INVESTIGATION OF SNX3 IN COLON CANCER

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ABSTRACT

INVESTIGATION OF SNX3 IN COLON CANCER

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Sorting Nexin 3 (SNX3) is part of the retromer complex that recycles cargo receptors back to plasma membrane or to Trans Golgi Network. WNT ligand carrier protein Wntless (WLS) is a known SNX3 cargo protein. Our earlier data suggested over expression of SNX3 in colon cancer cells. Considering its importance in receptor recycling, we hypothesized SNX3 to be a potential modulator of cancer related receptors. To begin understanding the role of SNX3, we developed RNAi models of SNX3 in SW480 colon cancer cells and investigated biochemical and phenotypical outcomes of SNX3 knock down. Here we present evidence that SNX3 might regulate several receptors important in colon cancers and decreased SNX3 expression alters motility and migration of colon cancer cells by downregulating important signaling pathways with implications in epithelial to mesenchymal transition.

Keywords: SNX3, WLS, EGFR, EMT

ÖZ

KOLON KANSERİNDE SNX3'ÜN ARAŞTIRILMASI

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Retromer kompleksinin bir parçası olan SNX3, kargo reseptörlerini hücre zarına ya da golgi aygıtı ağına geri kazandırır. WNT ligandını taşıyan protein Wntless (WLS) ise SNX3'ün kargo proteini olarak bilinir. Önceki verilerimiz, SNX3'ün kolon kanseri hücrelerinde fazla ifade edildiği fikrini vermiştir. Reseptör geri kazanımının önemi düşünüldüğünde SNX3'ün kanserle ilişkilendirilmiş reseptörleri düzenlediği hipotezini kurduk. SNX3'ün rolünün anlaşılmasına başlamak için, kolon kanser hücre hattı olan SW480 hücrelerinde RNAi modeli geliştirdik ve SNX3'ün susturulmasının sebep olduğu biyokimyasal ve fenotipik etkileri araştırdık. Elde ettiğimiz bulgular SNX3'ün kolon kanseri vakalarında önem taşıyan pek çok reseptörü regüle edebileceğini, azalan SNX3 ifadesi ise epitelden mezenkimale geçiş ile ilişkili önemli sinyal yolaklarini etkileyerek kolon kanseri hücrelerinin hareketliliğini ve göçünü etkileyebileceğini desteklemektedir.

Anahtar Kelimeler: SNX3, WLS, EGFR, EMT

To Hamide Tokol and Ercüment Tokol

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

AP2	Clathrin adaptor protein 2
APA	Alternative polyadenylation
BAR	Bin/Rvs/Amphiphysin
BSA	Bovine Serum Albumin
CI-MPR	Cation Independent Mannose 6 Phosphate Receptor
CME	Clathrin Mediated Endocytosis
DMT1	Divalent Metal Transporter 1
Dvl	Dishevelled
EE	Early Endosome
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
ERK1/2	Extracellular Signal-Regulated Protein Kinases 1 and 2
FERM	4.1/Ezrin/Radixin/Moesin
GSK-3β	Glycogen synthase kinase 3
MAPK	Mitogen-Activated Protein Kinase
MIT	Microtubule-Interacting and Trafficking
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NT	Non-Target

PCP	Planar Cell Polarity
PI3K	Phosphoinositide 3-Kinase
PI3P	Phosphatidylinositol 3-Phosphate
plgR	Polymeric Immunoglobulin Receptor
PM	Plasma Membrane
PX	Phox Homology
PXA	PX-associated domain A
PXC	PX- Associated domain C
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNX3	Sorting Nexin 3
SNXs	Sorting Nexins
TGN	Trans-Golgi Network
TWIST1	Twist Family BHLH Transcription Factor 1
VPS26	Vacuolar Protein Sorting 26
VPS29	Vacuolar Protein Sorting 29
VPS35	Vacuolar Protein Sorting 35
WASH	Wiskott–Aldrich Syndrome Protein and SCAR Homolog
WLS	Wntless
ZEB1	Zinc finger E-box Binding Homeobox1
ZO-1	Zonula Occludens 1

CHAPTER 1

INTRODUCTION

1.1. SNXs

Sorting nexins (SNXs) are a large group of proteins consisting of more than 30 members [1].

SNX family members are classified according to their domain structures. Members of sorting nexins have a combination of the following domains: Bin/Rvs/Amphiphysin domain (BAR), Phox homology domain (PX), 4.1/Ezrin/Radixin/Moesin-like domain (FERM), PXA (PX-associated domain A), RGS (Regulator of G-protein signaling), PXC (PX- associated domain C) and MIT (microtubule-interacting and trafficking molecule domain) [2] (Figure1.1).

Among these domains, PX domain is important for membrane trafficking, cell signaling and organelle motility. This domain interacts with phosphatidylinositol 3-phosphate (PI3P) [4]. PI3P is a phospholipid and like other phosphoinositide, it has a central role in membrane trafficking, motility and cellular organization [5]. PI3P is present in cell membranes as well as endosomes.

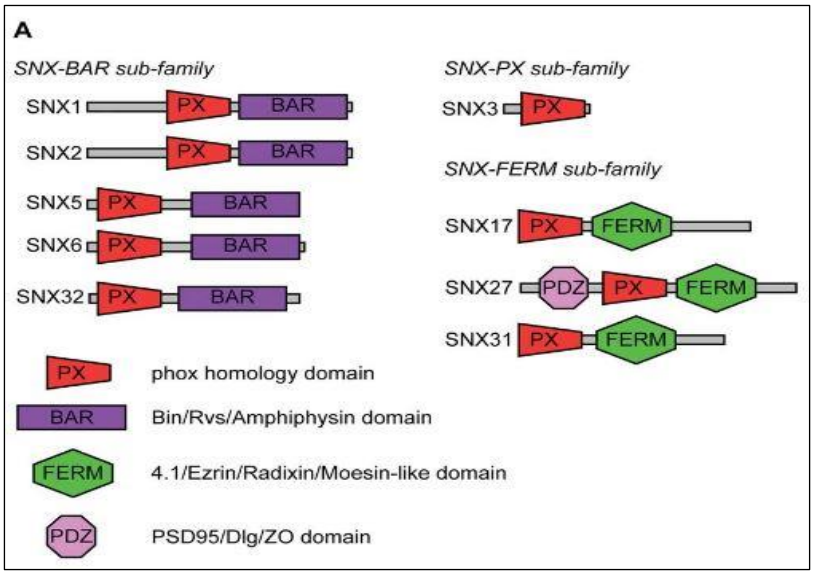

Figure 1.1. Domains of SNX family.

Four domains that SNX family belongs are illustrated. These domains are required to associate with retromer. (Figure taken from Gallon, 2015) [3]

Sorting nexins are generally associated with the retromer complex, which has a role in endosomal protein sorting and recycling of proteins. Retromers harbor additional structural proteins including Vacuolar protein sorting 26 (VPS26), Vacuolar protein sorting 29 (VPS29) and Vacuolar protein sorting 35 (VPS35) proteins which are part of the cargo recognition complex. VPS26, VPS29 and VPS35 trimer and SNX proteins generally form a complex to recognize cargo molecules with in the retromer.

Retromer complex recruited to the endosome carries cargo proteins to the trans-Golgi network (TGN) or plasma membrane (PM). Cargo proteins are generally membrane associated proteins and they ultimately have two fates. The first fate is lysosomal degradation after endocytosis, and the second one is recycling back to PM or to TGN. If cargo proteins are recycled by the retromer complex, they evade lysosomal

degradation. The balance between degradation and reuse of cargoes are crucial for regulation of biological processes.

Considering that, activated receptor molecules or cargo receptors are recycled to continue their functions, it is important to understand how retromer structures are formed and what provides the specificity of this process.

1.1.1. SNX3

SNX3 is a small protein that belongs to SNX family and it has only the PX domain. SNX3 maps to chromosome 6 in human genome and encodes a 162 amino acid long peptide. Molecular weight of the protein is 18762 Da.

Recent findings suggest the importance of VPS proteins along with SNX proteins to provide specific recognition of cargo molecules. Among various SNX proteins, SNX3 is starting to gain more attention due to its specificity in cargo selection. It was shown that among SNXs, only SNX3 is indispensable for recognition and recycling of WLS (Wntless) in Drosophila [6]. WLS is a cargo receptor that carries WNT ligands from TGN throughout the secretory pathway [7]. WLS is one of the well-studied cargo proteins that is recycled by SNX3 [8]. On the other hand, crystallographic analysis showed the binding of DMT1-II, SNX3 and retromer complex [9].

Structural studies started to reveal the significance of SNX3 in cargo specificity. For association of VPS26 and VPS35, specific sequence 106PRLYL110 is required. [10]. After association of VPS26 and VPS35, SNX3 is required for binding of VPS26 and VPS35 association complex to membranes [9]. PI3P is enriched at endosomal membranes, which is an essential phospholipid for binding PX containing proteins [11]. For efficient recruitment of retromer proteins to the endosomes, SNX3 binding to PI3P on endosome through its PX domain is required. In the meantime, RAB7A GTPase is also recruited to the endosome. Consequently, VPS35, VPS26 and VPS29 trimer interacts with the endosome. SNX3 almost functions a bridge by interacting with the PI3P and the VPS trimer (Figure1.2). Binding of retromer to the SNX3 is

thought to cause a conformational change on retromer complex which creates a binding surface for cargo proteins [3].

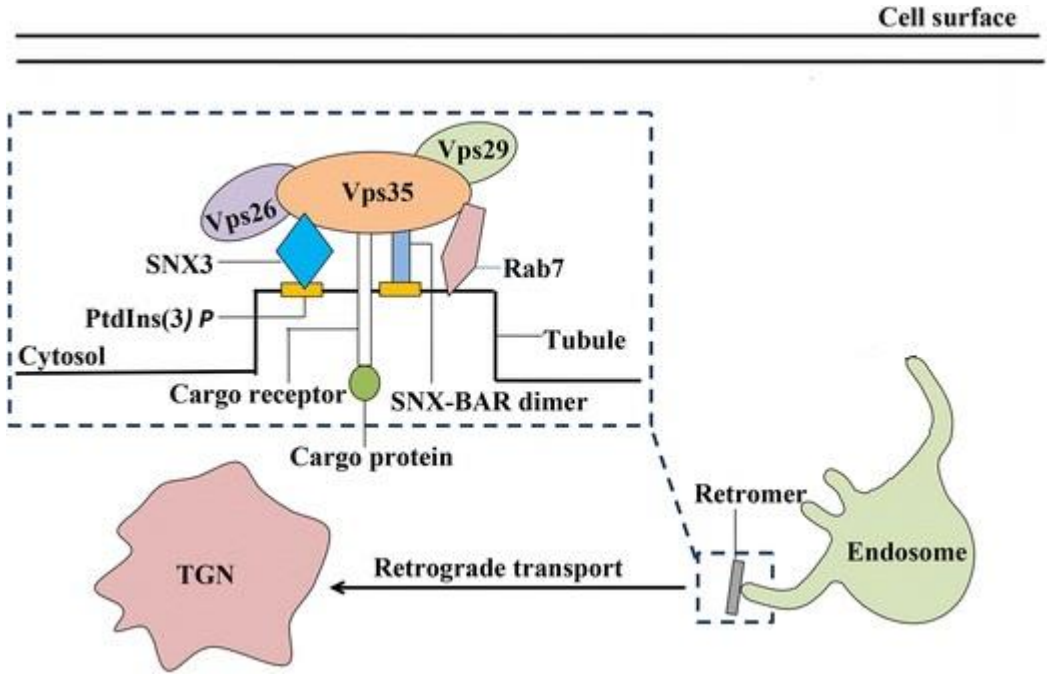

Figure 1.2. Retromer complex and SNX3.

In cargo selective process, VPS35 creates a surface for binding VPS26 and VPS29. SNX3 bonded with PtdIns(3)P (PI3P) and Rab7 physically links retromer complex to the endosome. These associations are required for cargo binding and retrograde transport to TGN. (Figure taken from Zhang, 2016) [12]

Earlier we identified SNX3 to be alternatively polyadenylated (APA) to generate a shorter 3'UTR isoform in breast cancers [13]. Our lab also showed shorter 3'UTR isoform to produce protein more efficiently than the longer isoform, suggesting overabundance of the protein through APA. These findings suggested that SNX3 might function as an oncogene. SNX3 was already shown to be important for the recycling of WLS back to TGN. When we searched the literature, we found evidence

that other plasma membrane proteins such as EGFR might also be recycled by SNX3 dependent retromer pathway [14]. Interestingly, in this study aspirin was shown to enhance recruitment of SNX3 to the early endosome where EGFR is recycled.

In short, considering that WNT signaling and EGFR pathway are among highly deregulated pathways in colon cancers, we hypothesized that deregulated SNX3 may have diverse effects on these pathways.

For sorting, specific signals are required. Most of the sorting signals in cargo proteins are on cytoplasmic tails. The ØX(L/M) motif is thought to be required for interaction with SNX3 association complex. Association of SNX3, VPS26 and VPS35 creates binding site for ØX(L/M) motif which is found in cargo proteins [9].

Wntless (WLS), divalent metal transporter 1 (DMT1-II), cation independent mannose 6 phosphate receptor (CI-MPR) and polymeric immunoglobulin receptor (plgR) are examples of proteins that have this motif [9].

Potential cargo receptors that harbor this motif have vital and broad range of role in embryonic development, tissue homeostasis [15], iron homeostasis in cell [16], lysosomal enzyme trafficking [17] as well as immune response [18].

1.2. WNTLESS

One of the known cargo proteins that interact with SNX3 and retromer complex is WLS [6]. WLS is a member of G-protein coupled receptor family. It is also known as Evenness, Interrupted/Sprinter in Drosophila, MOM-3/ Mig-14 in *C. elegans* and GPR177 in mammals.

WLS has a long N terminal region. It also has 7 or 8 transmembrane segment and like GPCR superfamily, C terminus tail falls into cytoplasm [15]. There are many motives in WLS, but important ones are indicated in (Figure 1.3). WLS is a WNT ligand carrier receptor. WNT binding domain is found in N terminus next to second transmembrane region. This domain is essential for interaction with WNT. The relation between WNT binding domain and WNT1, WNT3 and WNT5a was shown [19]. WNT ligands are

produced in ER and post translationally modified. Porcupine leads to palmitoylation of cysteine rich residues and C-terminus serine 209 residue [20]. Palmitoylation is an essential process for binding WNT ligands to WLS. If Porcupine cannot add palmitoyl group to the Serine 209, there are defects in WNT secretion from cells (15). Therefore, WLS is indispensable for WNT secretion. WNT signaling pathway plays role in embryonic development and homeostasis [21]. Therefore, dysregulation in WNT signaling pathway leads to many human diseases like embryonic development dysfunction and cancer [22]. WLS takes palmitoylated WNT ligands, from endoplasmic reticulum and carries it to the plasma membrane. After WNT ligand is secreted to the extracellular matrix, WLS is internalized to the early endosome (EE) [23]. YXXθ is an important motif that plays role in endocytosis of WLS (Figure 1.3). This motif acts as a signal for clathrin adaptor protein 2 (AP2) to internalize WLS from plasma membrane [24].

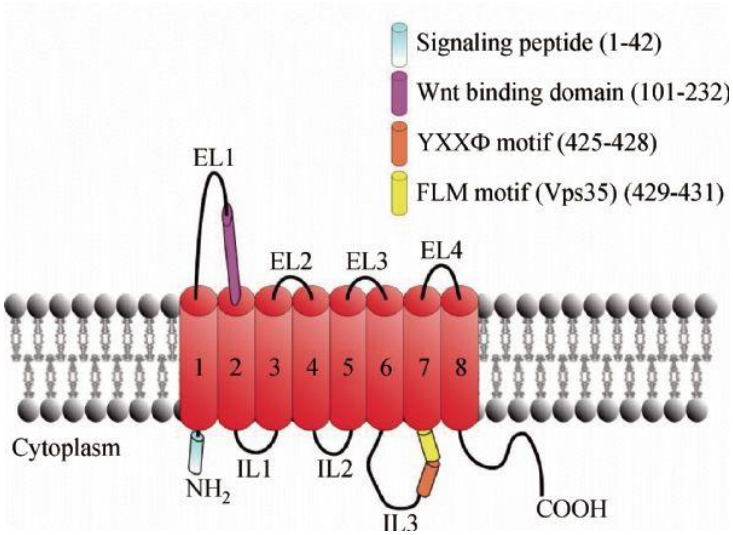

Figure 1.3. Structure of WLS.

Important domains and motives for protein-protein interaction are illustrated. (Figure taken from Das, 2012) [15]

Clathrin mediated endocytosis is a general internalization process of receptors, nutrients and cell surface materials. Clathrin triskelion protein is an essential scaffold protein that makes a tubule formation from inside face of the cytoplasm. PIP2 and AP2 accumulation is essential for formation of clathrin complex. For growth of the clathrin coated pit, proteins that contains BAR domains are needed. Some member of SNX family with BAR domains may have roles at this endocytosis stage [25].

Endocytosed WLS is recycled from early endosome to the TGN (Figure 1.4) by SNX3-retromer complex [6] or directly to the cell membrane [7]. This recycling saves WLS from lysosomal degradation and eventually WLS goes back to TGN where it starts to help WNT secretion again [7]. Therefore, recycling of WLS is important for efficient WNT secretion [8]. It appears only that only SNX3 is indispensable for WLS recycling [26].

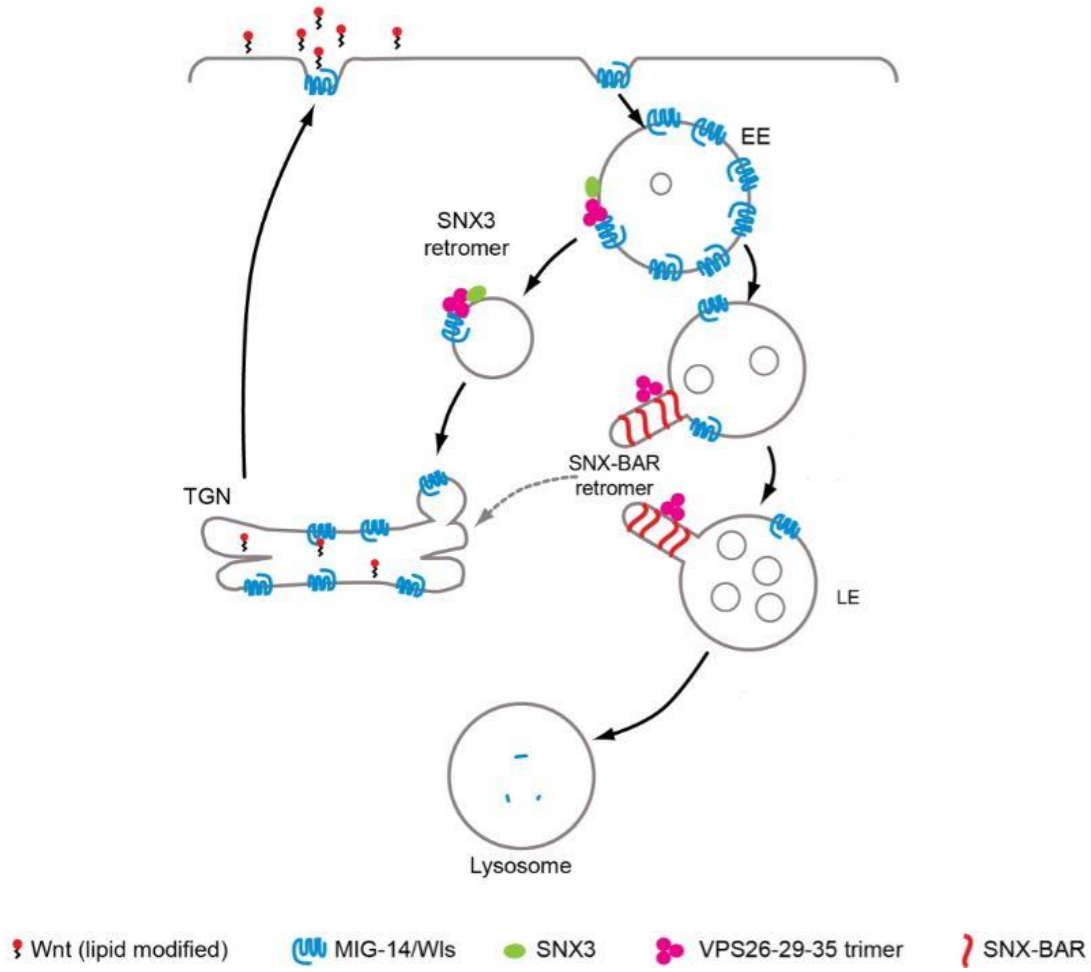

Figure 1.4. Secretion of WNT ligands and recycling of WLS.

WNT ligands are taken from TGN and carried to the plasma membrane by WLS. After disassociation of WLS and its cargo, WLS is endocytosed from plasma membrane. In the early endosome (EE), WLS is recycled by SNX3 and retromer complex association to TGN. (Figure taken from Lorenowicz, 2014) [27]

1.2.1. Downstream of WNT Secretion

After WLS aids secretion of WNT ligands to the extracellular matrix, canonical WNT pathway is triggered by interaction of WNT ligand with its receptors which are low density lipoprotein receptor related proteins LRP5 and LRP6. In the absence of WNT ligands, β-catenin and E-cadherin destruction complex APC/Axin/GSK-3β leads to degradation of β-catenin and E-cadherin by ubiquitylation. In the presence of the WNT ligands, WNT pathways are triggered, and Dishevelled (Dvl) is activated. When Dvl is activated, GSK-3β is phosphorylated and leads to disfunction of β-catenin destruction complex. Eventually it leads to accumulation of β-catenin in cytoplasm. Accumulated β-catenin is translocated to the nucleus where it associated with TCF/LEF that are transcription factors [28].

