A.E., SNX3 (Sorting Nexin 3), Atlas of Genetics and Cytogenetics in Oncology and Haematology, 2019, DOI: 10.4267/2042/70783.

ORAL PRESENTATIONS

1. Circir Hatil A, Akbulut O, Oyken M, Sahin O, Erson Bensan AE. SNX3'ün Meme Kanserindeki Rollerinin *In vitro* ve *In vivo* Sistemlerde Incelenmesi. Onkolojide İz Birakanlar Zirvesi 2019, Antalya, TR, 2019. Abstract 0084, Bildiri S-70

POSTERS

1. Circir Hatil A, Erson Bensan AE. WLS Expression in ER+ Breast Cancers. MolBiyoKon'18, Izmir, TR, 2018. PT021
2. Tefon B. E. , Çırçır Hatıl A., Özcengiz E., Özcengiz G. International Symposium on Biotechnology: Developments and Trends, Ankara, Türkiye, 27 - 30 Eylül 2009, ss.95

CERTIFICATES

1. 6th International Congress of the Molecular Biology Association of Turkey (2018) - Molecular Biology Association of Turkey
2. 5th National Environmental Engineering Congress (2014) - ODTÜ Biyoloji ve Genetik Topluluğu (BİYOGEN)
3. Certificate of Animal Use in Experimental Research (2011) - GATA Center for Research and Development
4. 1st International Symposium on Genetically Modified Organisms (2008) – UCTEA