INVESTIGATING SNX3 BINDING MOTIF ON EGFR

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ABSTRACT

INVESTIGATING SNX3 BINDING MOTIF ON EGFR

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Sorting Nexin 3 (SNX3) is a protein that is involved in retromer complex and has a role in recognition and trafficking of retromer cargo proteins. SNX3-retromer provides the recycling of their cargo proteins back to the plasma membrane or to Trans Golgi Network. Recent studies reveal the specificity of SNX3 in cargo recognition by acting on a recycling motif on receptor cytoplasmic tails. Considering the importance of SNX3 in receptor recycling, we hypothesized that EGFR can be one of the cargo proteins that is recognized and recycled by the SNX3-retromer. We used site-directed mutagenesis, Co-IP techniques to investigate a potential link between SNX3 and EGFR. Preliminary findings from this work will guide future experiments to further investigate protein-protein interaction.

Keywords: SNX3, Retromer, EGFR,

ÖZ

EGFR ÜZERİNDEKİ SNX3 BAĞLANMA MOTİFİNİN ARAŞTIRILMASI

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SNX3, retromer komplesi içersinde yer alan bir proteindir ve kargo proteinlerinin tanınması ve trafik edilmesinde rol oynar. SNX3 retromeri, kargo proteinlerinin ya hücre zarına ya da golgi aygıtı ağına geri kazanımını sağlar. Son çalışmalar, SNX3'ün kargo proteinini, kargonun sitoplasmik ucunda bulunan geri kazanım motifi ile spesifik olarak tanıdığını göstermektedir. SNX3'ün reseptör geri kazanımındaki önemini düşünüldüğünde, EGFR'ın da SNX3 retromeri tarafından tanınan ve geri kazanımı sağlanan proteinlerden bir tanesi olacağı hipotezini kurduk. EGFR ile SNX3 arasındaki potansiyel ilişkinin araştırılması amacıyla bölgeye özgü mutagenez ve immunopresipitasyon yöntemleri kullanılmıştır. Bu çalışmadan elde edilen ön bulgular, protein-protein ilişkisinin ortaya konmasına yönelik ileri araştırmalara öncülük edecektir.

Anahtar Kelimeler: SNX3, Retromer, EGFR,

To my family…

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LIST OF ABBREVIATIONS

ABBREVIATIONS

β2AR	β2 Adrenergic Receptor
BAR	Bin/Rvs/Amphiphysin
BSA	Bovine Serum Albumin
CI-MPR	Cation Independent Mannose 6 Phosphate Receptor
Co-IP	Co-Immunoprecipitation
CXCR 2	C-X-C Chemokine Receptor Type 2
DMT1	Divalent Metal Transporter 1
EGFR	Epidermal Growth Factor Receptor
ERBB2	Receptor Tyrosine Protein Kinase erbB-2
FACS	Fluorescence Activated Cell Sorting
FERM	4.1/Ezrin/Radixin/Moesin
FGFR	Fibroblast Growth Factor Receptor
GFP	Green Fluorescent Protein
HpIgR	Human Polymeric Immunoglobulin Receptor
IGFR1	The insulin-like Growth Factor 1
IL1R1	Interleukin 1 Receptor Type 1
LRP8	Low-Density Lipoprotein Receptor Related Protein 8
NT	Non-Target
NOTCH2	Neurogenic Locus Notch Homolog Protein 2

PtdIns(3)P	Phosphatidylinositol-3-phosphate
PtdIns3,5P2	Phosphatidylinositol 3,5-phosphate
PDZ	PSD95, Dlg1, Zo-1
PDZbms	PDZ-binding motifs
PGFRA	Platelet Derived Growth Factor Receptor Alpha
PM	Plasma Membrane
PX	Phox Homology
RET	Proto-oncogene Tyrosine Protein Kinase Receptor
siRNA	small interfering RNA
SNX3	Sorting Nexin 3
SNXs	Sorting Nexins
TGFBR1	Transforming Growth Factor Beta Receptor 1
TGN	Trans-Golgi Network
TNPO1	Transportin 1
VPS26	Vacuolar Protein Sorting 26
VPS29	Vacuolar Protein Sorting 29
VPS35	Vacuolar Protein Sorting 35
WLS	Wntless

CHAPTER 1

INTRODUCTION

1.1. Retromer in Endosomal Sorting

Cell membrane proteins impact cell function and homeostasis through regulating cell signaling networks. Control over the levels of such proteins is mainly provided by the expression, recycling and degradation pathways. For recycling and degradation, the protein coat complex of recycling endosomes, called retromer, is an important determiner to facilitate a balance between recycling and degradation.

Retromer is a protein complex that becomes functional when it is recruited and associated with the cytoplasmic side of the endosome membranes. Endosomes provide the trafficking of transmembrane proteins, such as signaling receptors, transporters, ion channels and other proteins into three destinations: 1) to lysosomes via late endosomes, 2) back to the plasma membrane (PM) via recycling pathway and 3) to trans-Golgi Network (TGN) via retrograde pathway (Johannes and Wunder 2016).

Mammalian retromer complex is composed of Vacuolar Protein Sorting 26 (VPS26), Vacuolar Protein Sorting 29 (VPS29) and Vacuolar Protein Sorting 35 (VPS35) heterotrimer, known as the retromer trimer. This structure further interacts with various combinations of sorting nexins (SNX) proteins (Carlton et al. 2004).

Structurally; VPS35-VPS29-VPS26 retromer trimer shows interaction of elongated proteins in which VPS29 and VPS26 bind to N- and C- terminal part of VPS35, respectively (Figure 1.1) (Gershlick and Lucas 2017). The retromer trimer has a role in recognition of cargo proteins. In case of VPS35 depletion, an essential component

of the retromer structure, the metal transporter 1 (DMT1-II) ends up in late endosomes and then in lysosomes (Tabuchi et al. 2010).

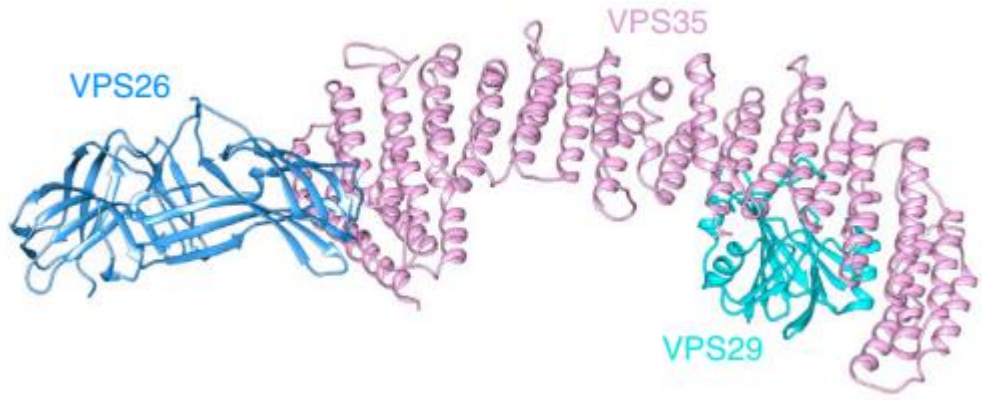


Figure 1.1. The structural illustration of VPS26-VPS29-VPS35 heterotrimer. VPS29 and VPS26 interacts with N- and C- terminal part of VPS35, respectively (Figure taken from Gershlick and Lucas 2017).

1.2. Sorting Nexins (SNXs)

Sorting nexins is a protein family sharing a phospholipid binding phox-homology (PX) domain. PX domain allows interaction with phosphatidylinositol-3-phosphate (PtdIns(3)P) and also with phosphatidylinositol 3,5-phosphate (PtdIns3,5P2) which are enriched in early and late endosomes (Purushothaman and Ungermann 2018). Other domains, not found in all SNX proteins, such as Bin, Amphiphysin, and Rvs (BAR) domains enable membrane to be curved and generate tubular structure in which cargo proteins are carried (Kvainickas et al. 2017). Another important domain; 4.1/ezrin/radixin/moesin (FERM) is responsible for the recognition of cargo protein via the NPxY/NxxY recycling signal (Ghai et al. 2013). PSD95, Dlg1, zo-1 (PDZ) domain of SNX27 also interacts with cargo proteins with PDZ-binding motifs (PDZbms) (S/T-x-Φ) (Gallon et al. 2014).

Given the diversity of domains, SNXs are classified in three sub-groups based on absence or presence of these domains (Figure 1.2) (Lucas et al. 2016);

1) SNX-PX subfamily: SNX3 has only the PX domain

2) SNX-BAR subfamily: SNX1, SNX2, SNX5, SNX6, SNX32 has both BAR and PX domain

3) SNX-FERM subfamily: SNX27 has PDZ and FERM domains

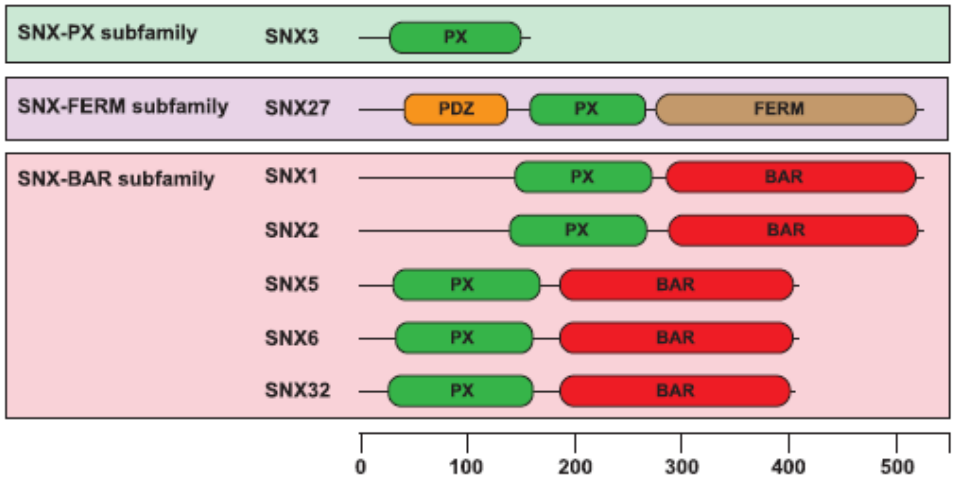


Figure 1.2. *The domains of SNX protein sub-families. SNX-PX subfamily has only PX domain. SNX-FERM subfamily has PDZ, PX and FERM domains. Moreover, SNX-BAR sub family has PX and BAR domains (Figure taken from Lucas et al. 2016).*

Incorporation of a distinct set of SNX proteins into the retromer complex provides different features to the retromer. For example, SNX-BAR subtype proteins can drive membrane deformation and form helical-shaped tubules through their BAR domains (Van Weering et al. 2010). SNX-BAR dependent retromer sorting is provided by discrete tubular profiles and tubulovesicular protein assembly (Simonetti et al.2017). Cargo specificity or cargo fate are other regulated features of the retromer structure (Figure 1.3) (Lucas and Heiro, 2015).