There are non-canonical WNT signaling pathways as well, which are called β-catenin independent pathways. These pathways are Planar cell polarity pathway (PCP) and WNT-Ca2+ pathway. PCP pathway couples short term cytoskeletal reorganization and control planar cell polarity by activating Ras and RAC1 gene family. Ultimately, they affect RHOA and JNK to control cytoskeleton and gene expression [29].

WNT-Ca2+ is the other non-canonical WNT signaling pathway. In this pathway, WNT receptor activates heterotrimeric G proteins. These G proteins activate PLC CamK2 and PKC. Effectors of this pathway control transcription factors that determine cell fate and migration [30].

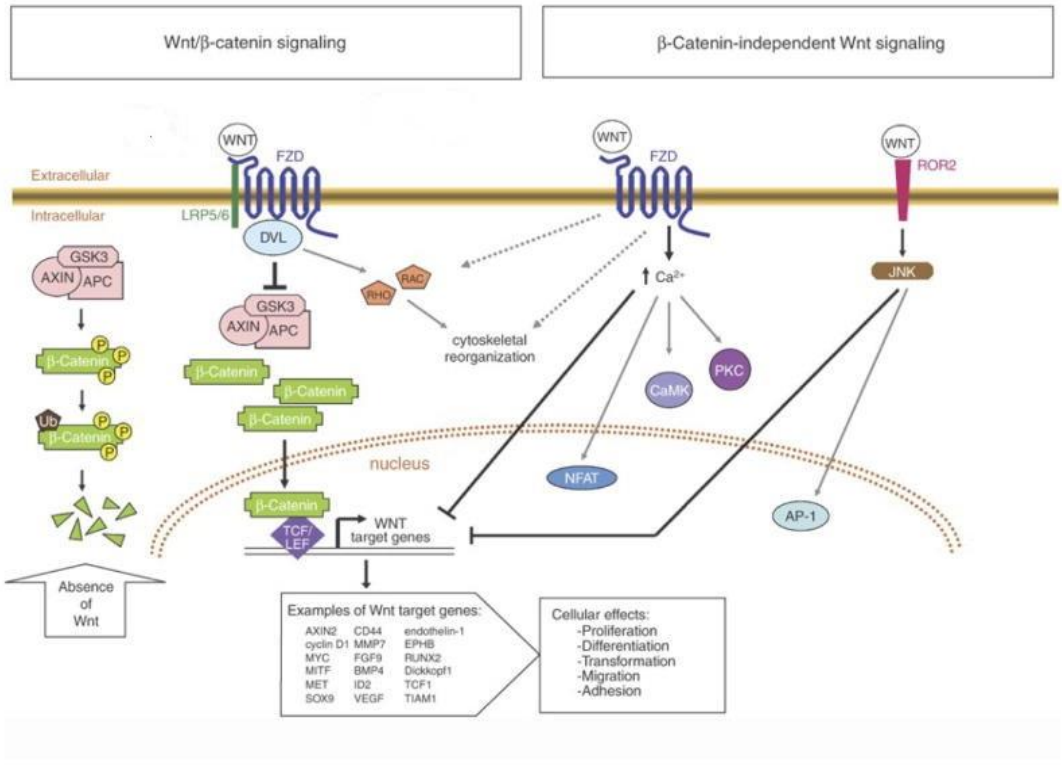

Figure 1.5. Canonical and non-canonical WNT signaling pathway.

Figure represents β-Catenin dependent and β-Catenin independent signaling pathways. In the absence of WNT ligands, β-Catenin destruction complex (GSK3-AXIN-APC) phosphorylate β-Catenin and leads to degradation. In the presence of WNT ligands, DVL inhibits β-Catenin destruction complex and leads to accumulation of β-Catenin in cytoplasm. Accumulated β-Catenin translocate to the nucleus and leads to regulate WNT target genes. WNT-Ca2+ and PCP pathway also regulates WNT target genes. These genes have role in proliferation, differentiation, transformation, migration and adhesion of cells. (Figure taken from Freese, 2010). [30]

To sum up, WLS is the only known WNT ligand carrier and is recycled by SNX3 dependent retromer complex [26]. Therefore, we hypothesize SNX3 to play an important role in WNT signaling pathway.

1.3. EGFR

Epidermal Growth Factor receptor (EGFR) is a receptor tyrosine kinase and belongs to ErbB receptor tyrosine kinases family [31]. Aberrant regulation of EGFR has been linked to various cancers (35).

Some pro-oncogenic signaling pathways are found downstream of the EGFR pathway. For example, activation of EGFR by EGF in Hela cells leads to phosphorylation of 2244 proteins at 6600 sites [32]. As a result, migration [33], proliferation [34], growth [35], and differentiation [36] of cells can be stimulated by the activation of EGFR signaling network.

Aberrant activation of EGFR may lead to increased transcriptional expression and gene amplification [119]. Elevated EGFR level may relate with poor prognosis in cancers such as colorectal cancer [120], lung cancer [121] and endometrial cancer [122]. EGFR level is a determinant factor for tumor size, patient prognosis and relapse of cancer.

For activation of EGFR, ligands are required. There are seven known EGFR ligands. They are EGF, TGF-α, AREG, EREG, BTC, HB-EGF and EPI [37]. Each ligand activates EGFR similarly. Following the ligand binding, EGFR dimerizes and is cross phosphorylated which initiates signaling cascades. Stimulated EGFR activates three main cascades;1) ERK MAPK, 2) AKT-PI3K and 3) PLC-γ1-PKC pathways (Figure 1.6). Activated EGFR is then internalized by endocytosis into early endosomes [38]. When EGFR is activated by EGF, it is internalized by either clathrin mediated endocytosis (CME) [39] and clathrin independent endocytosis which includes Caveolin mediated endocytosis and micropinocytosis [40]. Nevertheless, main EGFR endocytosis mechanism is CME [41]. Endocytosed EGFR carrying endosome either fuses with lysosomes or is transformed into a retromer structure [38] for recycling to cell surface [42]. CME leads EGFR to be found in endosomes that contain Rab5. After these endosomes become mature, they are recycled back to plasma membrane. If EGFR is found in late endosome which contain Rab7, it is ubiquitinated. Ubiquitinated

EGFR is directed to the intraluminal vesicles that forms multivesicular body where EGFR is degraded [43].

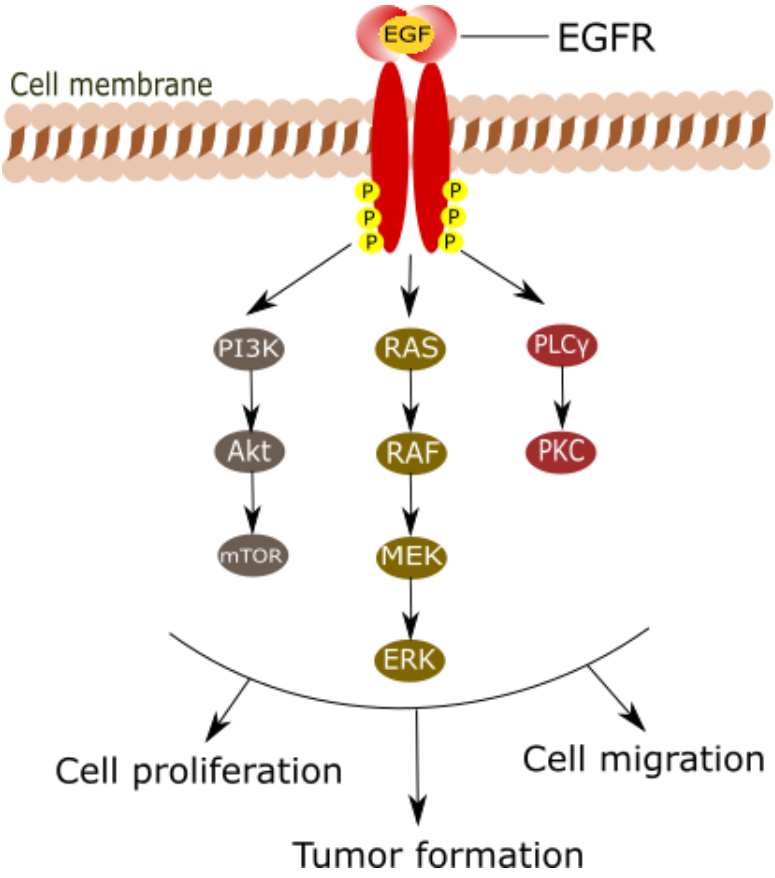

Figure 1.6. Overview of EGFR signaling pathway.

EGFR is activated by binding of EGF. EGF leads to homodimerization and cross phosphorylation of EGFRs. Stimulated EGFR can activate downstream signaling pathways like PI3K/AKT/mTOR, RAS/RAF/MEK/ERK and PLCγ/PKC. These activated pathways have role in cell proliferation, cell migration and tumor formation.

Retromer complex comes into stage while EGFR is sorting to the lysosome. Retromer complex which contain VPS35, VPS26, VPS29 and SNX3 has been linked to the WASH complex (Wiskott–Aldrich syndrome protein and SCAR homolog complex) which is necessary for sorting of EGFR to lysosome [44] [45]. It was also shown that

deletion of WASH1 from T cell of transgenic mice resulted defects of EGFR recycling from endosome to cell surface [46]. Close relation between WASH and retromer complex is putting SNX3 in a critical place for EGFR regulation. It was shown that downregulation of SNX3 led to increased EGFR degradation [14]. Although there are extensive studies about EGFR trafficking, the mechanism behind EGFR regulation by SNX3 is not clear yet.

1.4. Epithelial to Mesenchymal Transition

Transition of epithelial cells into motile mesenchymal cells known as epithelial to mesenchymal transition (EMT). EMT is a crucial process for some events like wound healing, development and cancer progression. EMT is also essential process for migration and cell fate.

Regulation or dysregulation of cell proliferation, motility and migration is not monopolized by one signaling pathway or just one molecule. In this context, crosstalk between signaling pathways becomes important.

Within the scope of this thesis, we investigated downstream effects of deregulated EGFR and WNT pathways, one of which is EMT.

1.4.1. EMT Transcription Factors

Key transcription factors during mesenchymal transition are SNAIL, SLUG, ZEB1 and TWIST. They control expression of each other. They also co-regulate target genes. As a result of transcriptional profile change, epithelial cells gain some mesenchymal properties while losing some of their epithelial characteristics (Figure 1.7).

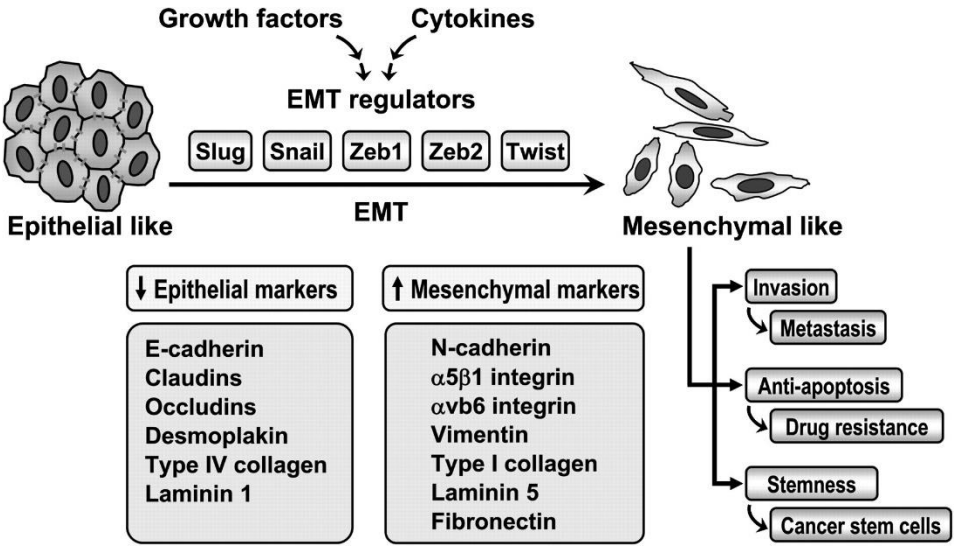

Figure 1.7. Overview of EMT markers.

Grow factors and cytokines trigger EMT regulators. Transcription factors Slug, Snail, Zeb1, Zeb2 and Twist lead to EMT and regulate epithelial and mesenchymal markers. These transitions can lead to metastasis, drug resistance and cancer stem cells. (Figure taken from Shih, 2011.) [47]

SNAIL which is a zinc finger transcription factor suppresses E-cadherin (Epithelial cadherin). Accumulation of SNAIL in nucleus is a key process to promote EMT. SNAIL directly binds the promoter region of E-cadherin and this binding represses the expression of E-cadherin [48]. Cells which have repressed E-cadherin will eventually go into mesenchymal transition. This transcription factor also plays role in wound healing and organogenesis. Like other EMT markers, SNAIL associated with metastasis especially in breast carcinoma MDA-MB-231 cells [49]. Overexpression of SNAIL is shown to increase metastasis in colorectal cancer [50].

SLUG, a member of SNAIL family, is another zinc finger transcription factor that is involved in EMT. SLUG also participates in organogenesis and wound healing. As is the case with SNAIL, SLUG also represses E-cadherin expression. [51]. It has been shown that SLUG expression is increased in colon cancer [52]. It is also reported that

SLUG expression is related with metastasis in colon cancer and expression of SLUG affects the survival of colon cancer patients [53].

Zinc finger E-box binding homeobox1 (ZEB1) also known as TCF8 is a transcription factor which promotes migration and invasion by inducing EMT. ZEB1, like SNAIL, is also regulated by signaling pathways like WNT, TGF-β and NFκB [54]. By binding E-box region on DNA, ZEB1 can regulate the target genes [55]. For example, E-box is found in the promoter region of the E-cadherin. Therefore, E-cadherin can be regulated by ZEB1 through binding the E-box region [56]. Downregulation of E-cadherin through ZEB1 and E-box interaction eventually leads to EMT induction. It was shown that overexpression of ZEB1 induce EMT in colon cancer [57]. Knocking down of ZEB1 inhibits epithelial-mesenchymal transition in breast, lung and colon cancer [58].

Another transcription factor that related to EMT is TWIST1. It belongs to a helix-loop-helix protein family. By regulating EMT, TWIST1 contributes to metastasis [59]. TWIST1 is also associated with poor prognosis in cancer. TWIST1 can also repressing E-cadherin [59].

1.4.2. EMT Cell Surface Protein

Cadherins are cell surface proteins that play roles in cell movement and tissue morphogenesis [60]. They are essential for adhesion. They are divided into three subfamilies as E (epithelial) -N (neural) and P (placental) cadherins. E-cadherin mediates epithelial cell to cell adhesion [61]. N-cadherin is subfamily of cadherins and known as EMT marker. During the EMT process, N- cadherin expression is increased, and this increase leads to alters cell adhesion [62]. Through homotypic N-cadherin interactions, cells tend to acquire more mesenchymal features and these interactions lead to migration and invasion [123]. Normally, N-cadherin is not expressed in epithelial cells however it may be expressed in some carcinomas. It has been shown that if there is a transition of expression level from E-cadherin to N-cadherin, it promotes migration and also invasion in cancer cells [63]. This is because of the

affected cell polarity since expression level of E-cadherin is decreasing and N-cadherin is increasing [64]. Higher level of N-cadherin expression is also associated with lower survival rate in colon cancer patients. This gives rise to N-cadherin to be used as prognostic marker on colon cancer [65].

Zonula occludens 1(ZO-1) known as tight junction protein and in normal epithelial cells, it is in a complex, which located at cell-to-cell adhesion membrane. When cell goes into EMT, ZO-1 is separated from complex and goes into cytoplasm and nucleus. The amount of disassociated ZO-1 from complex and translocation of it to nucleus depends on the grade of migration and differentiation [66]. ZO-1 has been linked to EMT in colorectal cancer [67]. In invasive cells, ZO-1 is required for matrix degradation [68].

1.4.3. Mesenchymal Protein

VIMENTIN is a filament protein and it has role in cytoskeleton [69]. It is upregulated during EMT and used as mesenchymal marker. This upregulation of VIMENTIN is necessary for EMT initiation [70]. Normally VIMENTIN is expressed in mesenchymal cells whereas expression level of VIMENTIN is low in epithelial cells. In mesenchymal cells integration of tissue and cells are retained by VIMENTIN [71]. VIMENTIN is associated with breast, melanoma, lung and prostate cancer and important target for cancer treatment [72]. Importantly, motility of cells in the edge of wound healing relies on VIMENTIN expression in mammary epithelial cells and breast cancer [73]. It is also known that when VIMENTIN is knocked down in fibroblast, motility and directional migration are attenuated [74].

1.4.4. Stemness

CD44 is a glycoprotein that found in transmembrane and has many isoforms due to alternative splicing [75]. It also known as cell adhesion molecule and EMT and stemness marker. CD44 participate many processes like cell adhesion, tumor

development and angiogenesis [76]. Especially in cancer development, CD44 plays crucial role. In colon cancer, like many other cancers such as liver prostate and gastric cancers, CD44 promotes EMT due to ability of downregulating epithelial markers and upregulating mesenchymal markers. [77]. CD44 is correlated with metastasis in colon and breast cancers because it enhances adhesion to endothelial cells [78]. It is also observed that adhesion ability decreased when CD44 is silenced in breast cancer cells. Surprisingly this decreased level of adhesion led to decreased invasion ability on these cells but had no effect on proliferation status [79]. Similarly, in colon cancer cell line SW480 cells, knocking down of CD44 leads to decreased ability of invasion and metastasis while overexpression of CD44 leads to increased ability of invasion and migration [80]. CD44 has a crucial role both during EMT and in migration/invasion in colon cancer [80].

1.4.5. EGFR and EMT

Recent studies have demonstrated that EGF and TGFβ1 can induce EMT. ERK1/2 and PI3K/Akt pathways can potently induce EMT [81], [82], [83], [84]. Expression of SNAIL which is a major transcription factor that leads to EMT is regulated by variety of signaling patterns like PI3K, MAPK, GSK-3β and NFκB pathways [85]. For example, EGFR pathway which is triggered by EGF induces SNAIL expression through preventing the GSK-3β activity [86]. GSK-3β phosphorylates SNAIL and leads to degradation through ubiquitination [87].

Increased CD44 expression level correlated with increased expression level of EGFR which activates PI3K/Akt in colon cancer cells [80]. In that way, CD44 was linked to invasion and migration.

It is known that, TWIST1 expression is regulated by signaling pathways components such as Akt and WNT-β Catenin [88]. AKT1 leads to increase the phosphorylation of TWIST1 which is required for degradation and ubiquitination [89]. Extensive work on EGFR and EMT indicates that there is a strong association between them.

1.4.6. WNT Signaling and EMT

WNT signaling can affect EMT from several aspects. For example, GSK3β phosphorylates SLUG and leads to its ubiquitination and proteasomal degradation which decrease EMT. However, upon activation of the canonical WNT pathway, GSK3β is inhibited causing increased availability of SLUG [90]. SLUG is an important transcription factor that is responsible for EMT. Increased SLUG levels repress E-cadherin expression. This eventually leads to increase mesenchymal properties of cell. In addition, WNT signaling activates transcription factor ZEB1 which is responsible for EMT [91].

It is known that CD44 is target of WNT signaling pathway [92] and it is upregulated by WNT signaling [93]. Studies on these EMT related proteins SLUG, ZEB1, N-Cadherin and CD44 links EMT and WNT signaling pathway.

1.5. Aim of the Study

Considering the important role of receptor recycling in maintaining cell signaling pathways in cancers, we focused on SNX3 which we identified earlier to be alternatively polyadenylated. Based on our preliminary data and existing literature, our hypothesis is that deregulated levels of SNX3 may modulate recycling of activated receptors such as EGFR and WLS. To address the hypothesis, we chose colon cancer as a model because of the significant role of EGFR and WNT pathways in colon cancers. We generated RNAi models of SNX3 in SW480 colon cancer cell lines and investigated how EGFR and WNT signaling cascades are altered.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Lines and Cell Culture

SW480 cells were a kind gift from Dr. Sreeparna Banerjee. Cells were grown in DMEM High Glucose (Biological Industries Cat #: 01-052-1A) which contains 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). Cells were incubated in 5% CO_2 incubator at 37^0C. Cells were stored in liquid nitrogen with 5% (V/V) dimethyl sulfoxide (DMSO) (Sigma, Cat#: 154938).

2.2. Transfections

SNX3 shRNA in pSUPER vector (designed and cloned by Merve Öyken, Erson Lab), NT shRNA (designed and cloned by İbrahim Özgül, Erson Lab) and pSUPER empty vector were stably transfected to SW480 cells. Transfection was done in growth medium which contains 4mg/ml G-418 (Roche, Cat#: 04727878001). After transfection, transfected cells were maintained in the 2 mg/ml G-418 containing medium. Transfections were performed with TurboFect (Thermo Scientific, Cat #: R0531) in 6-well plates. 2µg of DNA was diluted in 250 µl of serum free DMEM. 4µl TurboFect was added to the dilution and incubated for 20 minutes at room temperature. 4µl TurboFect, 2µg DNA and 250 µl DMEM containing dilution was added to the 1 well of 6-well.

SNX3 siRNA (20-50 nM) and Negative control siRNA (Qiagen, Cat#: 1027310) (20-50 nM) were transiently transfected to the SW480 cells. Transfection was performed with DharmaFECT (Horizon, Cat#: T-2004-02) according to manufacturer's manual.

SNX3 siRNA pool sequences and Negative Control siRNA target sequences are as follows:

Table 2.1. *siRNA pool sequences*

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

2.3. Protein Isolation

Total proteins were isolated from cell pellets with M-PER Mammalian Protein Extraction Reagent (Thermo, Cat#: 78501). 10 units of phosSTOP (Roche, Cat#: 04906837001) and 25X protease inhibitor (Roche, Cat#: 1187350001) were added to M-PER. Protein concentrations were measured on ice using Pierce BCA Protein Assay Kit (Thermo, Cat#: 23227) according to manufacturer's manual.

Cytoplasmic and nuclear proteins were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo, Cat#: 78835) according to manufacturer's manual.

2.4. Western Blot

50 µg protein was denatured in 6X Laemmli buffer at 100 ^{o}C for 10 minutes. Denatured proteins were run on a 5% stacking and 10% separating polyacrylamide gel. Proteins were transferred to the PVDF Western Blotting Membrane (Roche, Cat#: 03010040001) from gel. Transfer was performed in wet transfer system at 100V for 1 hour. Blocking of the membrane was done with 5% Bovine Serum Albumin (BSA) or non-fat milk in 1% Tris-Buffered saline-tween (TBS-T) for 1 hour at room temperature. Then membrane incubated with primary antibody for overnight. After that membrane was washed 3 times with TBS-T for 10 minutes.

For SNX3 protein, 5% BSA in 1%TBS-T was used to block the membrane. Then membrane was incubated with polyclonal SNX3 antibody (1:500 dilution, Cat #: 10772-1-AP, Proteintech). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For EGFR protein, 5% skim milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with EGFR antibody (1:500 dilution, Cat #: SC-373746, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-mouse antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For WLS protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with WLS antibody (1:500 dilution, Cat #: 655902, Biolegend). After that membrane was incubated with secondary anti-mouse antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour

For p-EGFR protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with p-EGFR antibody (1:500 dilution, Cat #: 2220, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For p-PLCγ1 protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with p-PLCγ1 antibody (1:1000 dilution, Cat #: 14008, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For PLCγ1 protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with PLCγ1 antibody (1:500 dilution, Cat #: 5690, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For p-AKT protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with p-AKT antibody (1:1000 dilution, Cat #: 4060, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For AKT protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with AKT antibody (1:500 dilution, Cat #: sc-8312, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For p-ERK 1/2 protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with p-ERK 1/2 antibody (1:500 dilution, Cat #: sc-16982, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For ERK 1/2 protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with ERK 1/2 antibody (1:200 dilution, Cat #: sc-514312, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-mouse antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For N-cadherin protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with N-cadherin antibody (1:1000 dilution, Cat #: 13116, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For Vimentin protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with Vimentin antibody (1:1000 dilution, Cat #: 5741, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For Twist1 protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with Twist1 antibody (1:1000 dilution, Cat #: 46702, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For ZO-1 protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with ZO-1 antibody (1:1000 dilution, Cat #: 8193, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For CD44 protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with CD44 antibody (1:1000 dilution, Cat #: 3570, CST). After that membrane was incubated with secondary anti-mouse antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For Snail protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with Snail antibody (1:1000 dilution, Cat #: 3879, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For Tcf8/Zeb1 protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with Tcf8/Zeb1 antibody (1:1000 dilution, Cat #: 3396, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For Slug protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with Slug antibody (1:1000 dilution, Cat #: 9585, CST). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For CTNNB1 protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with CTNNB1 antibody (1:500 dilution, Cat #: sc-133240, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-mouse antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For HDAC1 protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with HDAC1 antibody (1:1000 dilution, Cat #: sc-81598, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-mouse antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For α-Tubulin protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with α-Tubulin antibody (1:5000 dilution, Cat #: HRP-66031, Protein Tech). There is no secondary antibody for α-Tubulin since it is HRP conjugated.

For VPS35 protein, 5% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with VPS35 antibody (1:400 dilution, Cat #: ab97545, Abcam). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For GAPDH protein, 5% Skim Milk in 1% TBS-T was used to block the membrane. Then membrane was incubated with GAPDH antibody (1:2000 dilution, Cat #: sc-25778, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-rabbit antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

For ACTB protein, 3% BSA in 1% TBS-T was used to block the membrane. Then membrane was incubated with ACTB antibody (1:4000 dilution, Cat #: sc-47778, Santa-Cruz Biotechnology). After that membrane was incubated with secondary anti-mouse antibody (1:2000, Santa-Cruz Biotechnology) at room temperature for 1 hour.

We used Western ECL Blotting Substrates (BioRAD Clarity, Cat#: 1705060) to visualize the bands.

2.5. MTT Proliferation

Proliferation of cells was screened by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. NADPH dependent cellular oxidoreductase enzymes reduce MTT to formazan, which is insoluble and purple color. Enzyme activity correlates with the number of total cells, hence giving an indirect measure of cellular proliferation.

First, 10.000 cells were seeded to the each well of 96-well plate. 8-tehnical replicate were used for each cell type (SNX3 shRNA and NT shRNA transfected SW480 cells). Then MTT solution was prepared. 5mg Thiazolyl Blue Tetrazolium Bromide (Sigma, Cat#: M5655-1G) dissolved in 1ml Dulbecco's Phosphate Buffered Saline (Biowest, Cat#: L0615-500) and. After cells attached, 10µl MTT solution was added to each well and incubated for 4 hours at 37 ^{o}C. In the meantime, HCl-SDS solution was prepared. 1g Sodium Dodecyl Sulfate (SDS) (AppliChem, Cat#: A2263,0100) dissolved in 10ml 0.01M Hydrochloric acid (Merck, Cat#: 1.00314.2500). After incubation, 100µl HCl-SDS solution was added. Plates was incubated overnight, and Optical Density was measured at 570 nm with elisa reader.

2.6. Motility and Migration Experiments

To detect motility wound healing assay was performed.

To perform wound healing assay, 6 well plates were used. SNX3 shRNA and NT shRNA transfected SW480 cells were seeded to 6-well plates. After they reached 90% of confluency, tip of 1000 µl pipette was used to scratch cells. After scratching, debris were removed by washing 3 times with PBS. Each day, cells were captured with Olympus DP 72 microscope at 10X magnification. Washing step was repeated before every capture. After that images were analyzed with ImageJ program available at imagej.nih.gov. Scratched area was measured by wound healing tool in ImageJ program. All results were normalized to Day 0. Wound healing assay was repeated 3 times.

Migration assay was performed with xCELLigence real time cell analyzer- dual purpose (RTCA-DP). Cell invasion and migration (CIM) plate used for this experiment. CIM plate has gold sensors that reads the electric impedance to quantify cell migration in real time manner. $1x10^4$ SNX3 shRNA, NT shRNA and pSUPER empty vector transfected SW480 cells were seeded to each well. NT shRNA and pSUPER empty vector transfected SW480 cells were used as control. Migration was measured in real time at every 15 minutes for 96 hours. Assay was performed according to manufacturer protocol.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Stable and Transient Knock-down of SNX3 Gene

Earlier, we identified SNX3 to be alternatively polyadenylated in cancers including breast and colon cancers. To begin understanding the role of this switch in polyA site usage, we started looking into the function of SNX3. SNX3 is a retromer protein and takes role in receptor recycling [6]. Based on previous literature and our own experience, we think colon cancer related pathways may be highly sensitive to SNX3 dependent retromer recycling. One of the known targets of SNX3 is WLS which is a cargo receptor that carries WNT ligands from the Golgi apparatus for secretion [6]. WNT signaling plays a major role in colon tumorigenesis [94]. Another target of SNX3 is possibly EGFR [95] which involves in the development of tumor in colon cancer [96].

Given the importance of WNT and EGFR signaling in colon cancers, we started with the development of knockdown models of SNX3 in SW480 colon cancer cells which are epithelial cells derived from primary adenocarcinoma of the colon.

We cloned SNX3 shRNA and Non-target (NT) shRNA oligos into pSUPER.retro.neo+gfp vector (Oligoengine Cat#: VEC-PRT-0006) and generated stably transfected SW480 cells. Stable polyclonal cells were tested for SNX3 protein levels. Western blot analysis (Figure 3.1A) showed 25% silencing of SNX3 shRNA.

In parallel we used siRNAs (Dharmacon, Cat#: SO-2660749G) to target SNX3 and collected lysates from the transfected cells at 24 h, 48 h, 72 h and 96 h time points. We showed approximately 75% inhibition of SNX3 protein levels, determined by densitometry (Figure 3.1B). Non-targeting (NT) siRNA control had no effect on the SNX3 protein levels.

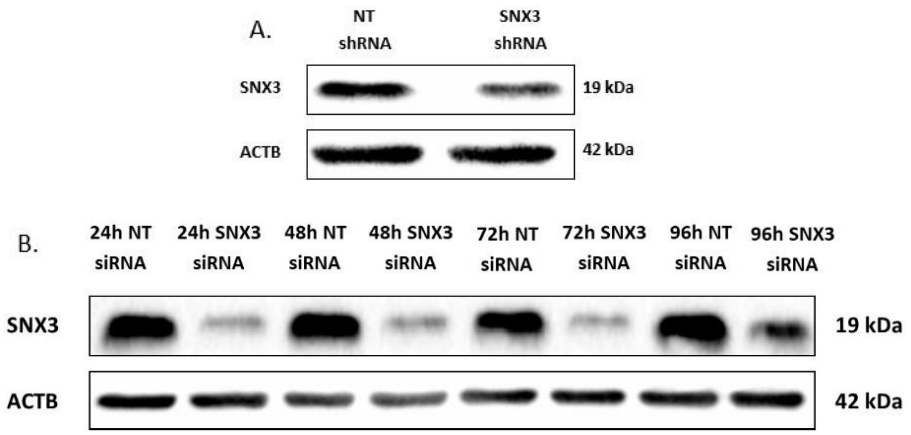

Figure 3.1. Protein expression level of SNX3 in shRNA and siRNA transfected SW480 cells.

(A) Western blot analysis of SNX3 expression in NT shRNA and SNX3 shRNA transfected SW480 cells. 50 µg protein was loaded to 10% gel. SNX3 AB: Proteintech polyclonal antibody. Cat #: 10772-1-AP. 1:500 dilution was prepared in 0.1% TBST with 5% BSA. ACTB AB: SantaCruz Cat#: SC-47778. 1:4000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control. (B) Western blot analysis of SNX3 expression in non-target siRNA and SNX3 siRNA transfected SW480 cells. Transient transfection was performed, and lysates were collected at 24, 48,72 and 96 h time points. ACTB was used as a loading control. (n=3).

Here we showed the silencing efficiency on both the shRNA and siRNA methods to target SNX3 expression.

3.2. EGFR and WLS Protein Levels in SW480 Cells

We checked the protein levels of WLS and EGFR proteins in shRNA stable-transfected SW480 cells and siRNA transfected SW480 cells with western blot analysis. EGFR and WLS protein levels were low in SNX3 shRNA cells (Figure 3.2.A). Interestingly, EGFR and WLS protein levels changed in a time dependent manner in siRNA transfected SW480 cells. For example, 24 hours after siRNA transfections EGFR and WLS levels were low, however, in time EGFR and WLS levels came back even though SNX3 was still silenced (at 96 hours). We think this recovery must be due to the dependency of the cells to EGFR and to that of as of yet unidentified receptors.

We then looked into p-EGFR which is phosphorylated at Tyr1086 site upon ligand activation [97] to understand the effect of EGFR loss on immediate downstream signaling cascades. At 24 hours where total EGFR protein was decreasing, p-EGFR(Tyr1086) was increasing. At 48 hours both total EGFR and p-EGFR(Tyr1086) protein levels did not change compared to the controls. At 72 hours, total EGFR and p-EGFR(Tyr1086) levels were high. At 96 hours where total EGFR protein level was high, p-EGFR(Tyr1086) was low. On the other hand, WLS was low at 24 and 48 h time points but it came back at 96 h time point.

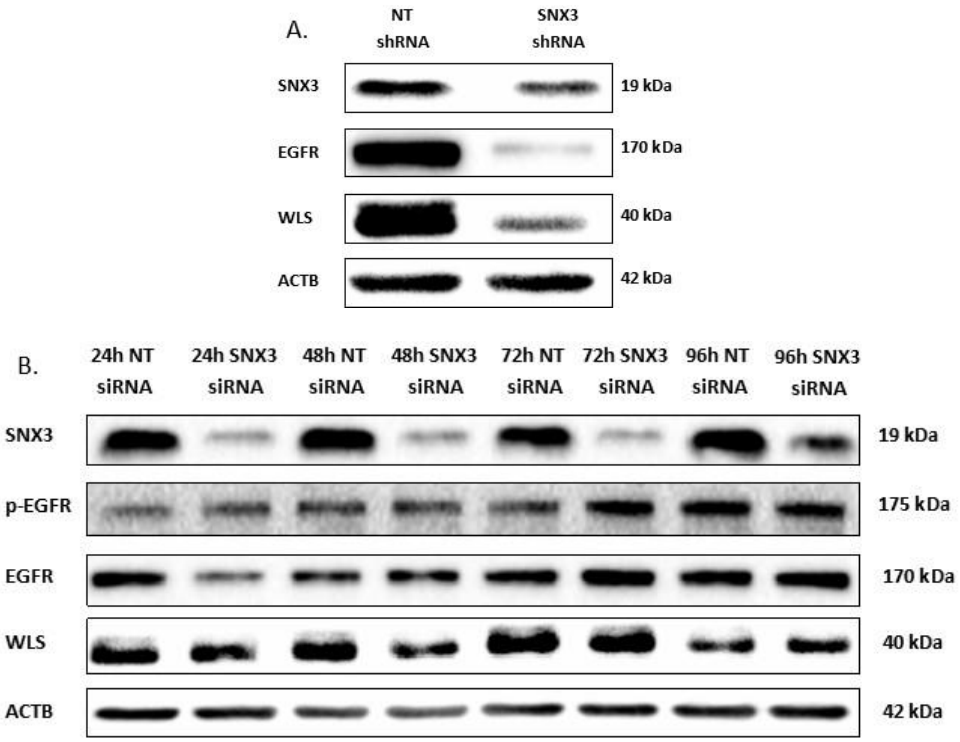

Figure 3.2. Protein expression level of EGFR and WLS in SNX3 shRNA and SNX3 siRNA transfected SW480 cells.

(A) Western Blot analysis of EGFR and WLS proteins expression in NT shRNA and SNX3 shRNA transfected SW480 cells. 50 µg protein was loaded to 10% gel. EGFR AB: SantaCruz. Cat#: SC-373746. 1:500 dilution was prepared in 0.1%TBS-T with 5% skim milk. WLS AB: Biolegend, Cat#: 655902. 1:500 dilution was prepared in 0.1% TBS-T with 5% BSA. ACTB AB: SantaCruz Cat#: SC-47778. 1:4000 dilution used in 0.1% TBS-T with 3%BSA. ACTB was used as a loading control. (B) Western blot analysis of total EGFR, p-EGFR(Try1086) and WLS expression in NT siRNA and SNX3 siRNA transfected SW480 cells. Transient transfection was performed in time dependent manner. 50 µg protein was loaded to the gel. Gel and use of antibody conditions explained above. ACTB was used as a loading control. (n=4).

This fluctuation of EGFR and WLS levels made us think that there may indeed be compensatory mechanism to maintain the survival of cells.

3.3. Proliferation of SW480 Cells

Aberrant expression of EGFR and WLS has been implicated in phenotypic outcomes in colon cancers [120]. Therefore, after we showed dramatic decrease of EGFR and WLS protein expression levels in SNX3 knocked down SW480 cells, we wanted to see the effect of these changes on cellular proliferation. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to see the effect of SNX3 knocking down on proliferation. SNX3 shRNA transfected SW480 cells were used. Interestingly, no significant change on proliferation at 0-24-48-72 and 96 h time points was observed in SNX3 shRNA stably transfected SW480 cells compared to NT shRNA transfected cells. MTT assay was also performed with SNX3 siRNA and NT siRNA transfected SW480 cells. There was no significant change in SNX3 siRNA transfected cells compared to NT siRNA transfected SW480 cells.

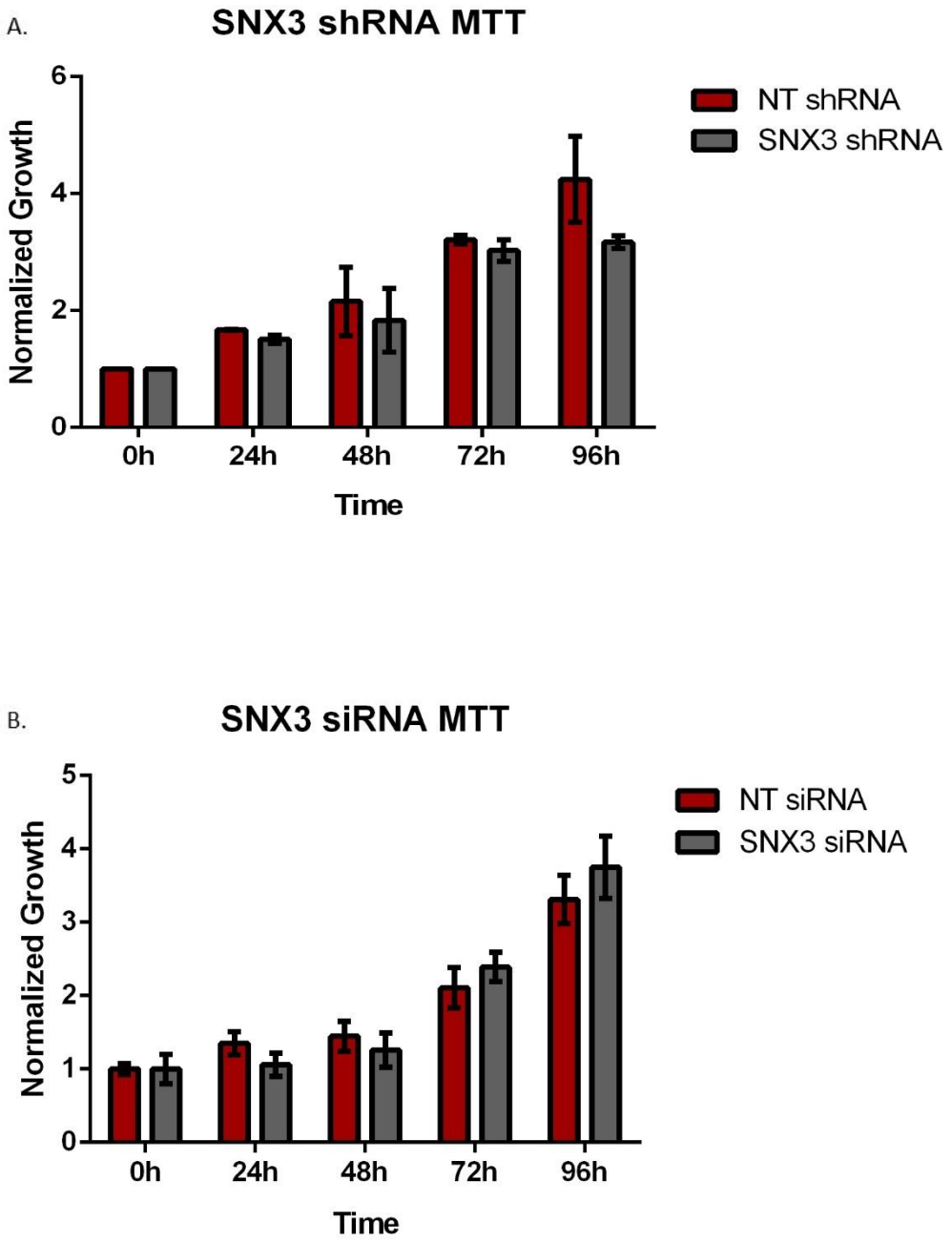

Figure 3.3. Proliferation of SNX3 siRNA and shRNA transfected SW480 cells.

(A) SNX3 shRNA and NT shRNA transfected SW480 cells growth determined by MTT assay. After tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduced to formazan, optical density was measured at 570 nm with Elisa plate reader. Viability of cells were measured after 24 h-48 h-72 h and 96 h incubation. Graph represents data from 3 independent assays n=3. It shows mean ± standard deviation. According to t-test, no significant change on proliferation. (B) SNX3 shRNA and NT shRNA transfected SW480 cells growth.

Despite EGFR and WLS protein level changes in SNX3-shRNA cells, there was no dramatic change in proliferation up until 96 hours. It is either that SNX3 regulated proteins are not directly involved in proliferation or that feedback mechanisms may compensate for the signaling pathways related to the proliferation. In fact, both of these speculations may be valid. EGF signaling has been linked to proliferation in certain cells but only to a migration phenotype in others [98]. Therefore, next, migration ability of SNX3 knocked down SW480 cells was tested.