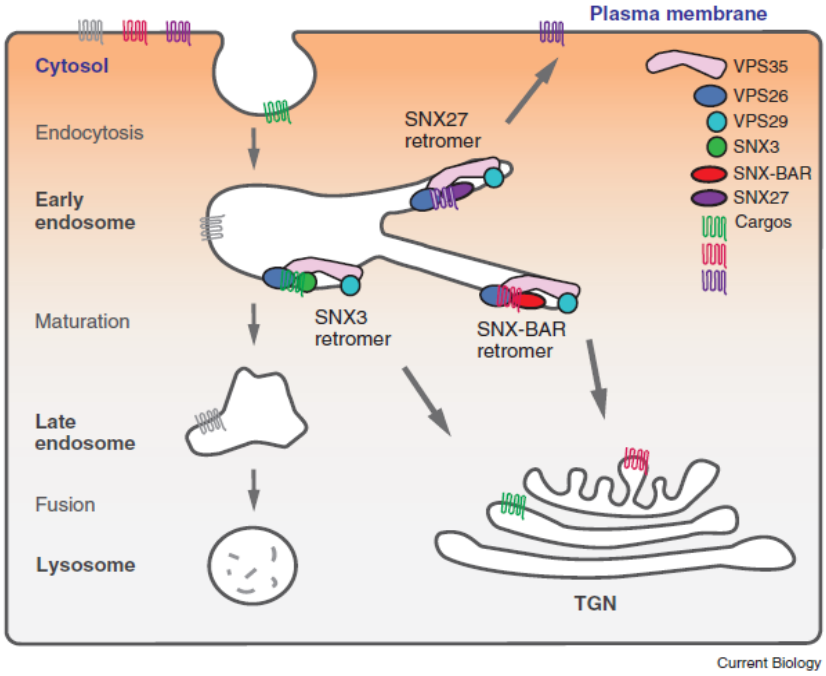


Figure 1.3. Different forms of retromers act on distinct mechanisms. (Figure taken from Lucas and Heiro, 2015).

A well understood example is the retrograde transport of cation-independent mannose 6-phoshate receptor (CI-MPR) recognized by the SNX-BAR retromer. CI-MPR is a TGN-resident receptor which transports newly synthesized lysosomal hydrolases from TGN to lysosomes. Its retrograde transport from endosome to TGN is required for another round of lysosomal hydrolase transport. Therefore, SNX-BAR retromer provides CI-MPR sorting from endosome to TGN (Simonetti et al. 2017).

Other well-studied examples are the Wnt signaling receptor Wntless (WLS) (Harterink et al. 2011) and divalent metal transporter 1 isoform II (DMT1-II) (Lucas et al. 2016) recycling from plasma membrane back to the TGN by the SNX3-retromer complex.

On the other hand, β2 adrenergic receptor (β2AR) transport is mediated by SNX27 dependent retromer from endosome to plasma membrane (Gallon and Cullen 2015).

Hence, the destination and sorting of cargo proteins depends on the retromer composition.

1.3. SNX3-retromer

Within the SNX protein family, SNX3 is a unique member that has only the PX domain. Through its PX domain it associates with endosomal lipid phosphatidylinositol-3-phosphate (PtdIns(3)P) of membranes (Purushothaman and Ungermann 2018). SNX3 has role in recruiting VPS26-VPS35-VPS29 retromer trimers to endosomal membrane (Lenoir et al. 2018). A retromer trimer associated with SNX3 is named the SNX3-retromer (Purushothaman and Ungermann 2018).

Based on recent findings, to associate with a SNX3-retromer for retrograde transport, cargo proteins should follow endosomal recycling pathway which begins with Rab5-positivite early endosomes. Gradually, maturation of early endosomes is achieved by recruiting the GTP-bound Rab7, which later recruits retromer complex onto its membrane and cargo is concomitantly recognized via its recycling signal, thus; sorted and transported towards TGN or to PM (Klinger et al. 2015) (Tabuchi et al. 2010).

Recent studies reveal the importance and specificity of SNX3-retromer for cargo recognition and trafficking. SNX3 plays a key role in the regulation of Wntless (WLS) seven-pass transmembrane protein that controls Wnt ligand secretion. Recycling of WLS protein from PM to TGN is controlled by the SNX3-retromer and is independent of other SNX proteins (Harterink et al. 2014). Another study demonstrated that null mutant SNX1 and SNX6 show no perceptible changes in Wnt secretion, on the other hand; null mutant of SNX3 led to wing defects due to lack of Wnt signaling in *Drosophila* (Zhang et al. 2011).

Absence of SNX3 has already been linked to defects in Wnt signaling associated with neurodegeneration such as Alzheimer and Parkinson's diseases (Wang et al. 2014).

Furthermore; DMT1-II, Sortilin, CI-M6PR and hpIgR are other known cargo proteins recycled by the SNX3-retromer (Lucas 2016).

1.4. SNX3-retromer for Cargo Recognition and Sorting

Structural analysis of retromer proteins is of interest to gain more insight into how the recognition mechanism takes place between cargo proteins and retromers. Lucas et al. 2016, suggested a structural mechanism of DMT1-II cargo recognition by SNX3-retromer. This study demonstrated the overall structure of VPS26-VPS35-SNX3 and DMT1-II by X-ray crystallographic analysis (Figure 1.4).

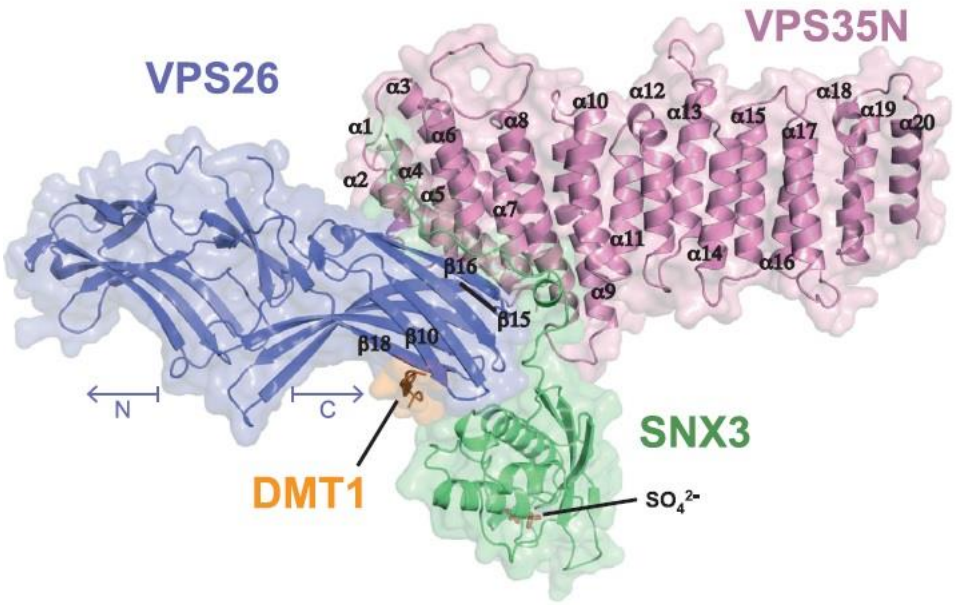


Figure 1.4. Overall structural illustration of SNX3 retromer with DMT1-II cargo protein. In this illustration, DMT1-II cargo protein interacts with the VPS26-SNX3 interface which is formed by concomitant interactions of retromer and cargo protein. N-terminal VPS26 associates both C-terminal VPS35 and N-terminal SNX3. Furthermore, SNX3 forms interactions with both VPS26 and VPS35 via its C-terminal part (Figure taken from Lucas et al. 2016).

According to proposed structural analysis, SNX3 interacts with N-terminal extension of VPS35 (VPS35N) and C-terminal tail of VPS26 (VPS26C). This interaction occurs via N-terminal part of SNX3. In addition, PX domain of SNX3 protein stands in the opposite side of VPS26C-VPS35N interaction surface (Lucas et al. 2016). In the structural configuration, the PX domain is represented as two sulfate ions (SO_4^{2-}).

Additionally, in the same study, a binding site at the interface between VPS26 and SNX3 is described. The exposure of this binding site occurs by the concomitant interaction of SNX3, VPS26 and cargo (Lucas et al. 2016). In the cargo protein, this site is the ØX(L/M) motif recognized by the retromer. Consequently, recognition of cargo protein recycling motif occupies with the membrane recruitment of retromer (Figure 1.5) (Johannes and Wunder 2016).

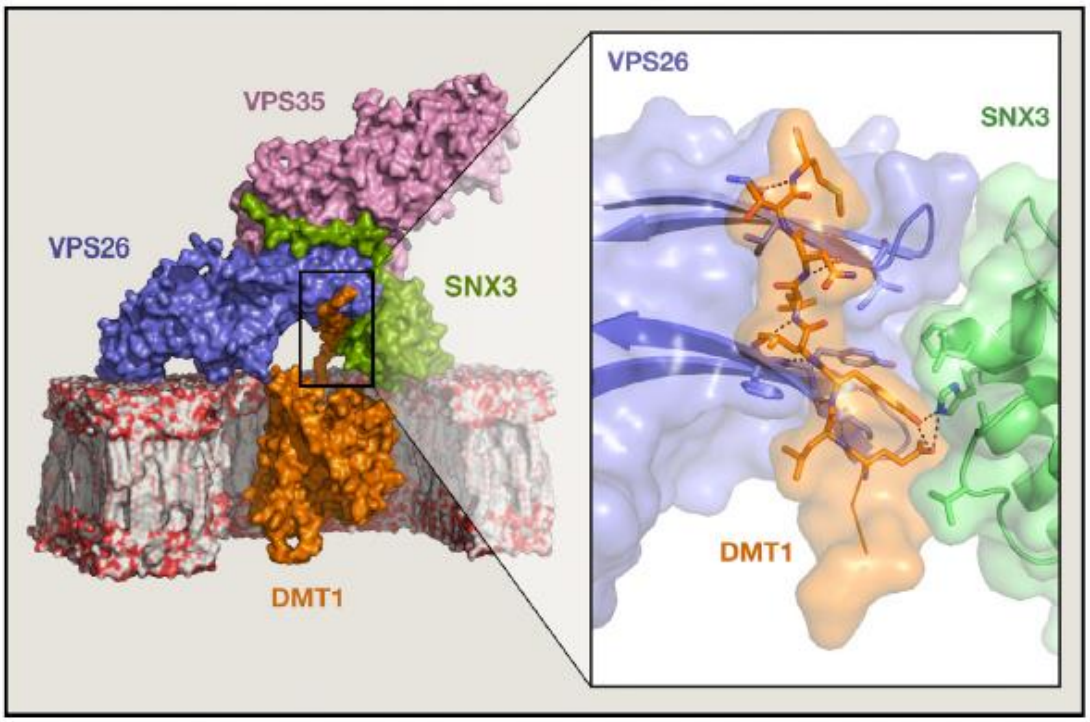


Figure 1.5. The structural illustration of SNX3-retromer complex on membranes for cargo recognition. The recognition of DMT1-II recycling motif achieved via binding site on VPS26-SNX3 interface (Figure taken from Johannes and Wunder 2016).

For the formation of retromer and cargo protein complex, cargo should have the ØX(L/M) signals in which Ø stands for bulky amino acids such as Tyrosine (Y), Tryptophan (W), and Phenylalanine (F); L stands for amino acid Leucine, and M stands for amino acid Methionine in its C-terminal tail.

For SNX3-retromer, this consensus motif is expanded and promoted with additional seven side residues (Figure 1.6) (Lucas et al. 2016).

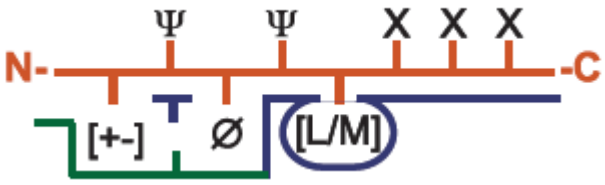


Figure 1.6. Schematic illustration of VPS26-SNX3 cargo binding motif. From C- to N-terminal: X represents any residue; L stands for amino acid Leucine, and M stands for amino acid Methionine; Ψ shows a hydrophobic or long aliphatic hydrocarbon residue; Ø stands for bulky aromatic amino acids, [+-] for any charged amino acids (Figure taken from Lucas et al. 2016).