3.4. Motility and Migration Experiments

Downstream of the EGFR signaling pathway has effect on motility and migration [96]. We first performed wound healing assay in SNX3 shRNA and NT shRNA transfected SW480 cells. SNX3 shRNA and NT shRNA transfected SW480 cells were grown to 90% confluency and scratched with a 1000 µl micropipette tip. Every day, cells were captured with Olympus DP 72 microscope at 10X magnification. Area that is covered by the motile cells were measured with ImageJ. Surprisingly on day 2, NT control cells completely recovered the scratched area but SNX3 knocked down cells were not able to recover the entire scratched area even after 2 days (Figure 3.4.A). Bar graph in Figure 3.4.B shows normalized scratched area measured with ImageJ. This result shows that SNX3 silencing had an effect on the motility of cells.

To further investigate this phenotype, we performed real time migration assay using the xCELLigence RTCA DP instrument. This device relies on sensors found in cell invasion and migration plate to monitor the number of cells that migrate, and sensors measure electric signals which is reduced by the migrated cells. Change in the signal correlates with migration of cells through the pores. SNX3 shRNA, pSUPER EV and NT shRNA transfected cells were monitored for 96 hours with 15-minutes interval data collection points.

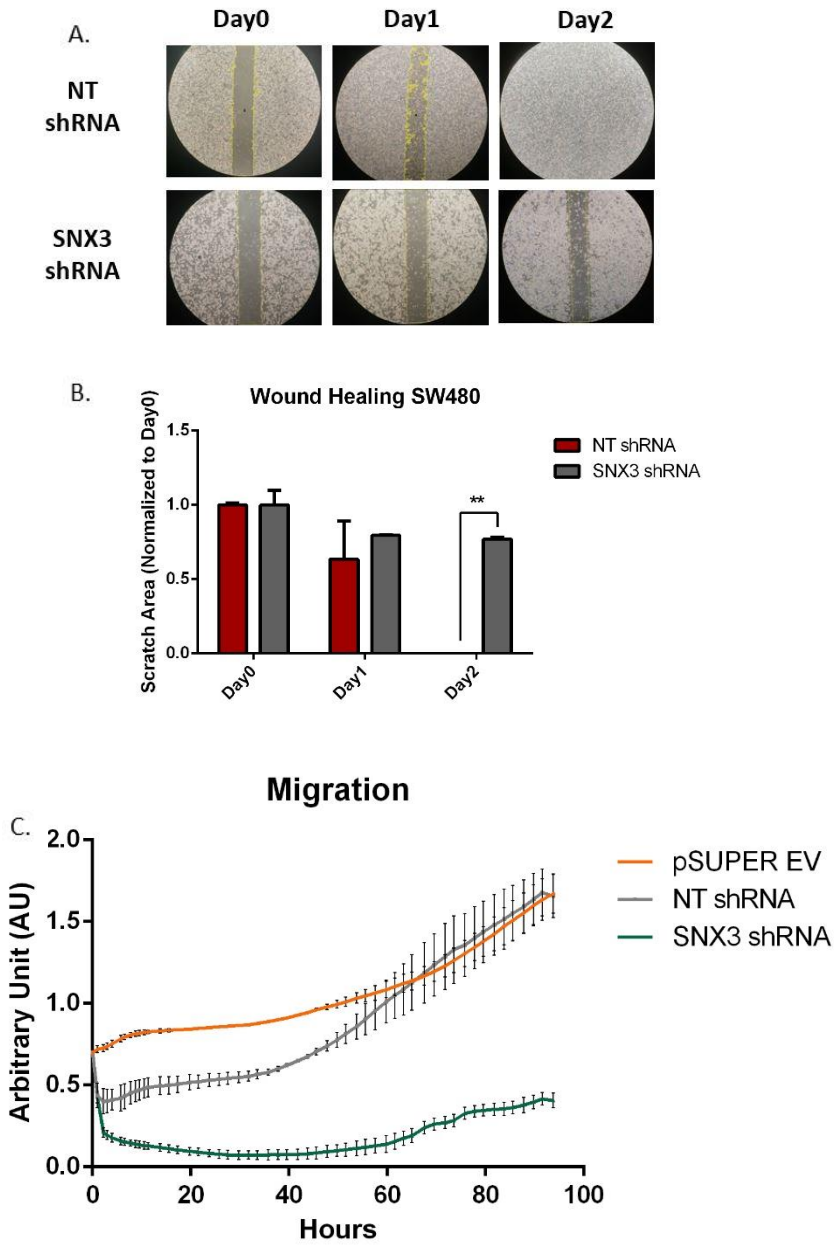

Figure 3.4. Motility and Migration of SNX3 shRNA and NT shRNA transfected SW480 cells.

(A) Wound healing assay was performed to assess the migration of SNX3 shRNA transfected SW480 cells. Cells were scratched in 6-well plate with 1000µl tip after 90% of confluency. Before capture images, cells were washed 3 times with PBS. Images were captured with Olympus DP 72 microscope at 10X magnification. (B) Scratched area was measured with ImageJ and bar graph represents normalized scratched area. At Day 2 closed scratched area is significant (*P<0.01)(n=3). (C) Real-time analysis of migration on SNX3 shRNA, NT shRNA and pSUPER EV stably transfected SW480 cells were assayed. Migration was done using the xCELLigence RTCA DP instrument with fetal bovine serum (FBS) as the chemoattractant. Medium containing 1% FBS was placed in the upper chamber, and medium containing 10% FBS was placed in the lower chamber of the CIM-plate. From 0h to 96 h, cell index was measured in every 15min.(n=2).

This result clearly showed that decreased protein level of SNX3 on SW480 cells had significant effect on migration ability. This may be due to decreased EGFR or WNT downstream signaling pathway as well as other unknown targets of SNX3. To illuminate the reason behind this significant decreased migration ability of SNX3 shRNA transfected cells, we continued with screening of downstream EGFR signaling pathway.

3.5. Downstream Analysis of EGFR pathway in SNX3 shRNA Transfected Cells

Ligand bound EGFR is cross phosphorylated (p-EGFR). From that point EGFR signaling can activate different cascades which are AKT, ERK, PLCγ, STAT and Src kinase pathways [99]. Of all these potential routes, AKT and PLCγ pathways have been linked to migration [100]. Figure 1.5.A shows that when SNX3 was knocked down with SNX3 shRNA, total EGFR and p-EGFR (Try1086) protein levels were downregulated. Similarly, total PLC gamma 1 and phosphorylated PLC gamma 1 (Try783) decreased. Interestingly, while total AKT is increasing, p-AKT (Ser473) is decreasing in SNX3 knocked down SW480 cells. In ERK1/2 case, total ERK1/2 is increasing while p-ERK1/2 (Thr202/Try204) is decreasing.

It is known that PLC gamma 1 plays active role in cell migration. Therefore, it may play a central role in our migration case because of dramatic decrease. Changes in AKT protein level draws a roadmap about migration since it is also associated with migration and invasion in many cancers. These results help us to answer the questions about decreased migration ability of the SNX3 knocked down SW480 cells.

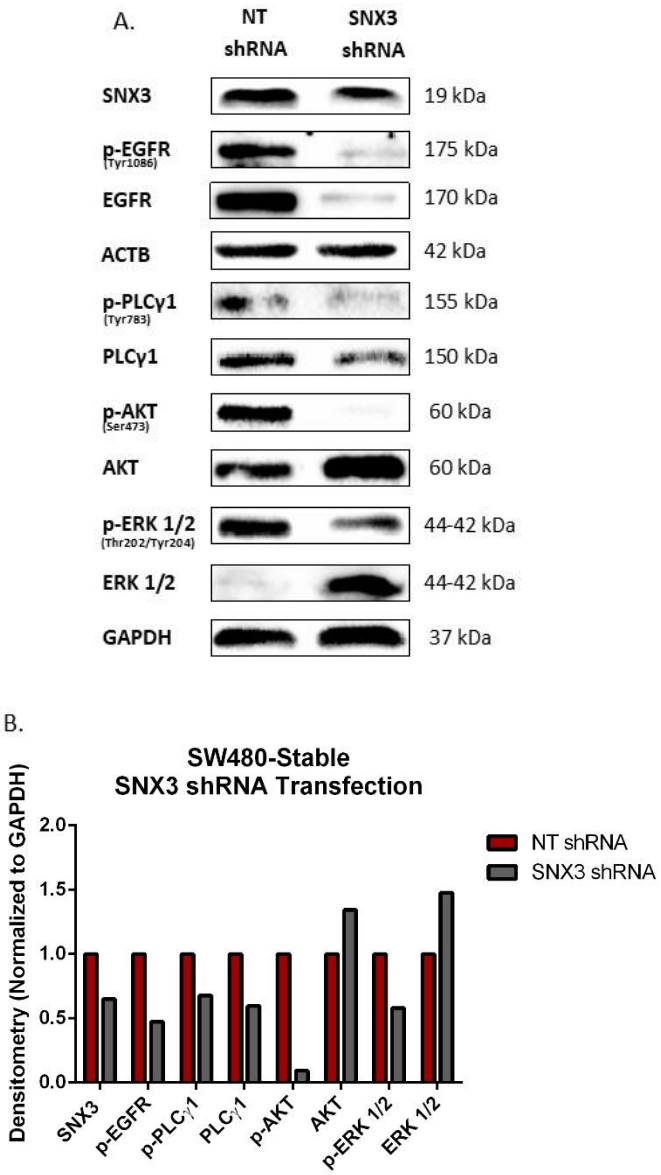

Figure 3.5. EGFR signaling pathway proteins in SNX3 shRNA transfected SW480 cells.

(A) Western Blot of SNX3, p-EGFR, p-PLCγ1, PLCγ1, p-AKT, AKT, p-ERK 1/2, ERK 1/2 protein expression in SNX3 shRNA and NT shRNA transfected SW480 cells. 50 μg protein was loaded to 10% gel. SNX3 AB: ProteinTech, Catalog #: 10772-1-AP, 1:500 dilution was prepared in 0.1% TBST with 5%BSA. p-EGFR AB: CST, Catalog #: 2220, 1:500 dilution was prepared in 0.1% TBST-T with 5% BSA. P-PLCγ AB: CST, Catalog #: 14008, 1:1000 dilution was prepared in 0.1% TBST with 5% Skim Milk. PLCγ AB: CST, Catalog #: 5690 1:500 dilution was prepared in 0.1% TBST with 5%BSA. p-AKT AB: CST, Catalog #: 4060, 1:1000 dilution was prepared in 0.1 %TBS-T with 5% BSA. AKT AB: SantaCruz, Catalog #: sc-8312, 1:500 dilution was prepared in 0.1% TBS-T with 5% Skim Milk. p-ERK 1/2 AB: SantaCruz, Catalog #: sc-16982, 1:500 dilution was prepared in 0.1% TBS-T with 5%Skim Milk. ERK 1/2 AB: SantaCruz, sc-514312, 1:200 dilution was prepared in 0.1%TBS-T with 5% BSA. GAPDH was used as loading control. n=2 (B) Graph represent densitometry results from A.

3.6. Downstream Analysis of EGFR Pathway in SNX3 siRNA Transfected Cells

While stable transfection of SNX3 shRNA is a long-term effect, siRNA transfection is short term. To test whether there is a difference between long term and short term SNX3 silencing, we used siRNAs to target SNX3 (Fig. 3.2.A), we screened EGFR signaling pathway proteins in SNX3 siRNA transfected SW480 cells (Figure 3.6.A.) Total PLC gamma 1 protein level was not significantly different through all time points. At 96 h time point PLC gamma 1 protein level decreased, but it was not significant. When we focused on total AKT protein level, it was low at 24 h and 96 h time points but it was high at 48 h and 72 h time points when compared to NT siRNA transfected SW480 cells. Phosphorylated AKT (Ser473) decreased sharply at 24 h time point but didn't change at 48 h and 72 h time points. Nevertheless, it was slightly high at 96 h time point. Phosphorylated ERK1/2(Thr202/Try204) was high while total ERK1/2 didn't change at 24 h time point. Both total and phosphorylated ERK1/2 didn't change at 48 and 72 h time points, on the other hand p-ERK1/2 was low while total ERK1/2 was high at 96 h time point.

For SNX3 siRNA transfected SW480 cells at early time points, results were different from the cells transfected with SNX3 shRNA but at 96 h time point some results were following similar pattern as SNX3 shRNA did. This situation may be explained with the late effect of SNX3 siRNA on SW480 cells.

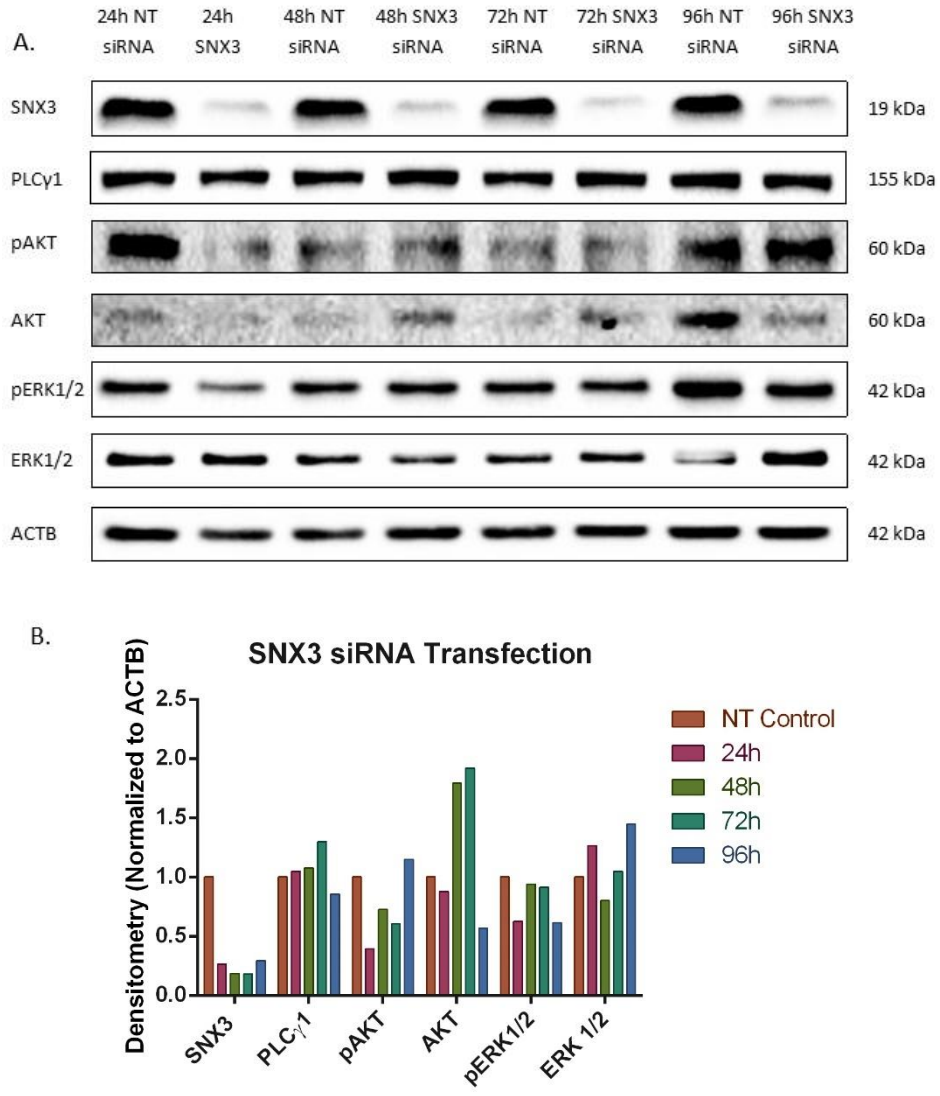

Figure 3.6. EGFR signaling pathway proteins expression in SNX3 siRNA transfected SW480 cells.

(A) Western Blot of SNX3, p-EGFR, p-PLCγ1, PLCγ1, p-AKT, AKT, p-ERK 1/2, ERK 1/2 protein expression in SNX3 siRNA and non-target siRNA transiently transfected SW480 cells. 50 µg protein was loaded to 10% gel. SNX3 AB: ProteinTech, Catalog #: 10772-1-AP, 1:500 dilution was prepared in 0.1% TBST with 5%BSA. p-EGFR AB: CST, Catalog #: 2220, 1:500 dilution was prepared in 0.1% TBST-T with 5% BSA. P-PLCγ AB: CST, Catalog #: 14008, 1:1000 dilution was prepared in 0.1% TBST with 5% Skim Milk. PLCγ AB: CST, Catalog #: 5690 1:500 dilution was prepared in 0.1% TBST with 5%BSA. p-AKT AB: CST, Catalog #: 4060, 1:1000 dilution was prepared in 0.1 %TBS-T with 5% BSA. AKT AB: SantaCruz, Catalog #: sc-8312, 1:500 dilution was prepared in 0.1% TBS-T with 5% Skim Milk. p-ERK 1/2 AB: SantaCruz, Catalog #: sc-16982, 1:500 dilution was prepared in 0.1% TBS-T with 5%Skim Milk. ERK 1/2 AB: SantaCruz, sc-514312, 1:200 dilution was prepared in 0.1%TBS-T with 5% BSA. ACTB was used as loading control. n=3 (B) Graph represent densitometry results from B.

Bar graph in Figure 3.6.B shows the densitometry result calculated from Figure 3.6.A western blots. This graph clearly shows that related protein levels fluctuated through time points.

Screening EGFR signaling pathway components in SNX3 shRNA and SNX3 siRNA transfected cells revealed that most of the EGFR signaling pathway protein levels were altered either at the protein level or their activated states. There were slight differences between early time points results of SNX3 siRNA transfection and SNX3 shRNA transfection. However, SNX3 siRNA transfected results were converging with SNX3 shRNA transfection results at 96 h time point in terms of some of the downstream EGFR signaling pathway proteins level. Overall, knocking down of SNX3 led to aberrant regulation of EGFR signaling pathway proteins.

In parallel, due to the decrease in WLS levels, we tested whether downstream WNT signaling pathways were affected from SNX3 knock-down.

3.7. Downstream Analysis of WNT Pathway

SNX3 knock down in SW480 cells caused a decrease in WLS protein levels (Figure 3.2.A and Figure 3.2.B). Because WLS plays a central role for secretion of WNT ligands, we wanted to check WNT signaling pathway. Beta-Catenin (β-Catenin) is a downstream effector of WNT signaling pathway. β-Catenin engages with important transcription factors like LEF1, TCF1, TCF2 to initiate WNT specific gene expression [101]. To evaluate the effect of SNX3 silencing and decreased WLS levels on WNT signaling, nuclear and cytoplasmic β-Catenin levels were determined. SNX3 shRNA (Figure 3.8.A) and SNX3 siRNA (Figure 3.8.B) transfected SW480 cells did not have any change in β-Catenin protein levels compared with control groups. These results showed that decreased protein level of WLS had no effect on canonical β-Catenin dependent WNT signaling pathway when SNX3 was knocked down in SW480 cells.

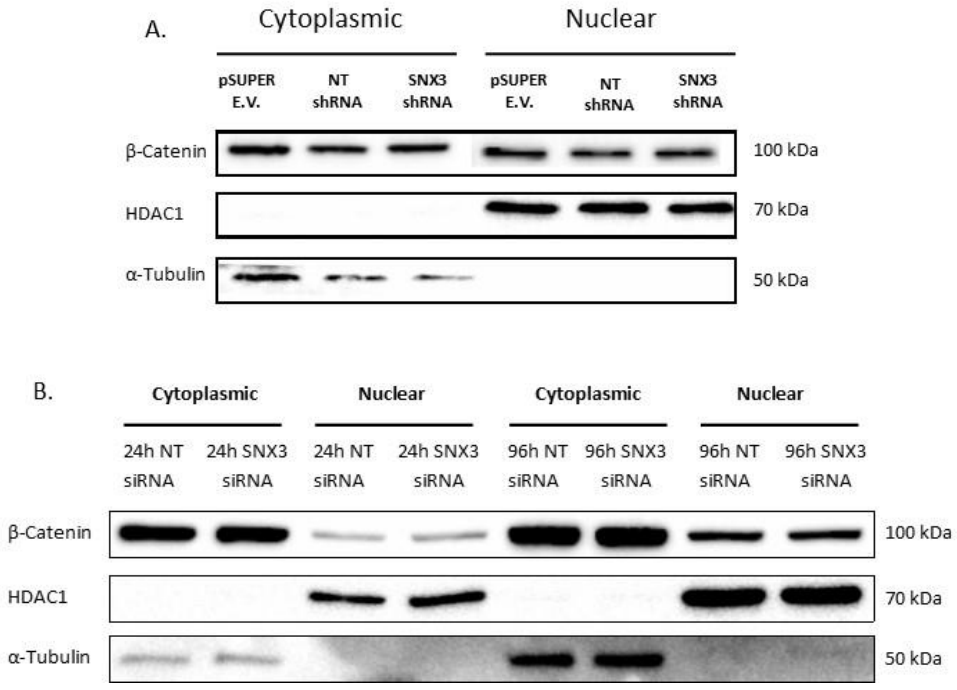

Figure 3.7. Protein expression level of B-Catenin in SNX3 shRNA and siRNA transfected cells.

30 µg cytoplasmic protein loaded and 10µg nuclear protein loaded to the 10% polyacrylamide gel. (A) Western blot of CTNNB1 cytoplasmic and nuclear protein expression in SNX3 shRNA, NT shRNA and pSUPER empty vector transfected SW480 cells. CTNNB1 antibody (1:500 dilution, Cat #: sc-133240, Santa-Cruz Biotechnology). HDAC1 (1:1000 dilution, Cat #: sc-81598, Santa-Cruz Biotechnology) is nuclear loading control and α-Tubulin (1:5000 dilution, Cat #: HRP-66031, Protein Tech) is cytoplasmic loading control.(n=2) (B) Western blot of CTNNB1 cytoplasmic and nuclear protein expression in SNX3 siRNA, Non-Target (NT) siRNA transfected SW480 cells. Proteins were isolated after 96 h transfection. CTNNB1 antibody (1:500 dilution, Cat #: sc-133240, Santa-Cruz Biotechnology). HDAC1 (1:1000 dilution, Cat #: sc-81598, Santa-Cruz Biotechnology) is nuclear loading control and α-Tubulin (1:5000 dilution, Cat #: HRP-66031, Protein Tech) is cytoplasmic loading control.(n=2).