In this identified SNX3-retromer binding motif, X represents any residue, Ø stands for bulky aromatic amino acids, Ψ shows a hydrophobic or long aliphatic hydrocarbon residue, and [+-] for any charged amino acids. For DMT1-II, this motif corresponds to ^{553}ELYLLNTM^{560} protein sequence. On this DMT1-II recycling motif, L557 residue fits the hydrophobic pocket on VPS26 which between β10 and β18 strands and this position is considered as a central position (P_0) of the motif. Furthermore, Y555 residue, which is found in two downstream residues from the central position and named a P_{-2} position, makes H-bonds with H132 residue of SNX3. Same residue of SNX3 (H132) also interacts and forms H-bonding interactions with E553 (P_{-4}) of

DMT1-II. Other residues of DMT1-II, which are aliphatic L554 (P_{-3}) and L556 (P_{-1}) residues, have a significant role in having specific interaction between cargo and VPS26-SNX3 binding site (Lucas et al. 2016). The interaction between DMT1-II cargo protein and SNX3-retromer complex occurs through comprehensive H bonding and hydrophobic interactions.

Overall, SNX3 emerges as a critical protein in determining the fate of a group of transmembrane proteins.

1.5. Aim of Study

Recent findings reveal the importance and specificity of SNX3-retromer for cargo protein recognition and sorting. In the light of new studies on SNX3-retromer and its cargo interactions, we aim to investigate whether SNX3 has a role in sorting of receptors in cancer cells. We predicted that Epidermal Growth Factor Receptor (EGFR) can be a candidate protein due to a potential SNX3-retromer binding motif on its C-terminal tail. Therefore, we hypothesized that there is a potential SNX3 recycling motif on EGFR. To investigate this hypothesis, we used several methods and examined the EGFR-SNX3 interaction.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Lines and Cell Culture

MDA-MB-231 and HEK293 cells were grown in DMEM High Glucose (Biological Industries, Cat #: 01-052-1A) containing 4.5g/l D-Glucose, 4mM L-Glutamine, 1mM sodium pyruvate (Biological Industries, Cat #: 03-042-1B), 10% Fetal Bovine Serum (FBS) (Biowest, Cat#: S1810-500) and 1% Pen-Strep (Biological Industries, Cat#: 03-031-1B). HeLa cells were maintained with MEM medium with Earle's salts (Biochrom, Cat#: F0385) that contains 1% NaHCO3, 2% L-Glutamine, 1% Pen-Strep and 10% Fetal Bovine Serum (FBS). Cells were incubated in 5% CO_2 incubator at 37^0C. Cells were stored in liquid nitrogen vapor with 5% dimethyl sulfoxide (DMSO) (Sigma, Cat#: 154938).

2.2. Protein Isolation

Total protein isolation was done by using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Cat#: 78501). M-PER reagent was with 10X phosSTOP (Roche, Cat#: 04906837001) and 25X protease inhibitor (Roche, Cat#: 1187350001). Protein concentrations were determined by using Pierce BCA Protein Assay Kit (Thermo Scientific, Cat#: 23227) according to manufacturer's manual.

2.3. Co-Immunoprecipitation (Co-IP) Assay

400 µg of total protein was completed up to 200 µl volume with cold Immunoprecipitation Buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA,

1% Tripton X-100, 0.5% NP-40, 10X phosSTOP, 25X protease inhibitor). For pre-clearing, 25 µl of Protein A Beads (NEB, Cat#: S1425S) were added to cell lysates and incubated at 4 °C for 1 hour using a rotator. Next, magnetic field was applied to pull beads to the side of microcentrifuge tube. Supernatant was pipetted to a clean 1.5 ml microcentrifuge tube and beads were discarded. For IP, 6 µg of EGFR antibody (Santa Cruz, Cat#: sc-373746) and TNPO1 antibody (Abcam, Cat#: ab10303) (as a negative control) were added IP lysates. Samples were gently vortexed and incubated at 4°C overnight with mild rotation. Then, 25 µl Protein A Magnetic Bead suspension was added samples were further incubated for 1 hour at 4°C. Following this incubation, magnetic field was applied, and beads were separated from the supernatants. Beads were washed twice with 350 µl of the Immunoprecipitation Buffer, bead pellets were resuspended in 30 µl of 3X SDS Sample Loading Buffer. Samples were incubated at 100°C for 5 minutes. After applying magnetic field to samples, supernatants were loaded on SDS-PAGE gel and electrophoresed.

2.4. GFP-Trap

For one immunoprecipitation reaction; 10^6 HEK293 cells, which stably and separately express EGFR-GFP and EV (pEGFP-N1), was used. Cells were lysated using 200 µl ice-cold lyse buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40) by pipetting. Then, 300 µl cold dilution/wash buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA) was added into the lysate and 30 µl diluted lysate were saved for immunoblot analysis using as an input. Meanwhile, 25 µl GFP-Trap®_MA beads were washed with 500 µl dilution/wash buffer twice and separated with magnetic fields. Equilibrated beads were added into diluted lysate and bead-lysate mix were tumbled end-over-end for 1 hour at 4°C. After incubation, beads were separated by magnetic field and resuspended in 500 µl ice-cold dilution/wash buffer. Wash step was done twice. Next, beads were resuspended in 30 µl of 6X SDS Sample Loading Buffer and incubated at 95°C for 10 minutes. After applying magnetic field to samples, supernatants were loaded on SDS-PAGE gel and electrophoresed.

2.5. Western Blotting

Lysates were boiled in 6X Laemmli buffer at 100°C for 10 minutes. Then, denatured proteins were loaded into SDS-PAGE gels prepared as 5% stacking and 10% separating gels. Subsequently, electrophoresed proteins were transferred into PVDF membranes (Roche). Wet transfer was done for 1 hour at 100V. For hybridization, membranes were first blocked in 5% skim milk or 5% Bovine Serum Albumin (BSA) in 1% Tris-Buffered saline-tween (TBS-T) for 1 hour at room temperature. Following the blocking step, western blot membrane was incubated with primary antibodies at 4°C overnight. EGFR antibody (1:500, Santa Cruz, Cat#: sc-373746), SNX3 antibody (1:500 dilution, Proteintech, Cat #: 10772-1-AP), GFP antibody (1:500, Santa Cruz, Cat#: sc-9996), B-actin antibody (1:2000, Santa Cruz, Cat#: sc-47778) were used. HRP-conjugated secondary anti-mouse antibody (1:2000, Santa-Cruz) and secondary anti-rabbit antibody (1:2000, Santa-Cruz) incubation of membrane was performed for 1 hour at room temperature. Washes were done using %1 TBS-T. For visualization of membrane, Western ECL Blotting Substrates (BioRAD Clarity, Cat#: 1705060) was used according to the manufacturer's instructions.

2.6. Transfections

EGFR-GFP was purchased from Addgene (Cat#: 32751; http://n2t.net/addgene:32751; RRID: Addgen_32751), transfected into HEK293 cells to generate stable EGFR-GFP expression cell line. Stable cells were selected by 0.5 mg/ml G-418 (Roche, Cat#: 04727878001) treatment. pEGFP-N1 (EV) (Clontech Laboratories, Inc. GenBank Accession Cat#: U55762) stably transfected into HEK293 cells and maintained with G-418.

Beta-1,4-galactosyltransferase1-mCherry (Golgi-marker fusion gene) in pcDNA 3.1 (designed and cloned by Hüseyin Evci, Son Lab) used for transient transfections into HEK293 and HeLa cells. Transfections were done by using 4 µl TurboFect (Thermo Scientific, Cat #: R0531) in 6-well plates.

SiRNA targeting SNX3 (20-50 nM) (Dharmacon, Cat#:SO-2660749G) and its negative control siRNA (20-50 nM) (Qiagen, Cat#: 1027310) transfections were done transiently into HEK293 and HeLa cells using TurboFect reagent with 3.35 µl for each well.

The information about the SNX3 siRNA and NT siRNA pool sequences are indicated (Table 2.1).

Gene Name	Target Sequence
siGENOME SMART pool siRNA D-011521-01, SNX3	UAGAGGAGAUGAUGGAAUA
siGENOME SMART pool siRNA D-011521-02, SNX3	GAACCUGAAUGACGCCUAC
siGENOME SMART pool siRNA D-01152-04, SNX3	GAUGUGAGCAACCCGCAAA
siGENOME SMART pool siRNA D-011521-17, SNX3	CCAGCAACUUCCUCGAGAU
Negative Control siRNA	AATTCTCCGAACGTGTCACGT

Table 2.1. The pool sequences of SNX3 siRNA and NT siRNA.

2.7. Cycloheximide Treatment

Cycloheximide (Abcam, Cat#: ab120093) was dissolved in 96% EtOH at 1 mg/ml stock concentration. Cycloheximide treatment was done into 6-well plated HEK293 and HeLa cells with the final concentration of 5 ug/ml in 2 ml medium for 24 hours.

2.8. Visualization of Cells with Spinning Disc Confocal Microscope

Cells were grown and transfected in 6-well plate and then transferred into 35 mm imaging chamber a day before the image acquisition. The cells were visualized by Leica DMI4000 B with Andor DSD2 Confocal Microscope with a 63x oil immersion

objective lens. The filters of Bright Field, EGFP and mCherry were used to collect images.

2.9. In Silico Motif Analysis using Scansite 4.0

To search a motif sequence on protein databases, the tool called *Scansite 4.0* (Scansite, https://scansite4.mit.edu/4.0/#scanSeq) was used. In this tool, there are empty boxes corresponding to different positions of motif residues. In these residues, single-letter amino acid codes that indicated as symbols in wildcard are entered. Moreover, optionally, search can be restricted in several ways such as organism class, keyword regular expression etc. Finally, entered input is submitted and searching one or more motif patterns on protein databases is completed.

For our investigation, we entered the amino acids codes corresponding to VPS26-SNX3 binding site motif, that is shown by previous study (Lucas et al., 2016), in input table (Table 2.2).

1. Enter sequence pattern below using single-letter amino acid code and wild cards. Check the boxes below the position specific pattern to indicate phosphorylation sites (optionally).

			#/&	$	@	$	L/M	X	X	X				
-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Pattern: [HKRDE][GAVILM][FYW][GAVILM][LM][A-Z][A-Z][A-Z]

Table 2.2 Input table indication from the Scansite 4.0. In the table; X stands for any residue, L/M represents the amino acids Leucine or Methionine, $ indicates Aliphatic residues (GAVILM), @ for aromatic residues (FYW), # code for positive residues (HKR) and & code for negative residues (DE).

The search was restricted by written "Human" in "Species Regular Expression" category and "Receptor" in "Keyword Regular Expression" category. Moreover, "Mammals" option was selected for organism class (Table 2.3).

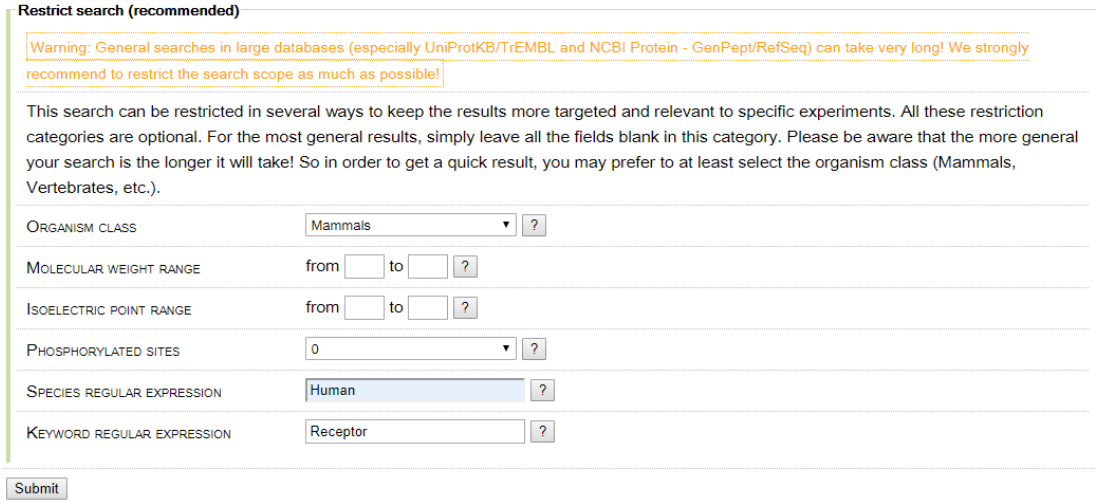


Table 2.3 Indication of search restriction from the Scansite 4.0.