This result suggests that diminished level of WLS protein has no effect on canonical WNT signaling pathway. However, we cannot exclude potential changes in the non-canonical WNT signaling pathways.

Given that EGFR related downstream pathways were affected from SNX3 knock-down more than canonical WNT signaling, we turned to potential other mechanism linking EGFR signaling and cell motility/migration. Migratory properties of cells are affected by EGFR signaling pathway and Epithelial to Mesenchymal Transition

(EMT). EGFR pathway and EMT mechanisms orchestrate the motility of cells [99]. Therefore, we screened EMT markers in our SNX3 knocked down models.

3.8. EMT Markers Screening in SNX3 shRNA Transfected SW480 Cells

Epithelial to mesenchymal transition is a biological process which is a source of the mesenchymal cells participating in tissue fibrosis, tumor invasiveness and metastasis [102]. Generally, these transitions make cells acquire more migratory properties. EGFR pathways stimulate EMT in breast cancer [103]. EMT and EGFR signaling pathway converge in AKT, MAPK, β-catenin and SMAD factors [104]. EGF induced EGFR signaling pathway upregulates Vimentin and Snail through ERK [84]. EGFR also downregulates E-cadherins which leads to increase mesenchymal proteins that leads to EMT [105]. Because EMT is a dynamic process regulated by many processes, we selected transcriptional regulators of EMT Zeb1, Snail, Slug and Twist, cell surface proteins as EMT marker ZO-1 and N-Cadherin, mesenchymal marker Vimentin as well as stemness markers CD44 to evaluate different aspects of EMT.

Figure 3.8.A showed SNX3 protein expression level in SNX3 shRNA and NT shRNA transfected SW480 cells. Figure 3.8.B showed the protein levels of transcriptional factors that were used as EMT marker. ZEB1 (Zinc Finger E-Box Binding Homeobox 1) also known as TCF8 is a transcription factor that repress E-cadherin and induces EMT [106]. ZEB1 was low in SNX3 knocked down SW480 cells compared to NT control.

SNAI2 (Snail Family Transcriptional Repressor 2) known as SLUG which is transcriptional repressor which represses E-Cadherin transcription and promotes invasion and metastasis [107]. SLUG protein level was low in SNX3 knocked down SW480 cells compared to NT control.

SNAI1 (Snail Family Transcriptional Repressor 1) also known as SNAIL which is transcriptional repressor and has role in EMT, survival and cell migration. It is related with Erk signaling pathway [108]. SNAIL protein level was high in SNX3 knocked down SW480 cells compared to NT control.

TWIST1 (Twist Family BHLH Transcription Factor 1) is a transcription factor which induces EMT and promotes cancer stem cells (CSCs). It is phosphorylated by MAPK [109]. Protein level of TWIST1 was slightly high in SNX3 knocked down cells in Figure 3.8.B.

Zonula occludens-1 (ZO-1) also known as Tight junction protein 1 is a cell surface protein which has role in cell integrity by forming networks between actin and integral tight junction proteins. [110]. It was also shown that ZO-1 bounds to EGFR and highly phosphorylated in in primary colorectal cancer but dephosphorylated in metastasized cancer [111]. It was also reported that downregulation of ZO-1 led to lower wound healing in lung cancer cells [112]. We showed that in Figure 3.8.C, ZO-1 was low in SNX3 knocked down SW480 cells compared to NT control.

Cadherin-2 (CDH2) also known as N-Cadherin is an EMT marker upregulated and associated with metastasis and poor prognosis of many cancers. It is also reported that N-cadherin upregulates p-ERK1/2 [113]. We showed that N-Cadherin protein level was low in SNX3 knocked down SW480 cells compared to NT control in figure 3.8.C.

VIM (Vimentin) is an EMT marker that ubiquitously expressed in mesenchymal cells and overexpressed in many epithelial cancers [72]. Vimentin was associated with migration. It was reported that Vimentin was upregulated in edge of the wound in mammary epithelial cells in breast cancer [70]. We showed in Figure 3.8.C. that Vimentin was low in SNX3 knocked down cells compared to NT control.

CD44 is a transmembrane glycoprotein that is used as both stemness marker and EMT marker [75]. Hyaluronic acid (HA) is a ligand of CD44 protein. It was shown that inhibition the interaction of HA and CD44 led to decrease tumor cell motility [114]. We showed that protein level of CD44 was low in SNX3 knocked down cell compared to NT control in figure 3.8.C.

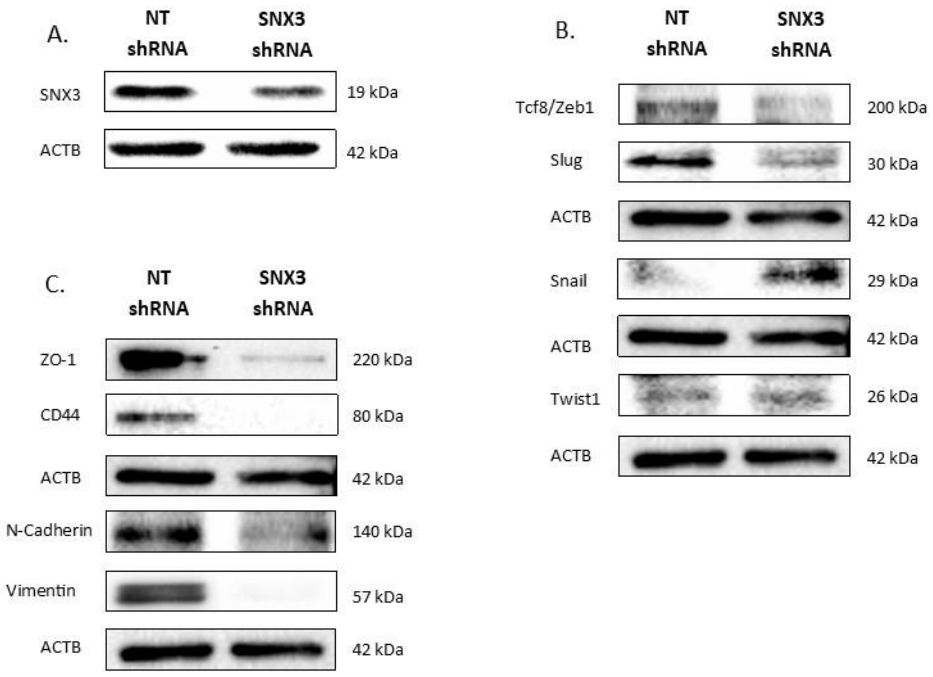

Figure 3.8. Protein expression levels of EMT markers in SNX3 shRNA transfected SW480 cells.

50 µg protein was loaded on to 10% polyacrylamide gel. (A) Western blot analysis of SNX3 (1:500 dilution, Cat #:10772-1-AP, Proteintech). (B) Western blot analysis of Tcf8/Zeb1 (1:1000 dilution, Cat #: 3396, CST) Slug (1:1000 dilution, Cat #: 9585, CST) Snail (1:1000 dilution, Cat #: 3879, CST) Twist1(1:1000 dilution, Cat #: 46702, CST) expressions.(n=2). (C) Western blot analysis of ZO-1 (1:1000 dilution, Cat #: 8193, CST), CD44 (1:1000 dilution, Cat #: 3570, CST), N-Cadherin (1:1000 dilution, Cat #: 13116, CST) and Vimentin (1:1000 dilution, Cat #: 5741, CST) expressions. ACTB (1:4000 dilution, Cat #: sc-47778, Santa-Cruz Biotechnology) was used as loading control.(n=2).

As a result, there were dramatic changes in EMT marker protein levels. In SNX3 shRNA transfected SW480 cells, Zeb1 and Slug transcription factors were downregulated, but Snail and Twist1 were upregulated. Cell surface proteins ZO-1 and N-Cadherin, mesenchymal marker Vimentin as well as stemness markers CD44 were also downregulated in SNX3 shRNA transfected SW480 cells compared to NT control. These results may be late response of SNX3 knocking down. To check the early response of these markers we also screened these proteins in SNX3 siRNA transfected cells.

3.9. EMT Markers in SNX3 siRNA Transfected SW480 Cells

We observed the protein level changes in stably transfected SNX3 shRNA in SW480 cells, we also wanted to see the changes in transiently transfected SNX3 siRNA in SW480 cells.

Transcription factor ZEB1 didn't change dramatically at 24 h, 48 h and 96 h time points but it was low at 72 h compared to NT siRNA control. On the other hand, other transcription factor SNAIL protein level was low in all time points when compared to NT control. Transcription factor Twist1 protein level changed through time points. It was high at 24h, 48 h and 96 h time points but low at 72 h time point.

Cell surface protein ZO-1 was high at 24 h and low at 72 h. It didn't change at 48 h and 96 h time points in SNX3 siRNA transfected SW480 cells compered to NT siRNA. Another cell surface protein N-Cadherin was high at 24 h and low at 48 h. Protein level of N-Cadherin didn't change at 72 h but it was slightly low at 96 h time points.

Mesenchymal marker Vimentin didn't change at 24 h and 96 h time points but it was high at 48 h time point and low at 72 h time point.

The last EMT marker that was screened in SNX3 siRNA transfected SW480 cells was CD44 which is also a stemness marker. Protein level of CD44 didn't change dramatically at 24 h, 48 h and 96 h time points. On the other hand, at 72 h time point, it was low when compared to NT siRNA transfected control cells.

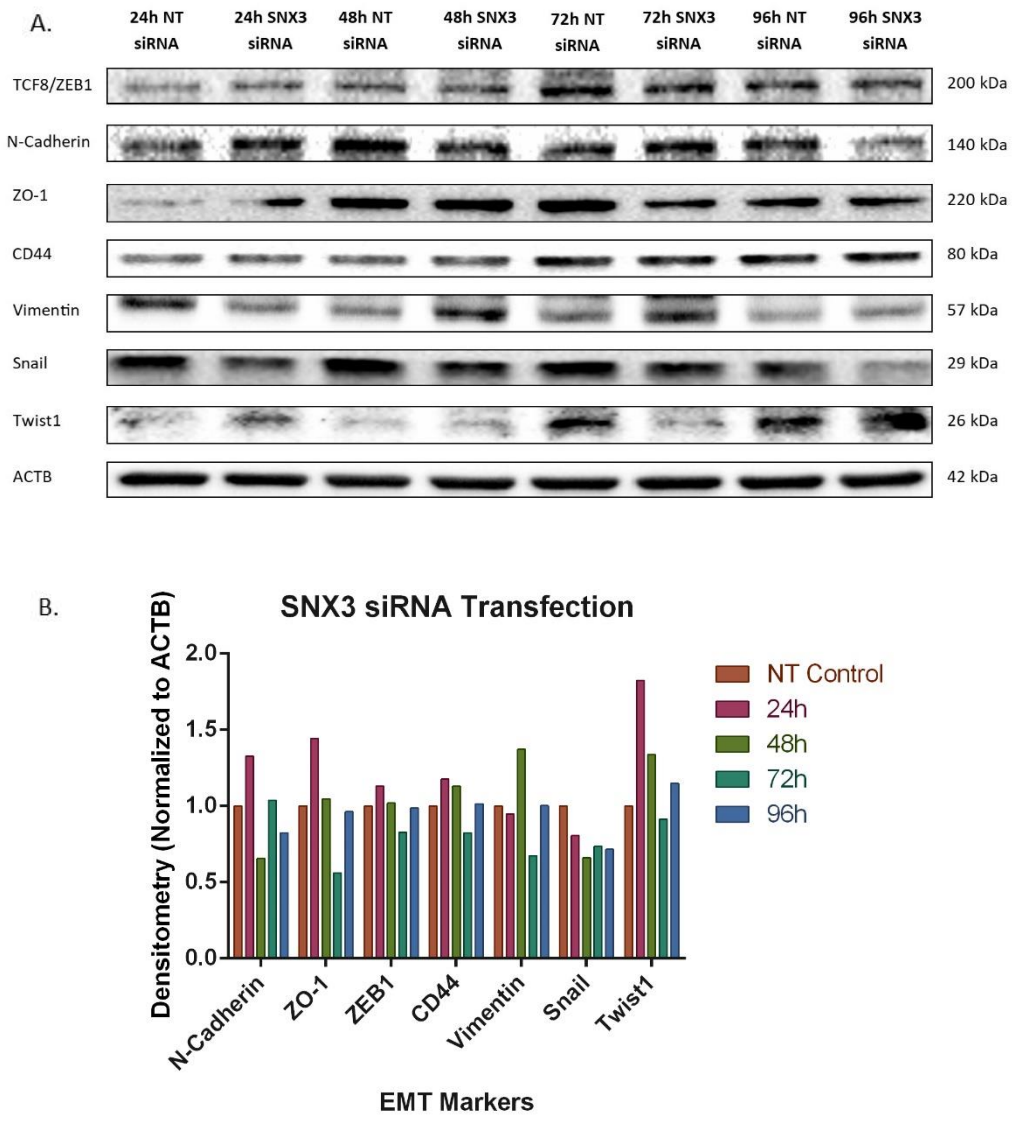

Figure 3.9. Protein expression levels of EMT markers in SNX3 siRNA transfected SW480 cells.

50 µg protein was loaded on to 10% polyacrylamide gel. Primary antibodies were used overnight (A) Western blot analysis of Tcf8/Zeb1 (1:1000 dilution, Cat #: 3396, CST) N-Cadherin (1:1000 dilution, Cat #: 13116, CST), ZO-1 (1:1000 dilution, Cat #: 8193, CST), CD44 (1:1000 dilution, Cat #: 3570, CST) Vimentin (1:1000 dilution, Cat #: 5741, CST) Snail (1:1000 dilution, Cat #: 3879, CST) and Twist1(1:1000 dilution, Cat #: 46702, CST) expression. ACTB (1:4000 dilution, Cat #: sc-47778, Santa-Cruz Biotechnology) was used as loading control. (B) Bar graph represents densitometry results of A.(n=2).

We observed that most of the EMT marker proteins such as N-Cadherin, Vimentin, ZO-1, CD44, Tcf8/Zeb1 and Slug were downregulated in SNX3 shRNA transfected SW480 cells, but interestingly protein levels of these markers fluctuated in SNX3 siRNA transfected SW480 cells compared to NT siRNA transfected SW480 cells. These results showed that knocking down of SNX3 changed the EMT profile of SW480 cells.

CHAPTER 4

CONCLUSION

In this study, we have investigated the role of SNX3 in colon cancer cell line SW480. For that purpose, we generated stably transfected SNX3 short hairpin RNA (shRNA) SW480 cells. In parallel, we also transfected SNX3 small interfering RNA (siRNA) into the SW480 cells. We observed that SNX3 shRNA and SNX3 siRNA transfection efficiently knocked down SNX3 in SW480 cells.

To begin understanding the consequences of SNX3 silencing, we investigated WLS protein levels, which is a known cargo molecule for SNX3 dependent retromer complex. Another important cargo molecule, EGFR, suggested to be regulated by SNX3 was the second focus of our experiments.

We observed that EGFR and WLS protein levels dramatically decreased in SNX3 shRNA transfected SW480 cells. Considering that, SNX3 regulates recycling of endocytosed receptors, we speculate the existence of EGF like ligands in the serum to induce EGFR activation and endocytosis and hence less recycling of the EGFR.

Interestingly, in SNX3 siRNA transfected cells, both WLS and EGFR levels changed in a time dependent manner. At 24 h time point after transfection, EGFR and WLS protein levels were reduced while at 96 h time point after transfection, the protein levels of EGFR and WLS came back even though SNX3 was still knocked down. Phosphorylated EGFR (pEGFR) was low at 24 h time point but it was high at 96 h time point compared to non-target (NT) controls in SNX3 siRNA transfected cells. We anticipate that there might be other compensatory mechanisms to upregulate these proteins to allow survival of the cells.

Next, to investigate the effect SNX3 knock down on cellular phenotypes, we performed an MTT assay. Interestingly, there was no significant proliferation change in SNX3 shRNA transfected cells up to 72 h time point compared with controls. At

96 h time point, proliferation of SNX3 shRNA transfected cells decreased significantly $p<0.05$ compared with NT shRNA transfected cells. Similarly, for SNX3 siRNA transfected cells there was no significant change on proliferation when compared to NT siRNA transfected control cells up to 96 h time point.

Since migration ability of cells is affected by aberrant regulation of EGFR [115] and WNT signaling pathway [116], we wanted to check whether there was a change on motility and migration ability of SW480 cells that have decreased EGFR and WLS proteins expression due to SNX3 knock-down. For that purpose, we performed wound healing and xCELLigence migration assays. Surprisingly, motility and migration of SNX3 knocked down cells decreased dramatically. This result showed that knocking down of SNX3 in SW480 cells may have role in motility and migration by downregulating EGFR and WLS expression.

Downstream EGFR signaling pathways are known to have role in motility and migration. Hence, we were focused on revealing which pathways were affected by knocking down of SNX3. First, we checked phosphorylated and total protein levels of three main EGFR downstream signaling pathways, which are AKT, ERK1/2 and PLCγ1.

We observed that while total AKT and ERK 1/2 levels were increased in SNX3 shRNA transfected cells when compared with NT control, active forms of these proteins, which are phosphorylated forms, were decreased. This showed that SNX3 knock-down decreased activity of AKT and ERK1/2, which play roles in motility and migration. Both phosphorylated and total forms of PLCγ1 expression were downregulated in SNX3 shRNA transfected SW480 cells. Since PLCγ1 have also been linked with migration [100], this downregulation affects migration of SW480 cells.

Same experiment was performed with SNX3 siRNA and NT siRNA transfected cells. Since siRNA transfection is transient and it gives us early effect of knocking down of SNX3, there were differences in the downstream signaling pathways. For example, there was no change in PLCγ1 expression level in SNX3 siRNA transfected cells. AKT expression level was fluctuated through time points but phosphorylated AKT

decreased when compared to NT control. Overall, most of the EGFR downstream signaling pathways were downregulated in SNX3 knocked down cells. We think this drastic change may have induced upregulation of other pathways to rescue the changes in these signaling molecules.

Next to delineate whether EGFR or WNT pathways have the determining role in decreased migration phenotype, we checked downstream WNT signaling pathway. In SNX3 siRNA and SNX3 shRNA transfected cells, there was no significant change in β-Catenin nuclear expression. This experiment showed that there is no correlation between β-Catenin dependent WNT pathway and decreased migration caused by knocking down of SNX3. However, we cannot eliminate activity of the non-canonical WNT pathway.

It is known that EMT is one of the major causes of migration of cells [117] and EMT is affected by some signaling pathways like EGFR [84] and WNT [118]. Therefore, we have checked the protein expression levels of EMT markers that are affected by knocking down of SNX3. We observed that protein expression levels of EMT transcription factors SNAIL and TWIST1 increased but SLUG and ZEB1 decreased. All EMT cell surface proteins, mesenchymal and stemness marker proteins decreased in SNX3 shRNA transfected SW480 cells. We observed similar pattern in SNX3 siRNA transfected SW480 cells. These findings suggest that SNX3 regulates motility and migration ability of SW480 cells through regulating EGFR signaling (an/or non-canonical WNT signaling) and EMT markers.

Given the significant changes in EGFR downstream pathways upon SNX3 silencing, we think we provided preliminary findings to point out the significance of SNX3 levels in cancer cells. The extent of these findings should be investigated further, and causative and mechanistic explanations between EGFR downregulation and decreased motility and migration will help us better understand the dynamics of SNX3 dependent recycling.

REFERENCES

[1] P. J. Cullen, “Endosomal sorting and signalling: An emerging role for sorting nexins,” Nature Reviews Molecular Cell Biology, vol. 9, no. 7. pp. 574–582, 2008.

[2] R. D. Teasdale and B. M. Collins, “Insights into the PX (phox-homology) domain and SNX (sorting nexin) protein families: structures, functions and roles in disease,” Biochem. J., vol. 441, no. 1, pp. 39–59, 2012.

[3] M. Gallon and P. J. Cullen, “Retromer and sorting nexins in endosomal sorting,” Biochem. Soc. Trans., vol. 43, no. 1, pp. 33–47, 2015.

[4] D. Karathanassis et al., “Binding of the PX domain of p47phoxto phosphatidylinositol 3, 4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction,” EMBO J., vol. 21, no. 19, pp. 5057–5068, 2002.

[5] A. C. Nascimbeni, P. Codogno, and E. Morel, “Phosphatidylinositol-3-phosphate in the regulation of autophagy membrane dynamics,” FEBS J., vol. 284, no. 9, pp. 1267–1278, 2017.

[6] P. Zhang, Y. Wu, T. Y. Belenkaya, and X. Lin, “SNX3 controls Wingless/Wnt secretion through regulating retromer-dependent recycling of Wntless.,” Cell Res., vol. 21, no. 12, pp. 1677–90, 2011.

[7] J. Yu, J. Chia, C. A. Canning, C. M. Jones, F. A. Bard, and D. M. Virshup, “WLS Retrograde transport to the endoplasmic reticulum during Wnt secretion,” Dev. Cell, vol. 29, no. 3, pp. 277–291, 2014.