As a result, list of human receptors that harbor predicted motif sites are listed (Table 2.4).

Predicted motif sites

Warning: Popup blockers may disable the links in the result table

Protein ID	Pattern: [HKRDE][GAVILM][FYW][GAVILM][LM][A-Z][A-Z][A-Z]	Protein Annotations	Molecular Weight [kDa]	Isoelectric Point	Motifs at expected sites	Evolutionary conservation
OX2R_HUMAN	Show 1 match	RecName: Full=Orexin receptor type 2; (click to expand)	50.70	8.99		
NECT2_HUMAN	Show 1 match	RecName: Full=Nectin-2; AltName: Full (click to expand)	57.75	4.74		
ERR2_HUMAN	Show 1 match	RecName: Full=Steroid hormone recepto (click to expand)	48.06	8.09		
LRP1_HUMAN	Show 6 matches	RecName: Full=Prolow-density lipoprot (click to expand)	504.67	5.16		
OR1D4_HUMAN	Show 1 match	RecName: Full=Olfactory receptor 1D4; (click to expand)	35.23	8.29		
FCGR3_HUMAN	Show 1 match	RecName: Full=High affinity immunoglo (click to expand)	32.24	8.87		
TMF1_HUMAN	Show 1 match	RecName: Full=TATA element modulatory (click to expand)	122.86	4.88		
OR2M2_HUMAN	Show 1 match	RecName: Full=Olfactory receptor 2M2; (click to expand)	39.18	7.07		
TSHR_HUMAN	Show 1 match	RecName: Full=Thyrotropin receptor; A (click to expand)	86.84	6.56		
BY55_HUMAN	Show 1 match	RecName: Full=CD160 antigen; AltName: (click to expand)	19.81	6.94		

Table 2.4. The output list from Scansite 4.0.

2.10. Site-Directed Mutagenesis

To introduce the mutations into the cytoplasmic domain of EGFR, the EGFR-GFP plasmid (Addgene, Cat#: 32751) was used for site-directed mutagenesis to create the deletion or alanine substitution. Two independent mutations were generated. The types of mutation and mutated amino residues on EGFR are indicated in Table 2.5.

Types of Mutation	**Mutated Residues**
Deletion of 3 amino acids	764**YVM**766
Deletion of 3 amino acids	1017**YLI**1019
Alanine Substitution	**M**766**A**

Table 2.5. The types of mutation and mutated residues on EGFR.

Site-directed mutagenesis was achieved by PCRs using EGFR-GFP plasmid as a template. PCRs for single amino acid mutations were run for 16 cycles of 40 s at 98°C and 40s at 68°C, followed by 8 min at 72°C. The resulting mutant plasmids were verified by DNA sequencing. Oligonucleotides primers used for site-directed mutagenesis are shown in Table 2.6.

Types of Mutation	**Sense Primer**	**Anti-sense Primer**
EGFR-GFP ΔYVM	5'-atcctcgatgaagccgccagcgtggacaac-3'	5'-gttgtccacgctggcggcttcatcgaggat-3'
EGFR-GFP ΔYLI	5'-ggatgccgacgagccacagcagggct-3'	5'-agccctgctgtggctcgtcggcatcc-3'
EGFR-GFP M766A	5'-cgatgaagcctacgtggccgccagcgtggacaacc-3'	5'-ggttgtccacgctggcggccacgtaggcttcatcg-3'

Table 2.6. Oligonucleotides primers used for site-directed mutagenesis.

CHAPTER 3

RESULTS AND DISCUSSION

A motif for recycling

SNX3 binding motif on cargo proteins on cytoplasmic tail is required for effective recognition and endosomal sorting by retromer complex (Strochlic et al. 2007). Given the availability of such a motif on cargo proteins, we were interested in identifying other cargo proteins that may be recognized by SNX3 (Table 3.1). While DMTII-1 and WLS carries perfect motif features, Sortilin another receptor recognized by SNX3 has V (valine) at the central position of motif besides L/M. Despite not having hydrophobic features; E, R, D amino acids stand at the position -3 for some cargo proteins. Therefore, by taking these exceptions into consideration, motif pattern may show some variances in different cargo proteins.

Cargo Protein	**NT**	**-4**	**-3**	**-2**	**-1**	**0**	**1**	**2**	**3**	**CT**
DM1-II	**553**	E	L	Y	L	L	N	T	M	**560**
CI-M6PR	**2367**	T	**E**	W	L	M	E	E	I	**2374**
Sortilin	**785**	G	**R**	F	L	**V**	H	R	Y	**792**
hpIgR	**744**	K	**D**	F	L	L	Q	S	S	**751**

Table 3.1. Alignment of cargo proteins' amino acids for their retromer binding motif (Lucas et al. 2016). Red color represents variations in amino acids at their motif positions.

For this purpose, we used the tool called Scansite 4.0 which searches protein databases for occurrences of one or more motif sequences. In this analysis, we entered the amino acids codes that correspond to VPS26-SNX3 binding site.

As a result, the list of predicted receptors with the SNX3-retromer binding motif are shown in Table 3.2. From the output list of the *Scansite 4.0*, indicated receptors on the list are selected since they harbor the binding motif on their cytoplasmic tails.

Receptors	
EGFR	ERBB2
PGFRA	NOTCH2
FGFR1	FGFR2
FGFR4	TGFBR1
CXCR2	CXCR3
IL1R1	RET
LRP8	IGFR1

Table 3.2. List of predicted receptors having SNX3-retromer binding motif on their cytoplasmic tails. The listed receptors are selected from the output list of Scansite 4.0.

From the receptors on the list, Epidermal Growth Factor Receptor (EGFR) was of interest because SNX3 was shown to go through alternative polyadenylation upon EGF treatment in breast cancers by our group (Akman et al. 2015). The possibility that SNX3 is regulated by the activation of EGFR pathway and that EGFR carries a potential recycling motif, we aimed to investigate whether this motif is functional.

3.1 SNX3 Protein Level in Tumorigenic and Non-tumorigenic Cell Lines

To begin investigating whether the potential recycling motif on EGFR is important for SNX3 interaction, we first tested which cell lines express SNX3 protein. We performed western blot in 22 breast cancer cell lines, and non-tumorigenic cell lines and analyzed their SNX3 protein levels (Figure 3.1).

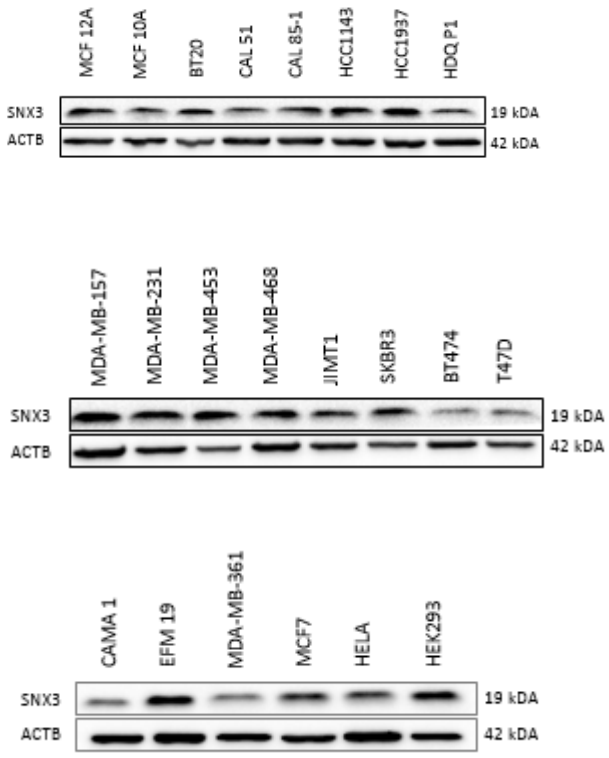


Figure 3.1. Western Blot of SNX3 protein expression in tumorigenic and non-tumorigenic cell lines. 50 µg protein was loaded to 10% gel. SNX3: ProteinTech polyclonal antibody, Cat #: 10772-1-AP, 1:500 dilution was prepared in 0.1% TBST with 5%BSA. ACTB: Santa Cruz Cat#: sc-47778 monoclonal antibody, 1:2000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control.

Regarding western blot result, we decided to use HEK293 cell line due to relatively high transfection potential while also expressing SNX3 protein.

3.2 Analysis of EGFR in SNX3 siRNA Transfected HEK293 Cells

We used siRNA (Dharmacon, Cat#: SO-2660749G) to knock down SNX3 levels. Transfection reagents TurboFect (Thermo Scientific, Cat #: R0531) and DharmaFECT (Horizon, Cat#: T-2004-02) were used for siRNA transfection to optimize transfection conditions of siRNA into HEK293. Cells were collected at different time points (24, 48, 72h) after transfection and analyzed by western blot (Figure 3.2 A, B).

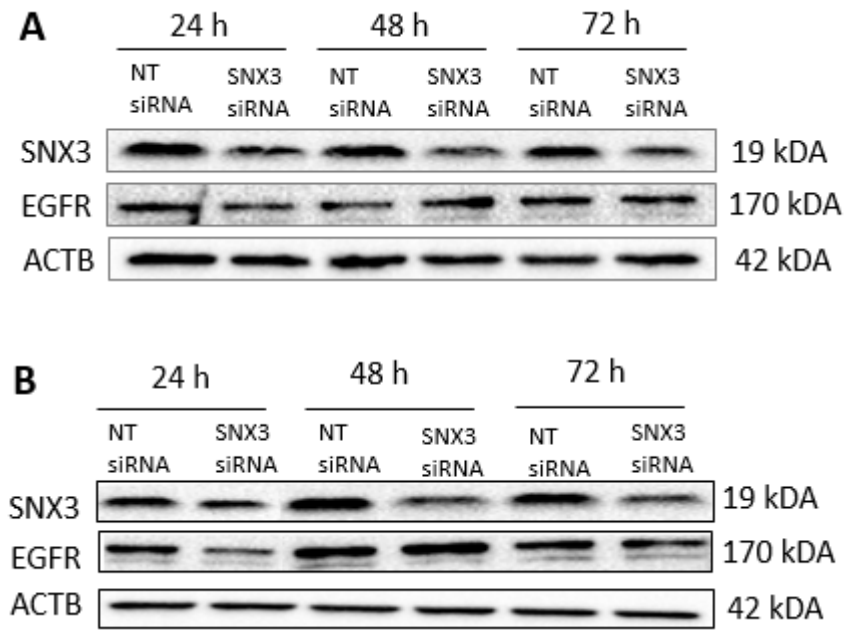


Figure 3.2. Western Blot analysis of SNX3, EGFR protein expressions in NT siRNA and SNX3 siRNA transfected HEK293 cells.

(A) Cells were transfected with 50 nM NT and SNX3 siRNA using TurboFect (Thermo Scientific, Cat #: R0531) and collected at different time points (24, 48, 72 h). 50 µg protein was loaded to 10% gel. SNX3: ProteinTech polyclonal antibody, Cat #: 10772-1-AP, 1:500 dilution was prepared in 0.1%

TBST with 5%BSA. EGFR: Santa Cruz Cat#: sc-373746 monoclonal antibody, 1:500 dilution was prepared in 0.1%TBS-T with 5% skim milk. ACTB: Santa Cruz Cat#: sc-47778 monoclonal 1:2000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control. (B) Cells were transfected with 50 nM NT and SNX3 siRNA using DharmaFECT (Horizon, Cat#: T-2004-02) and collected at different time points (24, 48, 72 h). 50 μg protein was loaded to 10% gel. SNX3: ProteinTech polyclonal antibody, Cat #: 10772-1-AP, 1:500 dilution was prepared in 0.1% TBST with 5%BSA. EGFR: Santa Cruz Cat#: sc-373746 monoclonal antibody, 1:500 dilution was prepared in 0.1%TBS-T with 5% skim milk. ACTB: Santa Cruz Cat#: sc-47778 monoclonal 1:2000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control.

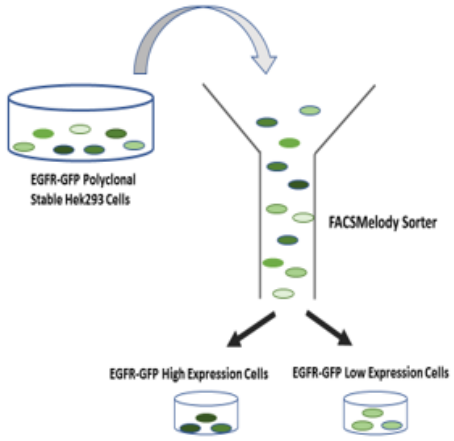
SNX3 knock down was confirmed at 24, 48 and 72 hours after transfection. Next, I checked EGFR levels in SNX3 knock-down cells. In both transfection conditions, EGFR protein level decreased in 24h along with SNX3 expression, and then compensated its protein expression in 48h and 72h.

To begin understanding how SNX3 siRNA effects EGFR protein levels, we decided to generate a stable HEK293 cell line using GFP tagged EGFR construct for further investigations.

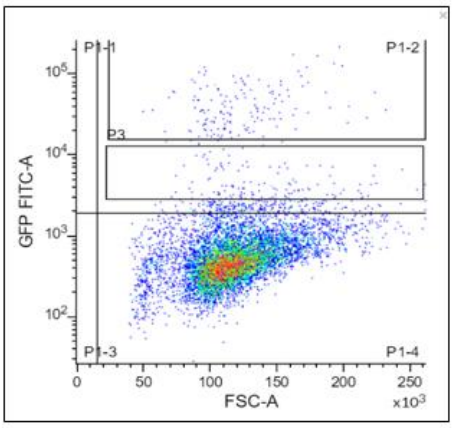
3.3 Cell Sorting of EGFR-GFP Polyclonal Stable HEK293 Cells

EGFR-GFP (Addgene Cat#: 32751) was transfected into HEK293 cells. We then sorted the high or low EGFR-GFP expressing cell populations. (Figure 3.3 A). For sorting cells, the BD FACSMelody cell sorter and FACS Chorus software were used (Figure 3.3 B). After sorting cells, EGFR-GFP high expression cells and EGFR-GFP low expression cells visualized with Floid Cell Imaging Station (Figure 3.3 C). EGFR-GFP protein levels were also detected and confirmed by western blot (Figure 3.3 D).

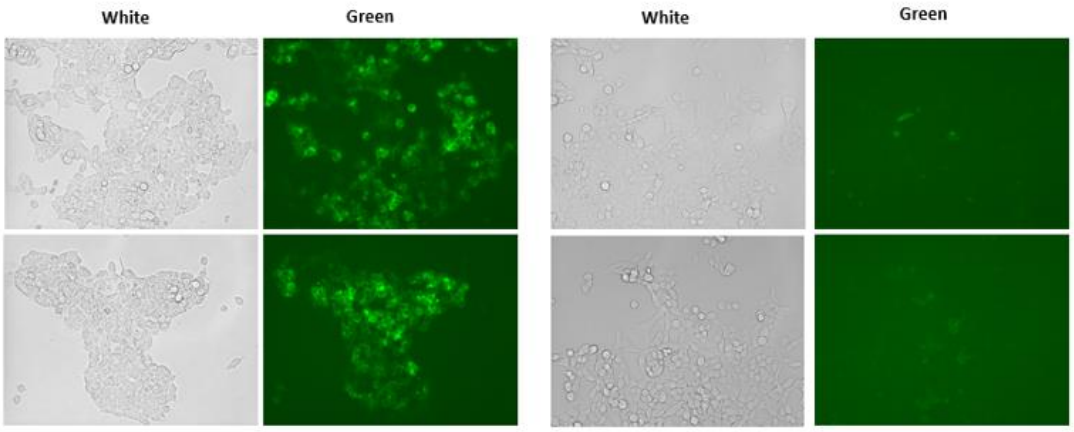
A


EGFR-GFP Polyclonal
Stable Hek293 Cells
FACSMelody Sorter
EGFR-GFP High Expression Cells
EGFR-GFP Low Expression Cells

B


P1-1
P1-2
P3
P1-3
P1-4
GFP FITC-A
FSC-A
×10³

C


EGFR-GFP High Expression stable HEK293 cells
EGFR-GFP Low Expression stable HEK293 cells
White
Green
White
Green

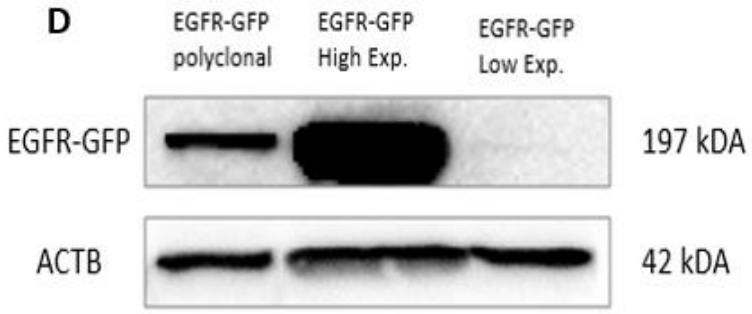


Figure 3.3. Cell Sorting of EGFR-GFP polyclonal HEK293 cells. (A) Scheme of EGFR-GFP polyclonal stable cells sorting. (B) Selection of GFP negative (bottom), GFP low positive (middle) and GFP high positive (top) HEK293 cells. (C) Images of EGFR-GFP high expression cells and EGFR-GFP low expression cells that are visualized by Floid Cell Imaging Station. (D) Western blot analysis of EGFR-GFP protein levels in EGFR-GFP High Expression and EGFR-GFP Low Expression HEK293 cells. 45 µg protein was loaded to 10% gel. GFP AB: Santa Cruz Cat#: sc-9996 1:500 dilution was prepared in 0.1% TBS-T with 5% skim milk. ACTB AB: Santa Cruz Cat#: sc-47778. 1:2000 dilution used in 0.1% TBS-T with 3% BSA. ACTB was used as a loading control.

Consequently, we decided to use these sorted cells for SNX3 siRNA transfections and analyze the cell distribution of GFP tagged EGFR and the changes of EGFR protein level.

3.4 Effects of SNX3 siRNA on EGFR Localization in EGFR-GFP Low Expression HEK293 Cells

EGFR-GFP (Low) HEK293 cells were transfected with SNX3 siRNA (Dharmacon, Cat#:SO-2660749G). Cells were treated SNX3 siRNA for 24, 48, 72h and were visualized with Leica Microsystem Fluorescent Microscope with 63x magnification (Figure 3.4 A, B). Images of cells were taken by using Andor Camera. After visualization, cells' lysates were collected and analyzed with western blot (Figure 3.4 C).

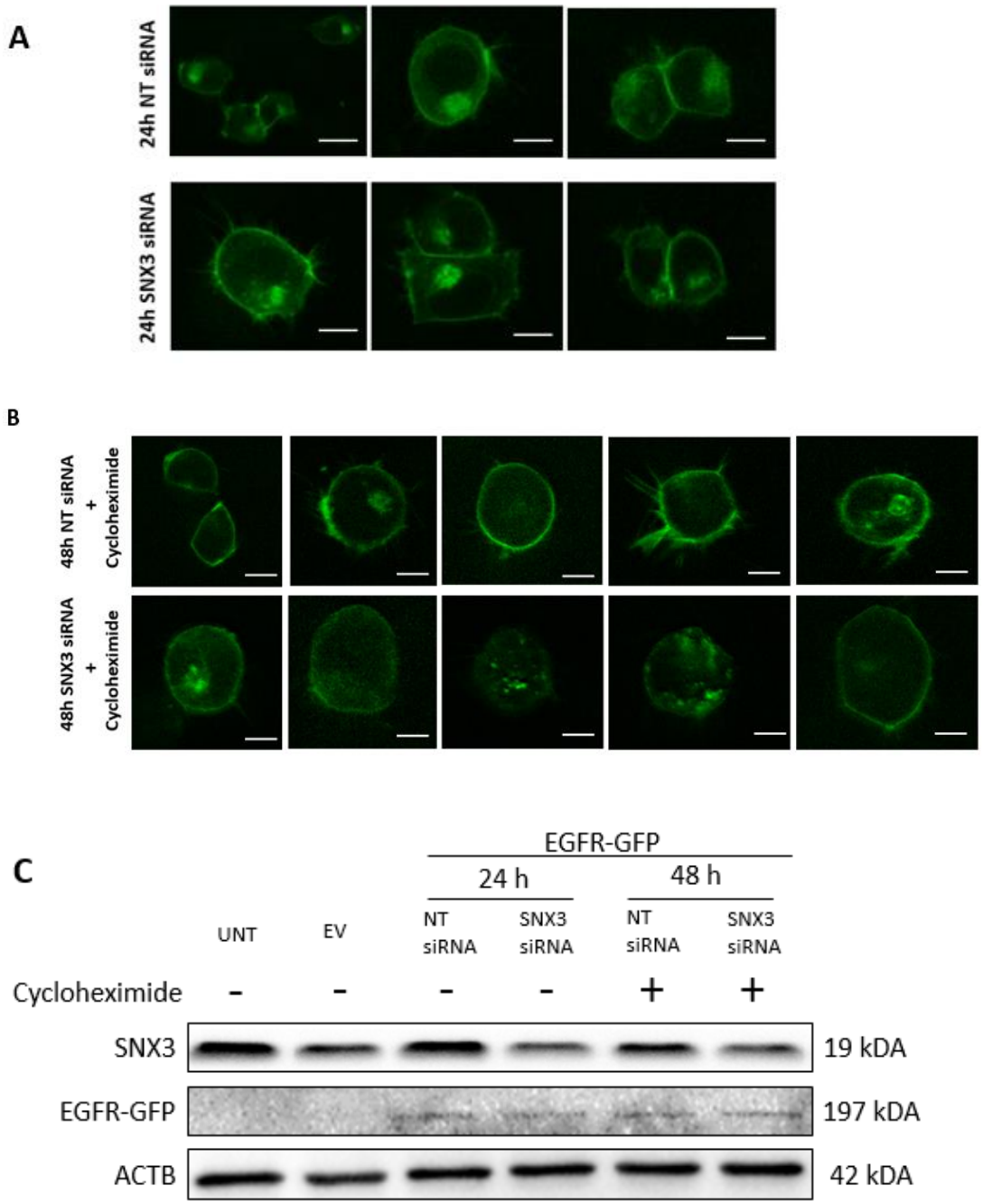


Figure 3.4. SNX3 siRNA transfection into EGFR-GFP low expression HEK293 cells. (A) Microscopic images of 24h SNX3 and NT siRNA transfected EGFR-GFP low expression HEK293 cells. Scale bar: 10 µm. (B) Microscopic images of 48h SNX3 and NT siRNA transfected EGFR-GFP low expression HEK293 cells. Cells were treated with 5 µg/ml cycloheximide for 24h. Scale bar: 10 µm. (C) Western blot analysis of EGFR-GFP expression in NT siRNA and SNX3 siRNA transfected EGFR-GFP low

expression HEK293 cells. Transient transfection was performed in time dependent manner. 50 µg protein was loaded to the gel. SNX3 AB: Proteintech polyclonal antibody. Cat #: 10772-1-AP. 1:500 dilution was prepared in 0.1% TBST with 5% BSA. GFP AB: Santa Cruz Cat#: sc-9996 1:500 dilution was prepared in 0.1% TBS-T with 5% skim milk. ACTB AB: Santa Cruz Cat#: sc-47778. 1:2000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control.