[8] M. Harterink et al., “A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion.,” Nat. Cell Biol., vol. 13, no. 8, pp. 914–23, 2011.

[9] M. Lucas, D. C. Gershlick, A. Vidaurrazaga, A. L. Rojas, J. S. Bonifacino, and A. Hierro, “Structural Mechanism for Cargo Recognition by the Retromer Complex.,” Cell, vol. 167, no. 6, p. 1623–1635.e14, Dec. 2016.

[10] S. Gokool, D. Tattersall, J. V Reddy, and M. N. J. Seaman, “Identification of a conserved motif required for Vps35p/Vps26p interaction and assembly of the retromer complex.,” Biochem. J., vol. 408, no. 2, pp. 287–295, 2007.

[11] C. Z. Zhou et al., “Crystal Structure of the Yeast Phox Homology (PX) Domain Protein Grd19p Complexed to Phosphatidylinositol-3-phosphate,” J. Biol. Chem., vol. 278, no. 50, pp. 50371–50376, 2003.

[12] Q. Y. Zhang, M. S. Tan, J. T. Yu, and L. Tan, “The Role of Retromer in Alzheimer’s Disease,” Mol. Neurobiol., vol. 53, no. 6, pp. 4201–4209, 2016.

[13] H. B. Akman, M. Oyken, T. Tuncer, T. Can, and A. E. Erson-Bensan, “3’UTR shortening and EGF signaling: Implications for breast cancer,” Hum. Mol. Genet., vol. 24, no. 24, pp. 6910–6920, 2015.

[14] K. H. Chiow et al., “SNX3-dependent regulation of epidermal growth factor receptor (EGFR) trafficking and degradation by aspirin in epidermoid carcinoma (A-431) cells,” Cell. Mol. Life Sci., vol. 69, no. 9, pp. 1505–1521, 2012.

[15] S. Das, S. Yu, R. Sakamori, E. Stypulkowski, and N. Gao, “Wntless in Wnt secretion: molecular, cellular and genetic aspects.,” Front. Biol. (Beijing)., vol. 7, no. 6, pp. 587–593, Dec. 2012.

[16] T. Skjørringe, A. Burkhart, K. B. Johnsen, and T. Moos, “Divalent metal transporter 1 (DMT1) in the brain: implications for a role in iron transport at the blood-brain barrier, and neuronal and glial pathology,” Front. Mol. Neurosci., vol. 8, no. June, pp. 1–13, 2015.

[17] T. Braulke and J. S. Bonifacino, “Sorting of lysosomal proteins,” Biochim. Biophys. Acta - Mol. Cell Res., vol. 1793, no. 4, pp. 605–614, 2009.

[18] H. Turula and C. E. Wobus, “The role of the polymeric immunoglobulin receptor and secretory immunoglobulins during mucosal infection and immunity,” Viruses, vol. 10, no. 5, pp. 1–15, 2018.

[19] J. Fu, M. Jiang, A. J. Mirando, H.-M. I. Yu, and W. Hsu, “Reciprocal regulation of Wnt and Gpr177/mouse Wntless is required for embryonic axis formation,” Proc. Natl. Acad. Sci., vol. 106, no. 44, pp. 18598–18603, 2009.

[20] H. Komekado, H. Yamamoto, T. Chiba, and A. Kikuchi, “Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a,” Genes to Cells, vol. 12, no. 4, pp. 521–534, 2007.

[21] H. Clevers, “Wnt/β-Catenin Signaling in Development and Disease,” Cell, vol. 127, no. 3, pp. 469–480, 2006.

[22] B. D. White, A. J. Chien, and D. W. Dawson, “Dysregulation of Wnt/β-catenin signaling in gastrointestinal cancers.,” Gastroenterology, vol. 142, no. 2, pp. 219–32, Feb. 2012.

[23] F. Port et al., “Wingless secretion promotes and requires retromer-dependent cycling of Wntless,” Nat. Cell Biol., vol. 10, no. 2, pp. 178–185, 2008.

[24] I. Gasnereau, P. Herr, P. Z. C. Chia, K. Basler, and P. A. Gleeson, “Identification of an endocytosis motif in an intracellular loop of Wntless protein, essential for its recycling and the control of Wnt protein signaling,” J. Biol. Chem., vol. 286, no. 50, pp. 43324–43333, 2011.

[25] J. R. T. van Weering, P. Verkade, and P. J. Cullen, “SNX-BAR proteins in phosphoinositide-mediated, tubular-based endosomal sorting.,” Semin. Cell Dev. Biol., vol. 21, no. 4, pp. 371–80, Jun. 2010.

[26] M. Harterink et al., “A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion,” Nat. Cell Biol., vol. 13, no. 8, pp. 914–923, 2011.

[27] M. J. Lorenowicz et al., “Inhibition of late endosomal maturation restores Wnt secretion in Caenorhabditis elegans vps-29 retromer mutants,” Cell. Signal., vol. 26, no. 1, pp. 19–31, 2014.

[28] B. T. MacDonald, K. Tamai, and X. He, “Wnt/beta-catenin signaling: components, mechanisms, and diseases.,” Dev. Cell, vol. 17, no. 1, pp. 9–26, Jul. 2009.

[29] A. M. Daulat and J. P. Borg, “Wnt/Planar Cell Polarity Signaling: New Opportunities for Cancer Treatment,” Trends in Cancer, vol. 3, no. 2, pp. 113–125, 2017.

[30] J. L. Freese, D. Pino, and S. J. Pleasure, “Wnt signaling in development and disease.,” Neurobiol. Dis., vol. 38, no. 2, pp. 148–53, May 2010.

[31] T. Sasaki, K. Hiroki, and Y. Yamashita, “The role of epidermal growth factor receptor in cancer metastasis and microenvironment.,” Biomed Res. Int., vol. 2013, no. 1, p. 546318, 2013.

[32] J. V. Olsen et al., “Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks,” Cell, vol. 127, no. 3, pp. 635–648, 2006.

[33] Y. Ohnishi, H. Yasui, K. Kakudo, and M. Nozaki, “Regulation of cell migration via the EGFR signaling pathway in oral squamous cell carcinoma cells,” Oncol. Lett., vol. 13, no. 2, pp. 930–936, 2016.

[34] S. Pennock and Z. Wang, “Stimulation of cell proliferation by endosomal epidermal growth factor receptor as revealed through two distinct phases of signaling.,” Mol. Cell. Biol., vol. 23, no. 16, pp. 5803–15, Aug. 2003.

[35] B. R. Voldborg, L. Damstrup, and H. S. Poulsen, “Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials,” Ann. Oncol. Off. J. Eur. Soc. Med. Oncol., no. Figure 1, pp. 1197–1206, 1997.

[36] K. Dumstrei, C. Nassif, G. Abboud, A. Aryai, A. Aryai, and V. Hartenstein, “EGFR signaling is required for the differentiation and maintenance of neural progenitors along the dorsal midline of the Drosophila embryonic head.,” Development, vol. 125, no. 17, pp. 3417–3426, 1998.

[37] B. Singh, G. Carpenter, and R. J. Coffey, “EGF receptor ligands: recent advances,” F1000Research, vol. 5, no. 0, p. 2270, 2016.

[38] K. Roepstorff et al., “Differential effects of EGFR ligands on endocytic sorting of the receptor,” Traffic, vol. 10, no. 8, pp. 1115–1127, 2009.

[39] F. Huang, A. Khvorova, W. Marshall, and A. Sorkin, “Analysis of Clathrin-mediated Endocytosis of Epidermal Growth Factor Receptor by RNA Interference,” J. Biol. Chem., vol. 279, no. 16, pp. 16657–16661, 2004.

[40] C. Le Roy and J. L. Wrana, “Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling,” Nat. Rev. Mol. Cell Biol., vol. 6, no. 2, pp. 112–126, 2005.

[41] S. Sigismund, E. Argenzio, D. Tosoni, E. Cavallaro, S. Polo, and P. P. Di Fiore, “Clathrin-Mediated Internalization Is Essential for Sustained EGFR Signaling but Dispensable for Degradation,” Dev. Cell, vol. 15, no. 2, pp. 209–219, 2008.

[42] L. Henriksen, M. V. Grandal, S. L. J. Knudsen, B. van Deurs, and L. M. Grøvdal, “Internalization Mechanisms of the Epidermal Growth Factor Receptor after Activation with Different Ligands,” PLoS One, vol. 8, no. 3, pp. 1–11, 2013.

[43] J. Bakker, M. Spits, J. Neefjes, and I. Berlin, “The EGFR odyssey – from activation to destruction in space and time,” J. Cell Sci., vol. 130, no. 24, pp. 4087–4096, 2017.

[44] M. N. J. Seaman, A. Gautreau, and D. D. Billadeau, “Retromer-mediated endosomal protein sorting: All WASHed up!,” Trends Cell Biol., vol. 23, no. 11, pp. 522–528, 2013.

[45] S. N. Duleh and M. D. Welch, “WASH and the Arp2/3 complex regulate endosome shape and trafficking.,” Cytoskeleton (Hoboken)., vol. 67, no. 3, pp. 193–206, Mar. 2010.

[46] J. T. Piotrowski, T. S. Gomez, R. A. Schoon, A. K. Mangalam, and D. D. Billadeau, “WASH Knockout T Cells Demonstrate Defective Receptor Trafficking, Proliferation, and Effector Function,” Mol. Cell. Biol., vol. 33, no. 5, pp. 958–973, 2013.

[47] J. Y. Shih and P. C. Yang, “The EMT regulator slug and lung carcinogenesis,” Carcinogenesis, vol. 32, no. 9, pp. 1299–1304, 2011.

[48] A. Villarejo, Á. Cortés-Cabrera, P. Molina-Ortíz, F. Portillo, and A. Cano, “Differential role of snail1 and snail2 zinc fingers in E-cadherin repression and epithelial to mesenchymal transition,” J. Biol. Chem., vol. 289, no. 2, pp. 930–941, 2014.

[49] D. Olmeda, G. Moreno-Bueno, J. M. Flores, A. Fabra, F. Portillo, and A. Cano, “SNAI1 is required for tumor growth and lymph node metastasis of human breast carcinoma MDA-MB-231 cells,” Cancer Res., vol. 67, no. 24, pp. 11721–11731, 2007.

[50] F. Fan et al., “Overexpression of Snail induces epithelial-mesenchymal transition and a cancer stem cell-like phenotype in human colorectal cancer cells,” Cancer Med., vol. 1, no. 1, pp. 5–16, 2012.

[51] Y. Toiyama et al., “Increased expression of slug and vimentin as novel predictive biomarkers for lymph node metastasis and poor prognosis in colorectal cancer,” Carcinogenesis, vol. 34, no. 11, pp. 2548–2557, 2013.

[52] R. Ganesan, E. Mallets, and J. Gomez-cambronero, “ScienceDirect The transcription factors Slug (SNAI2) and Snail (SNAI1) regulate phospholipase D (PLD) promoter in opposite ways towards cancer cell invasion,” Mol. Oncol., vol. 10, no. 5, pp. 663–676, 2015.

[53] M. Shioiri et al., “Slug expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients,” Br. J. Cancer, vol. 94, no. 12, pp. 1816–1822, 2006.

[54] P. Zhang, Y. Sun, and L. Ma, “ZEB1: At the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance,” Cell Cycle, vol. 14, no. 4, pp. 481–487, 2015.

[55] A. A. Postigo, “Opposing functions of ZEB proteins in the regulation of the TGF??/BMP signaling pathway,” EMBO J., vol. 22, no. 10, pp. 2443–2452, 2003.

[56] A. Eger et al., “DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells,” Oncogene, vol. 24, no. 14, pp. 2375–2385, 2005.

[57] S. Spaderna et al., “The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer,” Cancer Res., vol. 68, no. 2, pp. 537–544, 2008.

[58] Y. Li, L. Wang, L. Pappan, A. Galliher-Beckley, and J. Shi, “IL-1β promotes stemness and invasiveness of colon cancer cells through Zeb1 activation.,” Mol. Cancer, vol. 11, no. 77, p. 87, Nov. 2012.

[59] J. Yang et al., “Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis.,” Cell, vol. 117, no. 7, pp. 927–39, Jun. 2004.

[60] J. M. Halbleib and W. J. Nelson, “Cadherins in development: Cell adhesion, sorting, and tissue morphogenesis,” Genes Dev., vol. 20, no. 23, pp. 3199–3214, 2006.

[61] F. Van Roy and G. Berx, “The cell-cell adhesion molecule E-cadherin,” Cell. Mol. Life Sci., vol. 65, no. 23, pp. 3756–3788, 2008.

[62] M. Yilmaz and G. Christofori, “EMT, the cytoskeleton, and cancer cell invasion,” Cancer Metastasis Rev., vol. 28, no. 1–2, pp. 15–33, 2009.

[63] W. Shih and S. Yamada, “N-cadherin-mediated cell-cell adhesion promotes cell migration in a three-dimensional matrix,” J. Cell Sci., vol. 125, no. 15, pp. 3661–3670, 2012.

[64] O. W. Blaschuk, “N-cadherin antagonists as oncology therapeutics,” Philos. Trans. R. Soc. B Biol. Sci., vol. 370, no. 1661, pp. 8–11, 2015.

[65] X. Yan, L. Yan, S. Liu, Z. Shan, Y. Tian, and Z. Jin, “N-cadherin, a novel prognostic biomarker, drives malignant progression of colorectal cancer,” Mol. Med. Rep., vol. 12, no. 2, pp. 2999–3006, 2015.

[66] M. Polette et al., “β-catenin and ZO-1: Shuttle molecules involved in tumor invasion-associated epithelial-mesenchymal transition processes,” Cells Tissues Organs, vol. 185, no. 1–3, pp. 61–65, 2007.

[67] Hirakawa, “Localization of cortactin is associated with colorectal cancer development,” Int. J. Oncol., vol. 35, no. 06, pp. 8822–8828, Oct. 2009.

[68] B. Dekky, M. Ruff, D. Bonnier, V. Legagneux, and N. Théret, “Proteomic screening identifies the zonula occludens protein ZO-1 as a new partner for ADAM12 in invadopodia-like structures,” Oncotarget, vol. 9, no. 30, pp. 21366–21382, 2018.

[69] E. Russell, “Leadership Theories and Style : A Transitional Approach,” Gral. MacArthur Mil. Compet., vol. CGSC Class, no. September 2011, pp. 5034–5038, 2011.

[70] J. Ivaska, “Vimentin,” Small GTPases, vol. 2, no. 1, pp. 51–53, 2011.

[71] P. A. Coulombe and P. Wong, “Cytoplasmic intermediate filaments revealed as dynamic and multipurpose scaffolds,” Nat. Cell Biol., vol. 6, no. 8, pp. 699–706, 2004.

[72] A. Satelli and S. Li, “Vimentin in cancer and its potential as a molecular target for cancer therapy.,” Cell. Mol. Life Sci., vol. 68, no. 18, pp. 3033–46, Sep. 2011.

[73] K. Vuoriluoto et al., “Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer,” Oncogene, vol. 30, no. 12, pp. 1436–1448, 2011.

[74] B. Eckes et al., “Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts.,” J. Cell Sci., vol. 111 (Pt 1, pp. 1897–1907, 1998.

[75] H. Xu et al., “The role of CD44 in epithelial–mesenchymal transition and cancer development,” Onco. Targets. Ther., vol. 8, pp. 3783–3792, 2015.

[76] B. I. Jang, Y. Li, D. Y. Graham, and P. Cen, “The Role of CD44 in the Pathogenesis, Diagnosis, and Therapy of Gastric Cancer,” Gut Liver, vol. 5, no. 4, pp. 397–405, 2011.

[77] S. Y. Ju, S. H. Chiou, and Y. Su, “Maintenance of the stemness in CD44+HCT-15 and HCT-116 human colon cancer cells requires miR-203 suppression,” Stem Cell Res., vol. 12, no. 1, pp. 86–100, 2014.

[78] W. D. Hanley, M. M. Burdick, K. Konstantopoulos, and R. Sackstein, “CD44 on LS174T colon carcinoma cells possesses E-selectin ligand activity,” Cancer Res., vol. 65, no. 13, pp. 5812–5817, 2005.

[79] S. McFarlane et al., “CD44 increases the efficiency of distant metastasis of breast cancer,” Oncotarget, vol. 6, no. 13, pp. 11465–11476, 2015.

[80] S. H. Cho et al., “CD44 enhances the epithelial-mesenchymal transition in association with colon cancer invasion,” Int. J. Oncol., vol. 41, no. 1, pp. 211–218, 2012.

[81] J. P. Thiery, “Epithelial–mesenchymal transitions in tumour progression,” Nat. Rev. Cancer, vol. 2, no. 6, pp. 442–454, 2002.

[82] S. M. Ellerbroek et al., “Phosphatidylinositol 3-kinase activity in epidermal growth factor-stimulated matrix metalloproteinase-9 production and cell surface association,” Cancer Res., vol. 61, no. 5, pp. 1855–1861, 2001.

[83] S. Grünert, M. Jechlinger, and H. Beug, “Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis,” Nat. Rev. Mol. Cell Biol., vol. 4, no. 8, pp. 657–665, 2003.

[84] J. Kim, J. Kong, H. Chang, H. Kim, and A. Kim, “EGF induces epithelial-mesenchymal transition through phospho-Smad2/3-Snail signaling pathway in breast cancer cells,” Oncotarget, vol. 7, no. 51, pp. 85021–85032, 2016.

[85] B. De Craene, F. Van Roy, and G. Berx, “Unraveling signalling cascades for the Snail family of transcription factors,” Cell. Signal., vol. 17, no. 5, pp. 535–547, 2005.

[86] Y. Wu and B. P. Zhou, “Snail,” Cell Adh. Migr., vol. 4, no. 2, pp. 199–203, 2010.

[87] Q. Yu, B. P. Zhou, and Y. Wu, “The regulation of snail: on the ubiquitin edge,” vol. 4, no. 2, 2017.

[88] Z. Zhao, M. A. Rahman, Z. G. Chen, and D. M. Shin, “Multiple biological functions of Twist1 in various cancers,” Oncotarget, vol. 8, no. 12, pp. 20380–20393, 2017.

[89] C.-W. Li et al., “AKT1 Inhibits Epithelial-to-Mesenchymal Transition in Breast Cancer through Phosphorylation-Dependent Twist1 Degradation,” Cancer Res., vol. 76, no. 6, pp. 1451–1462, Mar. 2016.

[90] T. Zhan, N. Rindtorff, and M. Boutros, “Wnt signaling in cancer,” Oncogene, vol. 36, no. 11, pp. 1461–1473, 2017.

[91] H.-C. Chen, Y.-T. Zhu, S.-Y. Chen, and S. C. G. Tseng, “Wnt signaling induces epithelial-mesenchymal transition with proliferation in ARPE-19 cells upon loss of contact inhibition.,” Lab. Invest., vol. 92, no. 5, pp. 676–87, May 2012.

[92] L. N. Kwong and W. F. Dove, “APC and its modifiers in colon cancer.,” Adv. Exp. Med. Biol., vol. 656, no. 2, pp. 85–106, 2009.

[93] M. Schmitt, M. Metzger, D. Gradl, G. Davidson, and V. Orian-Rousseau, “CD44 functions in Wnt signaling by regulating LRP6 localization and activation,” Cell Death Differ., vol. 22, no. 4, pp. 677–689, 2015.

[94] A. W. Burgess, M. C. Faux, M. J. Layton, and R. G. Ramsay, “Wnt signaling and colon tumorigenesis - A view from the periphery,” Exp. Cell Res., vol. 317, no. 19, pp. 2748–2758, 2011.

[95] K. H. Chiow et al., “SNX3-dependent regulation of epidermal growth factor receptor (EGFR) trafficking and degradation by aspirin in epidermoid carcinoma (A-431) cells,” Cell. Mol. Life Sci., vol. 69, no. 9, pp. 1505–1521, May 2012.

[96] A. M. Krasinskas, “EGFR Signaling in Colorectal Carcinoma,” Patholog. Res. Int., vol. 2011, no. 2, pp. 1–6, Feb. 2011.

[97] L. Guo, C. J. Kozlosky, L. H. Ericsson, T. O. Daniel, D. P. Cerretti, and R. S. Johnson, “Studies of ligand-induced site-specific phosphorylation of epidermal growth factor receptor,” J. Am. Soc. Mass Spectrom., vol. 14, no. 9, pp. 1022–1031, 2003.

[98] C. Lauand, P. Rezende-Teixeira, B. A. Cortez, E. L. de O. Niero, and G. M. Machado-Santelli, “Independent of ErbB1 gene copy number, EGF stimulates migration but is not associated with cell proliferation in non-small cell lung cancer,” Cancer Cell Int., vol. 13, no. 1, pp. 1–15, 2013.

[99] S. Lindsey and S. A. Langhans, “Epidermal Growth Factor Signaling in Transformed Cells,” in Liver Transpl, vol. 3, no. 10, 2015, pp. 1–41.

[100] M. Scaltriti and J. Baselga, “The Epidermal Growth Factor Receptor Pathway: A Model for Targeted Therapy,” Clin. Cancer Res., vol. 12, no. 18, pp. 5268–5272, Sep. 2006.

[101] K. M. Cadigan and M. L. Waterman, “TCF/LEFs and Wnt signaling in the nucleus,” Cold Spring Harb. Perspect. Biol., vol. 4, no. 11, pp. 1–22, 2012.