As a result; for 24 h SNX3 siRNA transfected cells, we did not observe any significant changes of EGFR-GFP cell distribution in SNX3 siRNA cells when we did comparison with NT siRNA. In NT and SNX3 siRNA transfected cells, GFP tagged EGFR localization was seen both on cell membrane and inside the cells. For 48h siRNA transfection with 24h cycloheximide treatment, we again did not able to tell any difference about EGFR localization under SNX3 silenced conditions.

Next, to better distinguish subcellular structures that might be relevant to where EGFR might be found, we performed a double transfection of mcherry tagged golgi resident protein (Beta-1,4-galactosyltransferase 1) and SNX3 siRNA to stable EGFR-GFP low and high expressing HEK293 cells to identify where we might observe the intracellular EGFR signal.

3.4.1 Double Transfection of Golgi-mCherry Protein and SNX3 siRNA into sorted EGFR-GFP Stable HEK293 Cells

Mcherry tagged golgi localization protein (Beta-1,4-galactosyltransferase 1) and siRNAs targeting SNX3 double transfection was done into EGFR-GFP low and high expression HEK293 cells, separately. For EGFR-GFP low expression cells, 72h after double transfection, 24-hour cycloheximide treated cells were visualized under Spinning Disc Confocal Microscope (Figure 3.5 A).

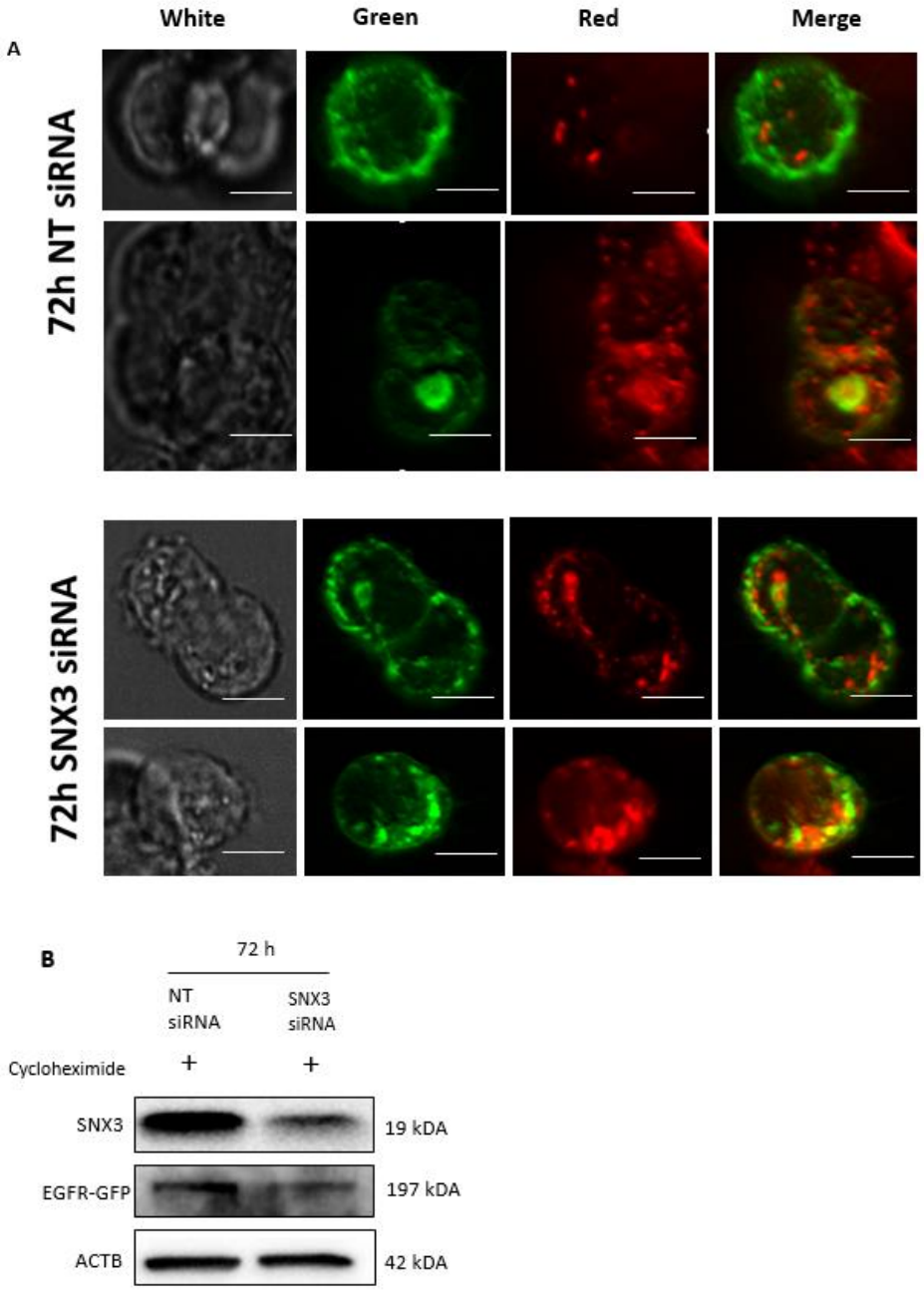


Figure 3.5. Double transfection into EGFR-GFP low expression HEK293 cells. (A) Microscopic images of double transfection of 72h SNX3 siRNA and mCherry tagged golgi location protein (Beta-1,4-galactosyltransferase 1) (red) into EGFR-GFP (green) low expression HEK293 cells. Cells were treated with 5 µg/ml cycloheximide for 24 h. Scale bar: 10 µm. (B) Western blot analysis of EGFR-GFP expression in 72h NT siRNA and SNX3 siRNA transfected EGFR-GFP low expression HEK293 cells. Transient transfection was performed e dependent manner. Cycloheximide treatment was done

for 24 h. 45 µg protein was loaded to the 10% gel. SNX3 AB: Proteintech polyclonal antibody. Cat #: 10772-1-AP. 1:500 dilution was prepared in 0.1% TBST with 5% BSA. GFP AB: Santa Cruz Cat#: sc-9996 1:500 dilution was prepared in 0.1% TBS-T with 5% skim milk. ACTB AB: Santa Cruz Cat#: sc-47778. 1:2000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control.

Based on our observation, for 72h SNX3 siRNA transfected EGFR-GFP low expression cells, we have seen decreased EGFR-GFP protein level in SNX3 siRNA cells with comparison with control siRNA (Figure 3.5 B). This reduction in EGFR-GFP protein level may be explained by recycling deficiency of EGFR in SNX3 silenced cells. Therefore, the internalized EGFR, that cannot be recycled by SNX3, may be transported to lysosome for degradation. However, despite the western blot result, we did not observe any changes of EGFR intensity and localization in the SNX3 and control siRNA transfected cells by microscopy.

As for; EGFR-GFP high expression cells, cells were visualized after 48h transfection with 24-hour cycloheximide treatment (Figure 3.6 A).

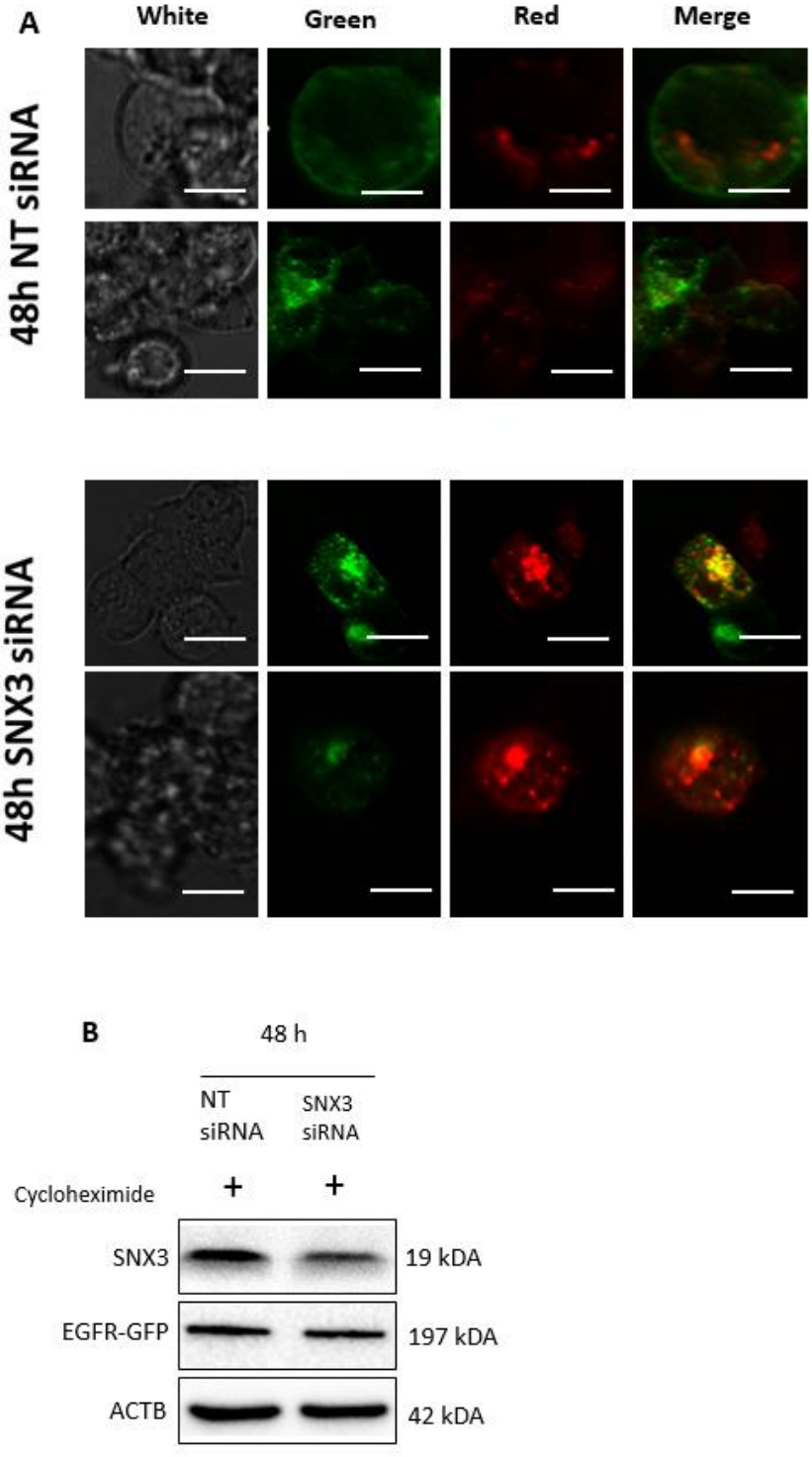


Figure 3.6. Double transfection into EGFR-GFP high expression HEK293 cells. (A) Microscopic images of double transfection of 48h SNX3 siRNA and mCherry tagged golgi location protein (Beta-

1,4-galactosyltransferase 1) (red) into EGFR-GFP (green) high expression HEK293 cells with 24h cycloheximide treatment. Scale bar: 10 µm. (B) Western blot analysis of EGFR-GFP expression in 48h NT siRNA and SNX3 siRNA transfected EGFR-GFP high expression HEK293 cells. Transient transfection was performed in time dependent manner. 35 µg protein was loaded to the 10% gel. SNX3 AB: Proteintech polyclonal antibody. Cat #: 10772-1-AP. 1:500 dilution was prepared in 0.1% TBST with 5% BSA. GFP AB: Santa Cruz Cat#: sc-9996 1:500 dilution was prepared in 0.1% TBS-T with 5% skim milk. ACTB AB: Santa Cruz Cat#: sc-47778. 1:2000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control.

We observed slightly decreased EGFR-GFP protein level in SNX3 silenced cells, however microscopy was not conclusive to suggest subcellular localization changes of EGFR in SNX3 siRNA transfected cells. This may be resulted from the disadvantages of using the HEK293 cell line, which appears as overlapping globular cells.

Next, we have decided to improve the EGFR expressing models by introducing mutations into the potential interaction motif with SNX3.

3.5 Site Directed Mutagenesis of Possible SNX3 Binding Motifs

To understand whether SNX3 and EGFR interact, we decided to do EGFR site-directed mutagenesis on its possible SNX3 binding motifs. First, we searched EGFR sequence to find possible candidate SNX3 binding motifs. To do this, we used *Scansite 4.0 silico* motif finder. According to motif features determined by Lucas et al. 2016, *Scansite 4.0 silico* motif finder detected a motif on EGFR protein sequence which corresponds to ^{762}EAYVMASV^{769} (Table 3.3). In this motif; M (Methionine) (P0) stands for central amino acid which is valid for being L/M in central amino acid position. Moreover; V (Valine) (P-1) and A (Alanine) (P-3) represents as hydrophobic

amino acids, Y (Tyrosine) (P-2) stands for being bulky amino acid and E (Glutamate) obey the motif description as being negatively charged amino acid.

EGFR_HUMAN	Show 1 match	RecName: Full=Epidermal growth factor (click to expand)	134.29	6.26

Matches of pattern [HKRDE][GAVILM][FYW][GAVILM][LM][A-Z][A-Z][A-Z] in protein EGFR_HUMAN (UniProtKB/Swiss-Prot)

```
    1 MRPSGTAGAA LLALLAALCP ASRALEEKKV CQGTSNKLTQ LGTFEDHFLS LQRMFNNCEV VLGNLEITYV QRNYDLsFLK TIQEVAGYVL IALNTVERIP
  101 LENLQIIRGN MyyENSyALA VLSNYDANKT GLKELPMRNL QEILHGAVRF sNNPALCNVE SIQWRDIVSS DFLSNMSMDF QNHLGSCQKC DPSCPNGSCW
  201 GAGEENCQKL TKIICAQQCS GRCRGKSPsD CCHNQCAAGC TGPRESDCLV CRKFRDEATC KDTCPPLMLY NPTTYQMDVN PEGKYSFGAT CVKKCPRNYV
  301 VTDHGSCVRA CGADSYEMEE DGVRKCKKCE GPCRKVCNGI GIGEFKDSLS INATNIKHFK NCTSISGDLH ILPVAFRGDS FTHTPPLDPQ ELDILKTVKE
  401 ITGFLLIQAW PENRTDLHAF ENLEIIRGRT KQHGQFSLAV VSLNITSLGL RSLKEISDGD VIISGNKNLC YANTINWKKL FGTSGQKTKI ISNRGENSCK
  501 ATGQVCHALC sPEGCWGPEP RDCVSCRNVS RGRECVDKCN LLEGEPREFV ENSECIQCHP ECLPQAMNIT CTGRGPDNCI QCAHyIDGPH CVKTCPAGVM
1:601 GENNTLVWKY ADAGHVCHLC HPNCTYGCTG PGLEGCPTNG PKIPsIAtGM VGALLLLLVV ALGIGLFMRR RHIVRKRtLR RLLQERELVE PLtPsGEAPN
  701 QALLRILKET EFKKIKVLGs GAFGtVyKGL WIPEGEKVKI PVAIKELREA TsPKANKEIL DEAyVMAsVD NPHVCRLLGI CLTSTVQLIT QLMPFGCLLD
  801 yVREHKDNIG SQYLLNWCVQ IAKGMNyLED RRLVHRDLAA RNVLVKTPQH VKITDFGLAK LLGAEEKEyH AEGGKVPIKW MALESILHRI yTHQSDVWSY
  901 GVTVWELMTF GSKPyDGIPA SEISSILEKG ERLPQPPICt IDVyMIMVKC WMIDADSRPK FRELIIEFSK MARDPQRyLV IQGDERMHLP sPtDsNFyRA
 1001 LMDEEDMDDV VDADEyLIPQ QGFFssPsTs RtPLLSsLsA tsNNstVACI DRNGLQsCPI KEDsFLQRys sDPtGALtED sIDDtFLPVP EyINQsVPKR
 1101 PAGsVQNPVy HNQPLNPAPs RDPHyQDPHs tAVGNPEyLN tVQPtCVNSt FDsPAHWAQK GsHQIsLDNP DyQQDFFPKE AKPNGIFKGs tAENAEyLRV
 1201 APQSSEFIGA
```

Table 3.3. Output table result from the Scansite 4.0. Blue colored letters represent the location of the motif that is found on the protein sequence.

In addition, we found an alternative motif on EGFR cytoplasmic tail which is ^{1015}DEYLIPQQ^{1022}. Regarding this motif, I (Isoleucine) found as a central amino acid. Despite of not being L/M, it shows hydrophobic features. Additionally, hydrophobic L (Leucine) (P-1), bulky amino acid Y (Tyrosine) (P-2) and negatively charged D (Aspartic Acid) (P-4) valid motif residues for motif description. There is an exception of E (Glutamate) (P-3), it shows charged amino acid features instead of being hydrophobic. However, this exception for this motif position is also seen for other cargo proteins.

After identifying possible SNX3 binding motifs on EGFR, we followed the Tabuchi et al. 2010 paper in which site-directed mutagenesis of DMT1-II retromer binding motif lead to mis-sorting of the receptor by preventing retromer and SNX3 interaction (Tabuchi et al. 2010). Hence, we aimed to do same mutagenesis on the possible binding motifs that we have found on EGFR.

Deletion of ^{555}YLL^{557} amino acids on DMT1-II, which corresponding to P0, P-1, P-2 positions of the motif, leads to complete mis-sorting of DMT1-II. In other words, this mutation dramatically decreases the ability of endosomal recycling DMT1-II. Moreover, DMT1-II binding affinity to retromer is reduced to <65% compared to wild type when the central amino acid Leucine substituted to Alanine (Tabuchi et al. 2010).

Therefore, we generated these mutations on the potential SNX3 interaction motif of EGFR by site-directed mutagenesis (Figure 3.7).

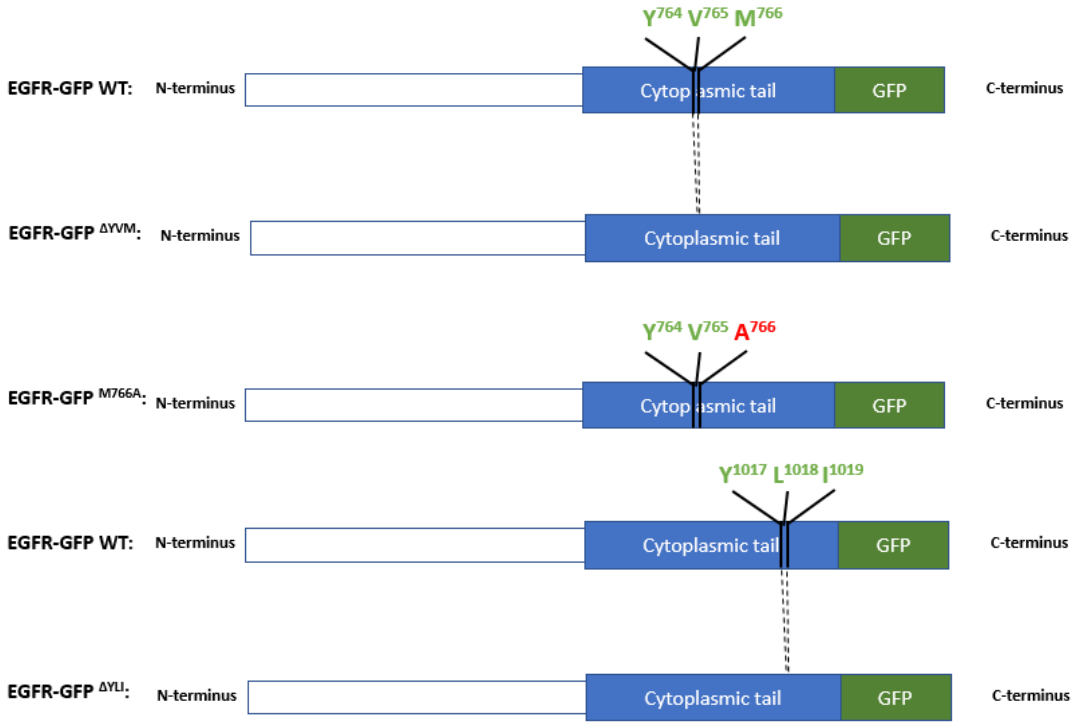


Figure 3.7. Schematic representation of EGFR-GFP mutants which are achieved by site-directed mutagenesis. Mutated residues are corresponding to possible SNX3-retromer binding motifs.

Because of the problems we had with HEK293 cells in terms of cell shape, these mutant constructs of EGFR-GFP were transfected into HeLa cells and visualized

under Leica Microsystem Fluorescent Microscope with 63x magnification (Figure 3.8).