[102] T. Brabletz, R. Kalluri, M. A. Nieto, and R. A. Weinberg, “EMT in cancer,” Nat. Rev. Cancer, vol. 18, no. 2, pp. 128–134, 2018.

[103] K. M. Hardy, B. W. Booth, M. J. C. Hendrix, D. S. Salomon, and L. Strizzi, “ErbB/EGF signaling and EMT in mammary development and breast cancer.,” J. Mammary Gland Biol. Neoplasia, vol. 15, no. 2, pp. 191–9, Jun. 2010.

[104] A. Mizumoto et al., “Induction of Epithelial-Mesenchymal Transition via Activation of Epidermal Growth Factor Receptor Contributes to Sunitinib Resistance in Human Renal Cell Carcinoma Cell Lines,” J. Pharmacol. Exp. Ther., vol. 355, no. 2, pp. 152–158, 2015.

[105] H. Lo et al., “Epidermal Growth Factor Receptor Cooperates with Signal Transducer and Activator of Transcription 3 to Induce Epithelial-Mesenchymal Transition in Cancer Cells via Up-regulation of TWIST Gene Expression,” Cancer Res., vol. 67, no. 19, pp. 9066–9076, Oct. 2007.

[106] Y. Liu, S. El-Naggar, D. S. Darling, Y. Higashi, and D. C. Dean, “Zeb1 links epithelial-mesenchymal transition and cellular senescence,” Development, vol. 135, no. 3, pp. 579–588, 2008.

[107] A. Gu, Y. Jie, Q. Yao, Y. Zhang, and E. Mingyan, “Slug Is Associated with Tumor Metastasis and Angiogenesis in Ovarian Cancer,” Reprod. Sci., vol. 24, no. 2, pp. 291–299, 2017.

[108] H. Yamaguchi, J. L. Hsu, and M.-C. Hung, “Regulation of Ubiquitination-Mediated Protein Degradation by Survival Kinases in Cancer,” Front. Oncol., vol. 2, no. February, pp. 1–9, 2012.

[109] Q. Q. Zhu, C. Ma, Q. Wang, Y. Song, and T. Lv, “The role of TWIST1 in epithelial-mesenchymal transition and cancers,” Tumor Biol., vol. 37, no. 1, pp. 185–197, 2016.

[110] M. Itoh, M. Furuse, K. Morita, K. Kubota, M. Saitou, and S. Tsukita, “Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins.,” J. Cell Biol., vol. 147, no. 6, pp. 1351–63, Dec. 1999.

[111] T. Kaihara et al., “Redifferentiation and ZO-1 reexpression in liver-metastasized colorectal cancer: Possible association with epidermal growth factor receptor-induced tyrosine phosphorylation of ZO-1,” Cancer Sci., vol. 94, no. 2, pp. 166–172, 2003.

[112] S. H. Lee, A. R. Paek, K. Yoon, S. H. Kim, S. Y. Lee, and H. J. You, “Tight junction protein 1 is regulated by transforming growth factor-ß and contributes to cell motility in NSCLC cells,” BMB Rep., vol. 48, no. 2, pp. 115–120, 2015.

[113] M. Wang et al., “N-cadherin promotes epithelial-mesenchymal transition and cancer stem cell-like traits via ErbB signaling in prostate cancer cells,” Int. J. Oncol., vol. 48, no. 2, pp. 595–606, 2016.

[114] K. N. Sugahara, T. Hirata, H. Hayasaka, R. Stern, T. Murai, and M. Miyasaka, “Tumor cells enhance their own CD44 cleavage and motility by generating hyaluronan fragments,” J. Biol. Chem., vol. 281, no. 9, pp. 5861–5868, 2006.

[115] Z. Lu, G. Jiang, P. Blume-Jensen, and T. Hunter, “Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase.,” Mol. Cell. Biol., vol. 21, no. 12, pp. 4016–31, Jun. 2001.

[116] A. E. Sedgwick and C. D’Souza-Schorey, “Wnt signaling in cell motility and invasion: Drawing parallels between development and cancer,” Cancers (Basel)., vol. 8, no. 9, pp. 1–15, 2016.

[117] H. Son and A. Moon, “Epithelial-mesenchymal transition and cell invasion,” Toxicol. Res., vol. 26, no. 4, pp. 245–252, 2013.

[118] S. Tanabe, “Wnt Signaling and Epithelial-Mesenchymal Transition Network in Cancer Wnt Signaling and Mesenchymal Transition Wnt Signaling in Cancer Epithelial-Mesenchymal Transition and,” pp. 2–3, 2018.

[119] R. Ali and M. K. Wendt, “The paradoxical functions of EGFR during breast cancer progression,” Signal Transduct. Target. Ther., vol. 2, no. November 2016, p. 16042, 2017.

[120] J. P. Spano et al., “Impact of EGFR expression on colorectal cancer patient prognosis and survival,” Ann. Oncol., vol. 16, no. 1, pp. 102–108, 2005.

[121] J. Brabender et al., “Epidermal Growth Factor Receptor and HER2-neu mRNA Expression in Non-Small Cell Lung Cancer Is Correlated with Survival Advances in Brief Epidermal Growth Factor Receptor and HER2-neu mRNA Expression in Non-Small Cell Lung Cancer Is Correlated with Surviv,” vol. 7, no. July, pp. 1850–1855, 2001.

[122] G. E. Konecny et al., “HER2 gene amplification and EGFR expression in a large cohort of surgically staged patients with nonendometrioid (type II) endometrial cancer,” Br. J. Cancer, vol. 100, no. 1, pp. 89–95, 2009.

[123] E. Theveneau and R. Mayor, “Cadherins in collective cell migration of mesenchymal cells,” Curr. Opin. Cell Biol., vol. 24, no. 5, pp. 677–684, 2012.

APPENDIX A

MTT DATA

Table A.1. *MTT OD Results of NT shRNA transfected cells BR 1*

NT shRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Rep6*	*Rep7*	*Rep8*	*Blank 1*	*Blank 2*
0 h	0.516	0.482	0.489	0.494	0.49	0.496	0.465	0.526	0.099	0.099
24 h	0.933	0.928	0.877	0.793	0.885	0.755	0.703	0.704	0.125	0.124
48 h	1.142	0.887	0.837	0.852	0.842	0.815	0.811	0.756	0.138	0.161
72 h	1.539	1.507	1.590	1.360	1.354	1.490	1.479	1.461	0.135	0.159
96 h	2.489	2.430	2.259	2.097	2.263	2.217	2.253	2.139	0.218	0.229

Table A.2. *MTT OD Results of SNX3 shRNA transfected cells BR 1*

SNX3 shRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Rep6*	*Rep7*	*Rep8*	*Blank 1*	*Blank 2*
0 h	0.382	0.376	0.419	0.393	0.398	0.434	0.412	0.445	0.124	0.112
24 h	0.543	0.688	0.582	0.656	0.687	0.541	0.598	0.604	0.125	0.124
48 h	0.72	0.704	0.66	0.601	0.627	0.63	0.651	0.708	0.145	0.14
72 h	0.859	1.127	1.328	1.162	1.176	1.009	0.945	0.999	0.167	0.159
96 h	1.431	1.607	1.773	1.627	1.564	1.517	1.539	1.474	0.301	0.301

Table A.3. *MTT OD Results of NT shRNA transfected cells br2*

NT shRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Rep6*	*Rep7*	*Rep8*	*Blank 1*	*Blank 2*
0 h	0.541	0.544	0.517	0.515	0.497	0.515	0.518	0.527	0.105	0.102
24 h	0.751	0.831	0.824	0.885	0.785	0.769	0.745	0.719	0.124	0.125
48 h	1.142	0.887	0.837	0.852	0.842	0.815	0.811	0.756	0.117	0.131
72 h	1.730	1.557	1.506	1.389	1.228	1.440	1.350	1.241	0.122	0.137
96 h	2.269	1.883	1.833	1.924	1.810	1.870	1.550	1.672	0.196	0.218

Table A.4. *MTT OD Results of SNX3 shRNA transfected cells br 2*

SNX3 shRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Rep6*	*Rep7*	*Rep8*	*Blank 1*	*Blank 2*
0 h	0.422	0.479	0.457	0.467	0.446	0.456	0.419	0.482	0.115	0.117
24 h	0.618	0.701	0.537	0.599	0.596	0.584	0.569	0.581	0.127	0.128
48 h	0.616	0.604	0.598	0.599	0.573	0.596	0.604	0.542	0.133	0.13
72 h	1.364	1.106	1.025	0.974	1.038	1.084	1.067	0.925	0.163	0.148
96 h	1.493	1.251	1.241	1.179	1.232	1.202	1.313	1.214	0.261	0.265

Table A.5. *MTT OD results of NT shRNA transfected cells br 3*

NT shRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Rep6*	*Rep7*	*Rep8*	*Blank 1*	*Blank 2*
0 h	0.482	0.481	0.492	0.479	0.467	0.492	0.481	0.52	0.136	0.129
24 h	0.782	0.746	0.771	0.804	0.758	0.729	0.883	0.696	0.114	0.116
48 h	0.963	0.833	0.754	0.840	0.891	0.788	0.737	0.713	0.12	0.136
72 h	1.503	1.324	1.389	1.262	1.214	1.271	1.375	1.213	0.125	0.140
96 h	1.904	1.773	1.589	1.848	1.716	1.641	1.814	1.599	0.19	0.246

Table A.6. *MTT OD results of SNX3 shRNA transfected cells br 3*

SNX3 shRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Rep6*	*Rep7*	*Rep8*	*Blank 1*	*Blank 2*
0 h	0.381	0.398	0.432	0.445	0.429	0.424	0.444	0.471	0.103	0.109
24 h	0.581	0.669	0.512	0.534	0.54	0.623	0.603	0.5	0.127	0.125
48 h	0.621	0.665	0.679	0.56	0.521	0.524	0.501	0.48	0.102	0.099
72 h	1.139	1.208	1.161	1.075	0.996	1.110	0.928	1.125	0.117	0.119
96 h	1.383	1.357	1.617	1.471	1.155	1.109	1.196	1.280	0.260	0.278

Table A.7. *MTT OD results of NT siRNA transfected cells*

NT siRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Blank1*	*Blank2*
0 h	0.631	0.607	0.575	0.554	0.54	0.108	0.106
24 h	0.78	0.771	0.723	0.636	0.638	0.12	0.114
48 h	0.691	0.646	0.828	0.752	0.857	0.119	0.123
72 h	1.763	1.205	1.057	0.916	1.017	0.121	0.130
96 h	1.296	1.502	1.583	1.493	1.805	0.131	0.159

Table A.8. *MTT OD results of SNX3 siRNA transfected cells*

SNX3 siRNA	*Rep1*	*Rep2*	*Rep3*	*Rep4*	*Rep5*	*Blank1*	*Blank2*
0 h	0.612	0.569	0.469	0.446	0.431	0.104	0.103
24 h	0.631	0.615	0.62	0.491	0.505	0.11	0.107
48 h	0.829	0.7	0.653	0.587	0.578	0.116	0.118
72 h	1.094	1.295	1.153	1.559	1.134	0.115	0.128
96 h	1.697	2.022	2.668	1.844	1.596	0.134	0.158

APPENDIX B

MIGRATION DATA

Table B.1. *xCELLigence migration cell index data*

Time (hh:mm:ss)	**pSUPER E.V.**	**pSUPER E.V.**	**pSUPER E.V.**	**NT siRNA**	**NT siRNA**	**NT siRNA**	**SNX3 siRNA**	**SNX3 siRNA**	**SNX3 siRNA**
00:00:00	0	0	0	0	0	0	0	0	0
00:46:23	0.035	0.0374	-0.1442	-0.171	-0.2766	0.0517	0.0744	-0.201	-0.2538
01:01:23	0.0043	0.035	-0.1864	-0.2103	-0.3154	0.0045	0.0275	-0.2554	-0.2996
01:31:25	-0.0024	0.0392	-0.2115	-0.2345	-0.3453	0.0082	0.003	-0.422	-0.4644
01:46:26	0.0042	0.0385	-0.2181	-0.2413	-0.3527	-0.0033	-0.0085	-0.4549	-0.4963
02:16:28	0.0122	0.042	-0.2196	-0.2441	-0.3532	-0.009	-0.0219	-0.4705	-0.5113
02:46:29	0.0183	0.0497	-0.2192	-0.2483	-0.3482	-0.0102	-0.0337	-0.4805	-0.5222
03:01:30	0.0238	0.0518	-0.2181	-0.247	-0.3466	-0.0097	-0.0351	-0.4868	-0.5241
03:31:32	0.0301	0.062	-0.2134	-0.2476	-0.3391	-0.0048	-0.044	-0.4949	-0.5331
04:01:34	0.0392	0.0683	-0.2071	-0.2443	-0.3327	-0.0033	-0.0529	-0.5041	-0.5412
04:31:36	0.0485	0.0735	-0.1996	-0.2362	-0.3612	-0.0014	-0.0593	-0.5159	-0.5501
04:46:37	0.0527	0.0793	-0.1964	-0.2353	-0.3584	0.0019	-0.0608	-0.519	-0.5516
05:16:39	0.0625	0.0897	-0.1856	-0.2275	-0.3468	0.0068	-0.0672	-0.523	-0.5549
05:46:41	0.0701	0.0987	-0.1793	-0.2224	-0.3359	0.0136	-0.073	-0.5249	-0.5577
06:01:42	0.0736	0.1024	-0.1763	-0.2218	-0.3313	0.0181	-0.0763	-0.527	-0.562
06:31:44	0.082	0.1074	-0.1712	-0.2129	-0.3212	0.0216	-0.079	-0.5309	-0.5609
07:01:46	0.0887	0.1124	-0.1694	-0.2063	-0.3104	0.0262	-0.0815	-0.5356	-0.5638
07:31:48	0.094	0.1162	-0.1651	-0.2012	-0.3004	0.0314	-0.0882	-0.536	-0.567

Table B.1 Cont'd

07:46:49	0.0966	0.1186	-0.1645	-0.1987	-0.296	0.0324	-0.087	-0.5396	-0.5671
08:16:51	0.1006	0.1205	-0.1615	-0.1949	-0.2892	0.0355	-0.0906	-0.5436	-0.5707
08:46:53	0.1038	0.1262	-0.1584	-0.1906	-0.2822	0.0401	-0.0961	-0.5435	-0.5748
09:01:54	0.1056	0.126	-0.1591	-0.1897	-0.2819	0.0412	-0.0972	-0.5459	-0.578
09:31:56	0.1073	0.1291	-0.1579	-0.1861	-0.2746	0.0439	-0.1003	-0.5483	-0.5794
10:01:58	0.1127	0.1288	-0.1548	-0.1796	-0.2657	0.0446	-0.1002	-0.5521	-0.5794
10:32:00	0.114	0.1335	-0.1567	-0.1794	-0.2626	0.0486	-0.105	-0.5527	-0.5843
10:47:00	0.1136	0.1331	-0.1557	-0.1747	-0.2609	0.0502	-0.1061	-0.5544	-0.5867
11:17:02	0.1181	0.133	-0.1534	-0.1719	-0.2551	0.0506	-0.1056	-0.5574	-0.5857
11:47:04	0.1177	0.137	-0.1553	-0.171	-0.2543	0.0553	-0.1104	-0.5587	-0.5898
12:02:05	0.1201	0.1364	-0.1522	-0.1688	-0.2512	0.0546	-0.1091	-0.5581	-0.5899
12:32:07	0.1204	0.1378	-0.1546	-0.1672	-0.2495	0.0574	-0.1119	-0.5626	-0.5935
13:02:09	0.1227	0.1399	-0.1511	-0.1661	-0.2459	0.0593	-0.114	-0.5626	-0.5938
13:32:10	0.1253	0.1407	-0.1503	-0.1633	-0.244	0.0603	-0.1141	-0.5645	-0.596
13:47:10	0.1237	0.142	-0.1519	-0.1659	-0.2438	0.0618	-0.1173	-0.5647	-0.5981
14:17:12	0.1255	0.1405	-0.1527	-0.1611	-0.2393	0.0639	-0.1183	-0.5674	-0.6002
14:47:13	0.1287	0.1396	-0.1503	-0.1616	-0.2375	0.0656	-0.1185	-0.5723	-0.6021
15:02:14	0.1284	0.1401	-0.1529	-0.1617	-0.2386	0.0677	-0.1209	-0.5711	-0.6029
15:32:16	0.1289	0.1424	-0.1513	-0.1627	-0.2379	0.0713	-0.1217	-0.5713	-0.6052
16:02:17	0.1327	0.1404	-0.1504	-0.1596	-0.2325	0.0721	-0.1203	-0.5757	-0.6044
16:32:19	0.1324	0.1425	-0.1502	-0.1608	-0.2325	0.0764	-0.1225	-0.5759	-0.6089
16:47:19	0.1323	0.1433	-0.1519	-0.1611	-0.2296	0.0769	-0.1236	-0.5767	-0.6086
17:17:21	0.1346	0.1425	-0.152	-0.1588	-0.2298	0.0783	-0.1263	-0.5792	-0.6123
17:47:23	0.1378	0.1404	-0.151	-0.157	-0.2256	0.078	-0.1253	-0.5821	-0.6132
18:02:24	0.1368	0.1418	-0.1494	-0.1567	-0.2245	0.0783	-0.1247	-0.5841	-0.6131
18:32:26	0.1368	0.1434	-0.1496	-0.1575	-0.2221	0.0814	-0.1276	-0.5823	-0.6168

Table B.1 Cont'd

19:02:28	0.1402	0.1427	-0.1473	-0.153	-0.2164	0.08	-0.1267	-0.5856	-0.6168
19:32:29	0.1416	0.1433	-0.146	-0.1518	-0.2148	0.0818	-0.1281	-0.5873	-0.6177
19:47:30	0.1422	0.1444	-0.1463	-0.1496	-0.2161	0.0809	-0.1299	-0.5876	-0.62
20:17:31	0.1433	0.1444	-0.1454	-0.148	-0.2126	0.0818	-0.1299	-0.5901	-0.6204
20:47:33	0.1416	0.1489	-0.1453	-0.1495	-0.2111	0.0858	-0.1316	-0.5892	-0.625
21:02:34	0.1445	0.1466	-0.1434	-0.1476	-0.2095	0.0846	-0.1309	-0.5927	-0.6236
21:32:34	0.1446	0.1507	-0.1437	-0.1498	-0.2079	0.0875	-0.1328	-0.5932	-0.625
22:02:36	0.1472	0.1511	-0.1423	-0.145	-0.2045	0.0875	-0.1325	-0.594	-0.626
22:32:38	0.1498	0.1507	-0.1392	-0.1443	-0.2021	0.087	-0.1339	-0.5973	-0.6285
22:47:38	0.1502	0.153	-0.1416	-0.1432	-0.2034	0.0893	-0.1361	-0.5956	-0.631
23:17:38	0.1516	0.1534	-0.1395	-0.1429	-0.1987	0.0887	-0.1352	-0.5983	-0.6312
23:47:39	0.152	0.1531	-0.1391	-0.1407	-0.1974	0.0892	-0.1367	-0.6015	-0.6323
24:02:40	0.1522	0.154	-0.14	-0.1416	-0.1972	0.091	-0.1363	-0.6002	-0.633
24:32:41	0.1538	0.157	-0.1407	-0.1411	-0.1958	0.092	-0.1386	-0.5998	-0.6354
25:02:41	0.1546	0.1574	-0.1383	-0.1403	-0.1922	0.0926	-0.14	-0.5998	-0.6383
25:32:42	0.1584	0.1561	-0.139	-0.1363	-0.1902	0.0922	-0.1418	-0.6034	-0.6394
25:47:42	0.1576	0.1574	-0.1391	-0.1386	-0.1908	0.094	-0.1402	-0.6015	-0.639
26:17:42	0.1613	0.1563	-0.1367	-0.1342	-0.187	0.0927	-0.1402	-0.6059	-0.6393
26:47:42	0.1616	0.1585	-0.137	-0.1335	-0.1857	0.095	-0.1436	-0.6037	-0.6447
27:02:42	0.1622	0.1582	-0.1352	-0.1319	-0.1846	0.0948	-0.1429	-0.6032	-0.6434
27:32:42	0.1629	0.1587	-0.1333	-0.1313	-0.183	0.0949	-0.1428	-0.6072	-0.643
28:02:42	0.1638	0.1604	-0.1338	-0.1327	-0.1825	0.0958	-0.1441	-0.6061	-0.6446
28:32:42	0.1647	0.1595	-0.1315	-0.131	-0.1802	0.0943	-0.1437	-0.6086	-0.6437
28:47:42	0.1637	0.163	-0.1332	-0.1329	-0.1795	0.0967	-0.1425	-0.6061	-0.6454
29:17:42	0.1666	0.1638	-0.1304	-0.1325	-0.1784	0.0949	-0.1433	-0.6077	-0.6456
29:47:42	0.1672	0.1634	-0.1277	-0.1284	-0.1756	0.0939	-0.1432	-0.6086	-0.6468