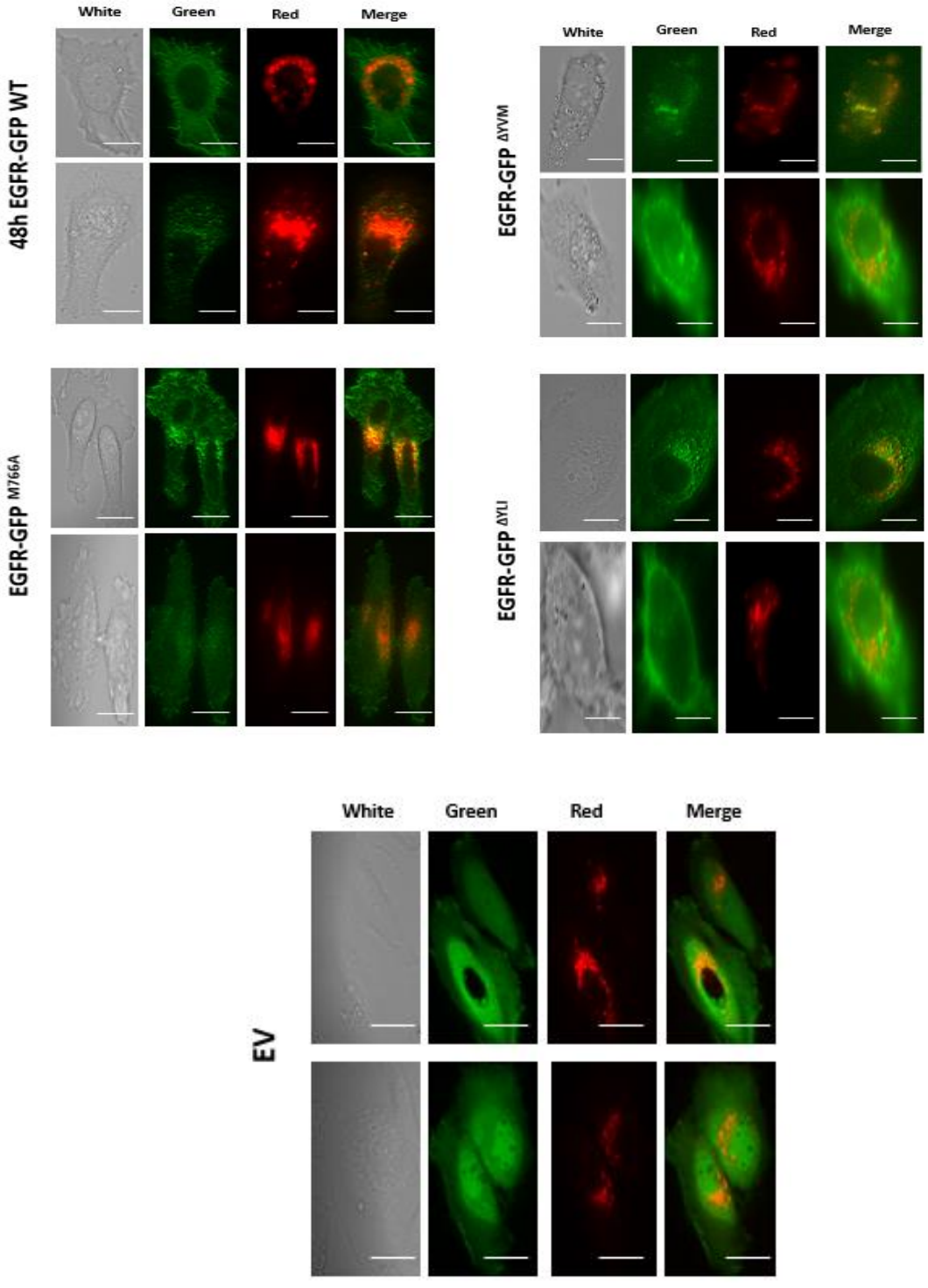


Figure 3.8. Microscopic images of double transfection of EGFR-GFP WT, EGFR-GFP mutants, EV (pEGFP-N1) (green) and mCherry tagged golgi location protein (Beta-1,4-galactosyltransferase 1) (red) into HeLa cells. Cells were transfected for 48h and images were taken at that time point. Scale bar: 10 μm.

Comparing images of WT and EGFR mutant transfected cells, we did not observe significant changes in EGFR localization when we generated mutations on possible motifs on EGFR. There can be several reasons. First, EGFR may use another recycling motif besides our identified motifs. Second, our identified motifs still can be valid, however; deletion of one of them can be compensated by the second motif. For future experiments, both motifs should be tested together.

Next, we took a different approach and aimed to study the interaction between SNX3 and EGFR by Co-Immunoprecipitation (Co-IP).

3.6 Investigation of Potential Interaction Between EGFR and SNX3

Investigating whether SNX3 is interacting with EGFR, Co-Immunoprecipitation (Co-IP) Assay was performed. At first, we did immunoprecipitation by using GFP-Trap® (Chromotek). To do this, we used stable EGFR-GFP polyclonal HEK293 cells.

Despite having clear results of EGFR and SNX3 on negative control lane and success in EGFR immunoprecipitation by GFP-Trap, we did not detect SNX3 in GFP IP sample (Figure 3.9).

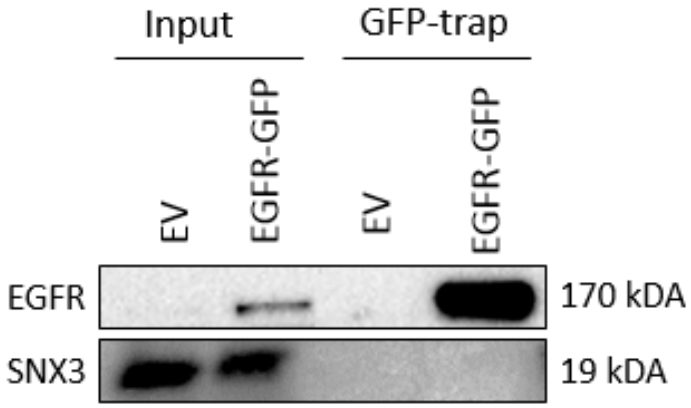


Figure 3.9. GFP-trap with EGFR-GFP in EGFR-GFP polyclonal HEK293 cells. Cells expressing EV (p-EGFP-N1) and EGFR-GFP were lysated and incubated with GFP-trap beads. Immunoprecipitated proteins were analyzed by western blotting method. EGFR: Santa Cruz Cat#: SC-373746 monoclonal antibody, 1:500 dilution was prepared in 0.1%TBS-T with 5% skim milk. SNX3: ProteinTech polyclonal antibody, Cat #: 10772-1-AP, 1:500 dilution was prepared in 0.1% TBST with 5%BSA.

We think the interaction between EGFR and SNX3 may be quite temporary and transient. Another possibility could be relatively low EGFR levels in the polyclonal HEK293 cells. We concluded that it would be better to repeat this experiment in higher EGFR expressing cells. Therefore, next, I used MDA-MB-231 cell line which is a triple negative breast cancer cell line with EGFR overexpression.

EGFR antibody was used to do immunoprecipitation proteins from MDA-MB-231 cells. Following Western blot analysis showed EGFR was immunoprecipitated as expected (Figure 3.10).

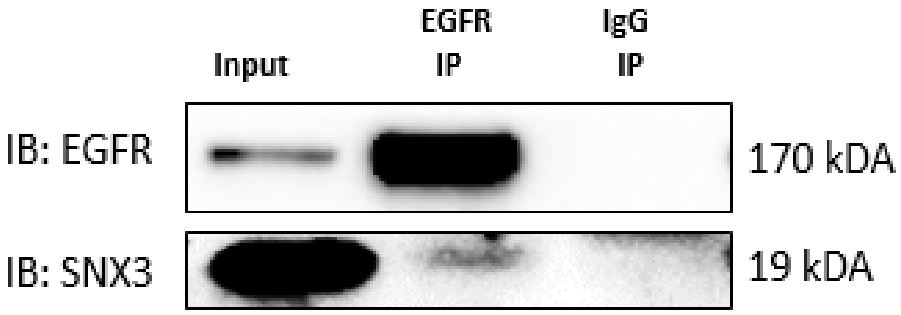


Figure 3.10. Cell lysates were immunoprecipitated and analyzed by western blotting method. EGFR antibody (1:500, Santa Cruz), and SNX3 antibody (1:500, PTG Lab) were used to detect EGFR and SNX3, respectively. IgG antibody (TNPO1, ab10303) was used as a negative control for Co-IP experiment. IP: immunoprecipitation; IB: immunoblot.

Finally, we were also able to observe SNX3 was pulled down with EGFR antibody.

This preliminary result may suggest that this interaction is very transient, and it is very much dependent on EGFR protein levels.

Overall, we obtained preliminary data suggesting EGFR and SNX3 might interact based on a Co-IP experiment. Our efforts with microscopy unfortunately did not yield conclusive results due to the selected cell line.

CHAPTER 4

CONCLUSION

SNX3 as a retromer protein has been linked to recycling of specific receptor proteins one of which is DMT1-II. DMT1-II and WLS, another known interacting partner with SNX3 share a common motif, thought to be important for cargo recognition by SNX3.

We identified other possible cargo proteins that harbor this SNX3 recycling motif on their cytoplasmic tails. To do that, we used in silico motif analysis tool; *Scansite 4.0*. Among identified candidate SNX3 cargo proteins, EGFR was selected because SNX3 was earlier shown to be regulated by EGF treatment (Akman et al. 2015). Therefore, in this study, we tested the hypothesis that SNX3 might interact with EGFR.

First, we generated tagged EGFR expressing cells with the prospect of microscopy studies. Then we used RNAi to silence SNX3 in HEK293 cells to test whether EGFR levels would be affected. Indeed, EGFR levels were decreased in 24h SNX3 siRNA transfected HEK293. Interestingly, EGFR levels were up again at 48h and 72h.

However, to observe more robust results, these polyclonal cells were sorted into low and high expression EGFR-GFP cells with using BD FACSMelody.

Sorted cells expressing EGFR-GFP (Low) were used in SNX3 siRNA transfection and cells were visualized under Leica Microsystem Fluorescent Microscope with 63x magnification. Based on the result, we have seen the EGFR-GFP signal on both cell membrane and intracellular in both SNX3 knock down and control cells.

To make a precise observation about EGFR-GFP localization, we did double transfection of SNX3 siRNA and mcherry tagged golgi localization protein (Beta-1,4-galactosyltransferase 1) into HEK293 cells expressing EGFR-GFP (Low and High) cells, separately. However; we were not able to reach a conclusion partly because of

the round shape and sticky nature of HEK293 cells. Despite of that, EGFR-GFP protein level was decreased in SNX3 silenced cells detected by western blotting. This result enabled us to propose that EGFR protein level might be low due to decreased recycling in case of SNX3 silencing.

We also did site-directed mutagenesis on possible SNX3 binding motifs of EGFR which we found via *Scansite 4.0* silico motif finder. Based on previous findings, we did deletion and amino acid substitution on the possible SNX3 binding residues to test receptor-retromer interaction. Unfortunately, we did not observe any differences in SNX3 silenced cells when we did comparison with WT EGFR-GFP and NT siRNA transfected cells. GFP tagged EGFR signal was detected on both cell membrane and within the cell. The reason why we were unable to see a difference in EGFR subcellular localization could be due to the fact that two potential motifs can be both functional and that deletion of one of them was not enough to prevent SNX3 and EGFR interaction. In addition, our tagged receptors can still dimerize with WT endogenous receptors and still be recycled.

Finally, we tested EGFR-SNX3 interaction by using Co-Immunoprecipitation (Co-IP) assay. We performed Co-IP experiments. We were able to see EGFR-SNX3 interaction in Co-IP experiments with using EGFR overexpressed MDA-MB-231 cells. These preliminary results based on our works should be investigated further to understand the EGFR-SNX3 interaction better. With the help of further investigations, we will able to propound the dynamics of EGFR recycling dependent on SNX3-retromer.

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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 dH2O

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

Running Buffer:

25 mM Tris base

190 mM Glycine

0.1% SDS

Transfer Buffer:

200 ml Methanol

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

Immunoprecipitation Buffer:

150 mM NaCl

10 mM Tris-HCl (pH 7.4)

1 mM EDTA

1% Tripton X-100

0.5% NP-40

10X phosSTOP

25X protease inhibitor

Lyse Buffer:

10 mM Tris/Cl (pH 7.5)

150 mM NaCl

0.5 mM EDTA

0.5% NP-40

Dilution/Wash Buffer:

10 mM Tris/Cl (pH 7.5)

150 mM NaCl

0.5 mM EDTA

B. CONFORMATION OF SITE-DIRECTED MUTAGENESIS

Table B.1. *DNA Blast Result of EGFR-GFP ΔYVM which shows the deleted 9 nucleotides that corresponding to YVM amino acids*

Table B.2. *DNA Blast Result of EGFR-GFP M766A which shows the substitution of Alanine (GCC) for Methionine(ATG)*

Table B.3. *DNA Blast Result of EGFR-GFP ΔYLI which shows the deleted 9 nucleotides that corresponding to YLI amino acids.*

C. MARKER

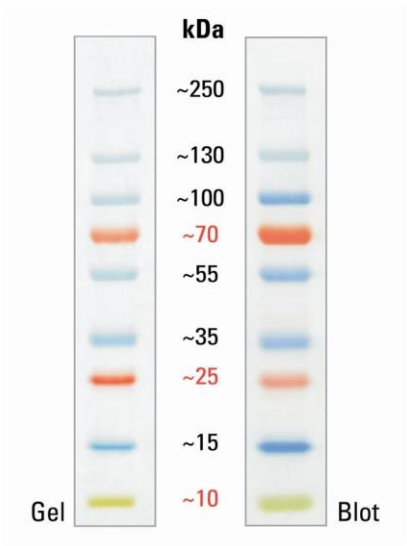

Figure C.1. Protein Ladder.

Thermo Scientific Page Ruler Plus Prestained 10-250 kDa Protein Ladder was used in Western Blot experiments.