Table B.1 Cont'd

30:17:42	0.1663	0.1635	-0.1256	-0.1272	-0.1737	0.0943	-0.1431	-0.6088	-0.6466
30:32:42	0.1659	0.1655	-0.1252	-0.1288	-0.1738	0.0956	-0.1399	-0.6092	-0.6461
31:02:42	0.1691	0.1643	-0.125	-0.1272	-0.1708	0.0961	-0.1409	-0.608	-0.648
31:32:42	0.1704	0.1657	-0.1203	-0.1243	-0.1688	0.0958	-0.139	-0.6102	-0.6464
31:47:42	0.1712	0.1673	-0.1218	-0.1237	-0.1665	0.0978	-0.1408	-0.6105	-0.6459
32:17:42	0.1721	0.1683	-0.1188	-0.121	-0.1642	0.0993	-0.1391	-0.6109	-0.6472
32:47:42	0.1749	0.1702	-0.1151	-0.1186	-0.1611	0.1011	-0.1376	-0.6109	-0.6469
33:17:42	0.1763	0.1748	-0.1146	-0.1167	-0.159	0.1047	-0.1371	-0.6085	-0.6482
33:32:42	0.1795	0.174	-0.1128	-0.1141	-0.1539	0.1048	-0.1374	-0.6085	-0.647
34:02:42	0.1798	0.1784	-0.1104	-0.1138	-0.1498	0.1087	-0.1345	-0.6078	-0.6482
34:32:42	0.1888	0.1805	-0.1089	-0.1112	-0.1464	0.1099	-0.1323	-0.6084	-0.6486
34:47:42	0.1915	0.18	-0.1059	-0.106	-0.1433	0.1102	-0.1329	-0.6099	-0.6475
35:17:42	0.1934	0.1804	-0.1016	-0.1058	-0.1383	0.1123	-0.1325	-0.6092	-0.6486
35:47:42	0.1943	0.1855	-0.0999	-0.1061	-0.1341	0.1176	-0.1291	-0.6047	-0.6486
36:17:42	0.1978	0.1876	-0.0965	-0.0993	-0.1286	0.1191	-0.1305	-0.6033	-0.6503
36:32:42	0.1994	0.1883	-0.0942	-0.0972	-0.1251	0.1195	-0.1297	-0.6048	-0.6476
37:02:42	0.2006	0.194	-0.0899	-0.0939	-0.1191	0.1242	-0.1281	-0.6038	-0.6502
37:32:42	0.2032	0.1983	-0.0836	-0.0915	-0.1144	0.1257	-0.125	-0.6038	-0.6482
37:47:42	0.2031	0.1984	-0.082	-0.0885	-0.1102	0.1269	-0.1245	-0.6047	-0.6467
38:17:42	0.2059	0.2006	-0.0767	-0.0842	-0.1044	0.13	-0.1226	-0.6038	-0.6468
38:47:42	0.2097	0.2057	-0.0741	-0.0814	-0.1006	0.1362	-0.1224	-0.5999	-0.6465
39:17:42	0.2141	0.2071	-0.0678	-0.0743	-0.0897	0.1385	-0.1225	-0.6025	-0.6471
39:32:42	0.2146	0.2083	-0.0651	-0.0718	-0.0849	0.1402	-0.1209	-0.6035	-0.6461
40:02:42	0.2167	0.2152	-0.0615	-0.0709	-0.0841	0.1448	-0.1189	-0.6006	-0.6466
40:32:42	0.2204	0.2197	-0.0587	-0.0631	-0.0742	0.1482	-0.1176	-0.6003	-0.6457
40:47:42	0.2212	0.2211	-0.0557	-0.0605	-0.0697	0.1507	-0.1178	-0.6015	-0.6439

Table B.1 Cont'd

41:17:42	0.2251	0.2252	-0.049	-0.053	-0.0591	0.1555	-0.118	-0.601	-0.6437
41:47:42	0.2286	0.2291	-0.0442	-0.0488	-0.0521	0.1608	-0.1166	-0.5999	-0.6442
42:17:42	0.2315	0.2329	-0.0413	-0.044	-0.0424	0.1668	-0.1159	-0.5963	-0.6461
42:32:42	0.2327	0.2354	-0.0386	-0.0427	-0.0382	0.1684	-0.1161	-0.5986	-0.644
43:02:42	0.2361	0.2417	-0.0332	-0.0374	-0.0291	0.175	-0.1146	-0.5943	-0.6447
43:32:42	0.2386	0.2479	-0.0263	-0.0317	-0.0185	0.1804	-0.1124	-0.5961	-0.6418
43:47:42	0.2401	0.2492	-0.0237	-0.0313	-0.0163	0.1837	-0.1103	-0.5955	-0.6425
44:17:42	0.2454	0.2539	-0.0194	-0.0238	-0.0052	0.1886	-0.1115	-0.5921	-0.6421
44:47:42	0.2471	0.2594	-0.0117	-0.0183	0.006	0.1935	-0.1085	-0.5913	-0.6392
45:17:42	0.249	0.2658	-0.0075	-0.0153	0.0135	0.2014	-0.1052	-0.5897	-0.6376
45:32:42	0.2548	0.2674	-0.0029	-0.0084	0.019	0.2033	-0.1062	-0.5877	-0.6396
46:02:42	0.2565	0.2731	0.0041	-0.0029	0.0289	0.2076	-0.1057	-0.5867	-0.6368
46:32:42	0.2593	0.2779	0.0097	0.0016	0.0385	0.2127	-0.1045	-0.5851	-0.6336
46:47:42	0.2594	0.2818	0.0119	0.0039	0.0427	0.2169	-0.1012	-0.5864	-0.6343
47:17:42	0.2648	0.2838	0.0191	0.0136	0.0553	0.222	-0.1008	-0.581	-0.6351
47:47:42	0.2672	0.2912	0.0241	0.0187	0.0636	0.2286	-0.098	-0.5786	-0.6319
48:17:42	0.2702	0.296	0.0307	0.0272	0.076	0.2339	-0.0986	-0.5789	-0.6306
48:32:42	0.2715	0.2976	0.0324	0.0301	0.0807	0.2364	-0.0969	-0.5776	-0.6309
49:02:42	0.2727	0.3035	0.0406	0.0345	0.0912	0.242	-0.0931	-0.5779	-0.6267
49:32:42	0.2767	0.3078	0.0469	0.0431	0.1036	0.2509	-0.0929	-0.5744	-0.6273
49:47:42	0.2778	0.3104	0.0511	0.0455	0.1087	0.2523	-0.0917	-0.5753	-0.6259
50:17:42	0.2798	0.3164	0.0541	0.0497	0.1212	0.2592	-0.0905	-0.5717	-0.6257
50:47:42	0.2837	0.3226	0.0605	0.0597	0.1351	0.2658	-0.0888	-0.5712	-0.6247
51:17:42	0.2888	0.3268	0.0672	0.0665	0.1503	0.2709	-0.0877	-0.5678	-0.623
51:32:42	0.2897	0.3295	0.0687	0.0717	0.1555	0.2765	-0.0859	-0.5661	-0.623
52:02:42	0.2939	0.3362	0.0765	0.0769	0.1672	0.2834	-0.0837	-0.5626	-0.6204

Table B.1 Cont'd

52:32:42	0.2985	0.3417	0.0831	0.0845	0.1766	0.2909	-0.0825	-0.5598	-0.6191
53:02:42	0.3024	0.3478	0.0927	0.0898	0.1924	0.2985	-0.0826	-0.5603	-0.6158
53:17:42	0.3052	0.3498	0.0951	0.0947	0.199	0.3029	-0.0823	-0.5564	-0.6172
53:47:42	0.3104	0.3546	0.1021	0.1036	0.2137	0.3088	-0.0821	-0.5548	-0.6171
54:17:42	0.3109	0.3577	0.1095	0.1099	0.2299	0.3158	-0.0799	-0.5545	-0.6132
54:32:42	0.3114	0.3617	0.1119	0.1157	0.2362	0.3217	-0.0775	-0.5555	-0.6119
55:02:42	0.3173	0.3666	0.1215	0.1261	0.2542	0.3275	-0.0777	-0.5488	-0.6121
55:32:42	0.3198	0.3726	0.1281	0.135	0.2695	0.3372	-0.075	-0.5462	-0.6108
56:02:42	0.3229	0.3789	0.1383	0.1436	0.287	0.3447	-0.0729	-0.5469	-0.607
56:17:42	0.3251	0.3812	0.142	0.1495	0.2949	0.3489	-0.0739	-0.5458	-0.6063
56:47:42	0.3292	0.3849	0.1517	0.1602	0.3094	0.3569	-0.0722	-0.5408	-0.6062
57:17:42	0.3324	0.3928	0.1596	0.1687	0.3263	0.3653	-0.0721	-0.5388	-0.6075
57:32:42	0.3323	0.3953	0.1629	0.1721	0.3346	0.3701	-0.0709	-0.5393	-0.6025
58:02:42	0.3357	0.4008	0.1714	0.1824	0.3479	0.3774	-0.0671	-0.5349	-0.6009
58:32:42	0.3384	0.406	0.1817	0.1936	0.3637	0.3837	-0.068	-0.5311	-0.6
59:02:42	0.3426	0.414	0.1904	0.2018	0.3798	0.3916	-0.0656	-0.5293	-0.5974
59:17:42	0.3436	0.4138	0.1975	0.2085	0.3872	0.396	-0.0655	-0.5272	-0.598
59:47:42	0.3483	0.419	0.2111	0.2188	0.4022	0.4011	-0.0664	-0.5233	-0.597
60:17:42	0.3532	0.4269	0.2171	0.226	0.4148	0.4103	-0.0617	-0.5208	-0.5925
60:32:42	0.3567	0.4277	0.2232	0.2312	0.4235	0.4119	-0.0627	-0.5191	-0.5923
61:02:42	0.3604	0.4335	0.2305	0.2411	0.4385	0.4205	-0.06	-0.516	-0.5884
61:32:42	0.3648	0.4393	0.2399	0.2484	0.4484	0.4284	-0.0591	-0.5098	-0.5787
62:02:42	0.3701	0.4461	0.2482	0.2535	0.462	0.436	-0.0581	-0.5087	-0.5734
62:17:42	0.3724	0.4499	0.2536	0.2607	0.4701	0.4402	-0.0578	-0.5074	-0.5714
62:47:42	0.3795	0.4526	0.2653	0.2696	0.487	0.4433	-0.0584	-0.501	-0.5679
63:17:42	0.3839	0.4618	0.2734	0.2746	0.4962	0.4535	-0.0553	-0.4939	-0.5543

Table B.1 Cont'd

63:32:42	0.386	0.463	0.2785	0.2808	0.5023	0.4562	-0.0551	-0.4883	-0.5491
64:02:42	0.3913	0.4675	0.2888	0.2874	0.5182	0.4632	-0.054	-0.4861	-0.5452
64:32:42	0.3953	0.4723	0.2969	0.2952	0.5319	0.4713	-0.0528	-0.4824	-0.5422
65:02:42	0.3989	0.4763	0.3093	0.3066	0.5469	0.4792	-0.0519	-0.4787	-0.5391
65:17:42	0.4022	0.4785	0.3135	0.3107	0.5512	0.4832	-0.0511	-0.4765	-0.5383
65:47:42	0.4065	0.4855	0.3255	0.3188	0.5661	0.4903	-0.0496	-0.4729	-0.5313
66:17:42	0.4131	0.4915	0.3384	0.3334	0.581	0.4994	-0.0462	-0.4596	-0.5204
66:32:42	0.4173	0.494	0.3451	0.3378	0.5908	0.5	-0.0461	-0.4556	-0.5157
67:02:42	0.4223	0.5023	0.3534	0.3477	0.6006	0.5119	-0.0427	-0.4447	-0.5023
67:32:42	0.4283	0.5078	0.3645	0.3556	0.6161	0.5205	-0.0402	-0.4337	-0.4866
68:02:42	0.4355	0.5134	0.3757	0.3671	0.6257	0.5241	-0.0408	-0.4234	-0.4757
68:17:42	0.4382	0.5174	0.3793	0.3703	0.6341	0.5303	-0.0381	-0.4209	-0.4727
68:47:42	0.4461	0.5236	0.3912	0.3804	0.6516	0.5382	-0.0384	-0.4178	-0.4686
69:17:42	0.4514	0.5347	0.4034	0.394	0.6617	0.5444	-0.0362	-0.4118	-0.4652
69:32:42	0.4535	0.5377	0.4096	0.3996	0.6684	0.5482	-0.0348	-0.4112	-0.4629
70:02:42	0.4574	0.5461	0.4215	0.4136	0.6835	0.5563	-0.0329	-0.4059	-0.46
70:32:42	0.4667	0.5544	0.4334	0.4246	0.7014	0.5618	-0.0356	-0.4045	-0.4564
71:02:42	0.4734	0.5645	0.4446	0.4334	0.7105	0.5678	-0.0344	-0.4079	-0.4594
71:17:42	0.4776	0.5657	0.4502	0.4394	0.7158	0.5708	-0.0343	-0.405	-0.4592
71:47:42	0.4822	0.5775	0.461	0.4454	0.7302	0.5814	-0.0303	-0.4032	-0.4553
72:17:42	0.4911	0.5859	0.4755	0.4589	0.7412	0.5857	-0.0294	-0.3974	-0.453
72:32:42	0.4952	0.5915	0.4826	0.4633	0.7469	0.5893	-0.0293	-0.3962	-0.4488
73:02:42	0.5022	0.6043	0.4936	0.4755	0.7615	0.5975	-0.0266	-0.3927	-0.4466
73:32:42	0.509	0.6151	0.501	0.4834	0.7735	0.6069	-0.0249	-0.3884	-0.4425
74:02:42	0.5172	0.6279	0.5111	0.4975	0.7847	0.614	-0.0217	-0.3847	-0.4381
74:17:42	0.5222	0.6335	0.519	0.497	0.7884	0.619	-0.0224	-0.3734	-0.4223

Table B.1 Cont'd

74:47:42	0.5302	0.6413	0.5285	0.5038	0.7803	0.6256	-0.0193	-0.3554	-0.4109
75:17:42	0.5375	0.6513	0.5421	0.5129	0.7846	0.6332	-0.018	-0.3478	-0.4065
75:47:42	0.5489	0.6618	0.5535	0.5205	0.7931	0.6405	-0.0177	-0.3467	-0.3977
76:02:42	0.5529	0.6645	0.5599	0.5283	0.7944	0.6445	-0.0165	-0.3414	-0.3988
76:32:42	0.561	0.6782	0.5657	0.5354	0.8014	0.6554	-0.0148	-0.3406	-0.3924
77:02:42	0.5721	0.6878	0.5778	0.5477	0.8137	0.6633	-0.0142	-0.3383	-0.3905
77:17:42	0.576	0.69	0.5851	0.5552	0.8199	0.6674	-0.0121	-0.3361	-0.3902
77:47:42	0.5863	0.7034	0.5938	0.5674	0.8357	0.6779	-0.0113	-0.335	-0.3837
78:17:42	0.5945	0.7166	0.6077	0.5776	0.8447	0.684	-0.0087	-0.3327	-0.3844
78:47:42	0.5998	0.728	0.6167	0.5882	0.8551	0.6933	-0.0071	-0.3306	-0.3804
79:02:42	0.6045	0.7348	0.624	0.5894	0.8611	0.6972	-0.0072	-0.3322	-0.3789
79:32:42	0.6149	0.7433	0.6372	0.6028	0.871	0.6996	-0.0048	-0.3279	-0.3788
80:02:42	0.622	0.7554	0.6512	0.6126	0.8805	0.7079	-0.0032	-0.3252	-0.3772
80:17:42	0.6257	0.7656	0.6553	0.6129	0.8876	0.7111	-0.0008	-0.3262	-0.3735
80:47:42	0.6346	0.7752	0.6709	0.6274	0.896	0.7173	-0.0019	-0.3241	-0.3741
81:17:42	0.6425	0.7847	0.6812	0.6385	0.9052	0.7247	-0.0006	-0.3254	-0.3715
81:47:42	0.6533	0.798	0.6925	0.6503	0.9112	0.7352	0.0037	-0.3211	-0.3738
82:02:42	0.6567	0.8026	0.7011	0.6558	0.9156	0.7378	0.0048	-0.3202	-0.3774
82:32:42	0.6665	0.8167	0.7123	0.6669	0.9186	0.7458	0.0074	-0.3197	-0.3737
83:02:42	0.6768	0.8294	0.7234	0.6753	0.9289	0.7523	0.0058	-0.3174	-0.3727
83:17:42	0.6803	0.8382	0.7274	0.6763	0.9339	0.7556	0.0101	-0.3171	-0.3739
83:47:42	0.6923	0.852	0.7391	0.6853	0.9459	0.7644	0.0104	-0.3176	-0.3724
84:17:42	0.7011	0.862	0.7501	0.6993	0.9503	0.7709	0.0122	-0.3175	-0.3734
84:47:42	0.7081	0.8735	0.7654	0.7153	0.9578	0.7763	0.0138	-0.3159	-0.3694
85:02:42	0.7133	0.878	0.7719	0.7199	0.9618	0.7796	0.0149	-0.3145	-0.3669
85:32:42	0.7213	0.8934	0.7859	0.7305	0.9708	0.7906	0.0185	-0.3109	-0.3647

Table B.1 Cont'd

86:02:42	0.727	0.903	0.8014	0.7479	0.9792	0.7986	0.0201	-0.3073	-0.3636
86:17:42	0.7327	0.9065	0.8104	0.7501	0.9803	0.7994	0.0206	-0.3027	-0.3643
86:47:42	0.7444	0.9172	0.8238	0.7552	0.989	0.8091	0.024	-0.2982	-0.3601
87:17:42	0.7561	0.9306	0.8401	0.7653	0.9994	0.8191	0.0263	-0.2952	-0.3561
87:47:42	0.7682	0.9408	0.8555	0.7775	1.0079	0.8252	0.0285	-0.2897	-0.3535
88:02:42	0.7756	0.9481	0.8637	0.7818	1.0168	0.8292	0.0279	-0.2879	-0.348
88:32:42	0.7847	0.957	0.8804	0.7962	1.0283	0.8336	0.0277	-0.2848	-0.3441
89:02:42	0.7979	0.9703	0.8961	0.8079	1.0366	0.8447	0.0313	-0.2805	-0.3392
89:17:42	0.8013	0.9756	0.9015	0.816	1.0378	0.8483	0.031	-0.2774	-0.3397
89:47:42	0.8107	0.9893	0.9155	0.8264	1.0493	0.855	0.0322	-0.2726	-0.3329
90:17:42	0.822	0.9986	0.929	0.8399	1.0577	0.8586	0.0314	-0.2684	-0.3249
90:47:42	0.829	1.0084	0.941	0.8536	1.0672	0.867	0.0349	-0.2622	-0.3224
91:02:42	0.8343	1.0136	0.9479	0.8586	1.0737	0.8699	0.0361	-0.2596	-0.3193
91:32:42	0.8446	1.025	0.9594	0.8764	1.0798	0.8735	0.035	-0.2559	-0.3117
92:02:42	0.854	1.0356	0.9772	0.8876	1.0895	0.8796	0.0376	-0.2488	-0.3048
92:17:42	0.8576	1.0399	0.9834	0.8876	1.0933	0.8839	0.0394	-0.245	-0.3059
92:47:42	0.863	1.0314	0.9701	0.8545	1.0487	0.8883	0.0331	-0.2665	-0.3344
93:17:42	0.873	1.0404	0.983	0.8563	1.0473	0.8903	0.0344	-0.2652	-0.329
93:47:42	0.8863	1.0558	0.9975	0.8645	1.0503	0.8957	0.0378	-0.2614	-0.3264
94:02:42	0.8901	1.0597	1.0045	0.8679	1.0542	0.8988	0.0394	-0.2589	-0.3284
94:32:42	0.8982	1.0767	1.0145	0.8792	1.0594	0.9019	0.0427	-0.2568	-0.3208
95:02:42	0.9095	1.0869	1.0252	0.8886	1.065	0.9091	0.0464	-0.2525	-0.3187
95:32:41	0.9147	1.0995	1.0349	0.898	1.0655	0.9107	0.049	-0.2474	-0.3131
95:47:41	0.9201	1.1087	1.0385	0.9004	1.0695	0.9165	0.0514	-0.2447	-0.3133
96:02:41	0.9226	1.1109	1.0464	0.9073	1.0719	0.9175	0.051	-0.2437	-0.3078
96:17:41	0.9262	1.1171	1.0513	0.9101	1.0742	0.9233	0.055	-0.2395	-0.3069

Table B.1 Cont'd

96:32:41	0.9326	1.1239	1.0576	0.9138	1.079	0.9253	0.0558	-0.2369	-0.3042
96:47:41	0.9353	1.1263	1.0665	0.9196	1.0824	0.9256	0.0538	-0.236	-0.2992

APPENDIX C

BUFFERS FOR EXPERIMENTS

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

PBS-T:

137 mM NaCl

2.7 mM KCl

10 mM Na2HPO4.2H2O

2 mM KH2PO4

0.1 % Tween 20

pH: 7.4

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

MTT Solution:

1 ml PBS

5 mg MTT powder

HCl-SDS Solution for MTT:

1 g SDS

10 ml 0.01 M HCl

APPENDIX D

MARKER

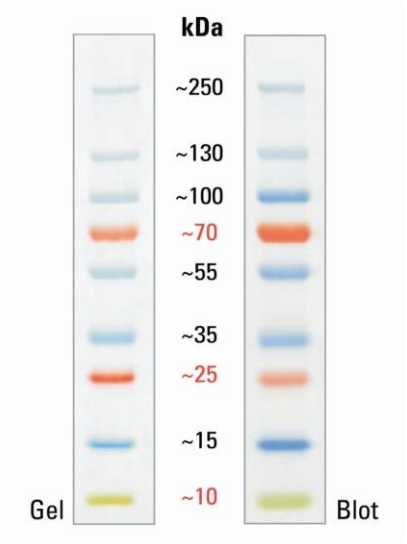

Figure D.1. Protein Ladder.

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