SORTING NEXIN 3 (SNX3) IS AN IMMEDIATE AND SUSTAINED TARGET OF EGF STIMULATION IN EGFR POSITIVE BREAST CELLS

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ABSTRACT

SORTING NEXIN 3 (SNX3) IS AN IMMEDIATE AND SUSTAINED TARGET OF EGF STIMULATION IN EGFR POSITIVE BREAST CELLS

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Sorting nexin 3 (SNX3) is a member of the sorting nexins family, which is involved in the endocytic trafficking network. SNX3 only has a PX domain, which helps endosomes bind to phosphatidyl inositol-3-phosphate and plays a role in the constitution of the retromer complex on early endosomes. Endocytosed receptors are internalized and recycled back to the plasma membrane, the Trans-Golgi Network, or are degraded in the lysosomes. The current understanding of SNX3 function in recycling Epidermal Growth Factor Receptor (EGFR), was demonstrated in an epidermoid carcinoma cell line (A431). Therefore, we hypothesized that SNX3 is an important component of the EGF/EGFR axis in breast cancers and investigated how SNX3 gene is regulated in EGFR positive cells in response to EGF stimulation. To test this hypothesis, we used EGFR (+) MCF10A cells which has been established as an EGF responsive cell line. We performed SNX3 stimulation in a time dependent setup, collected RNA and lysates from treated cells. We observed immediate and sustained upregulation of SNX3 protein upon EGF stimulation. Interestingly, SNX3 upregulation at early time points was independent of transcriptional regulation. EGF

stimulation improved protein half life. However, late response to EGF stimulation was due to transcriptional upregulation as was demonstrated by RT-qPCR. We also show that upregulation of SNX3 is also EGFR pathway specific as EGFR inhibitör AG1478 and other inhibitors that blocked EGFR downstream pathways. These observations were further confirmed in another cell line positive for EGFR; HEK293. Overall, these results show SNX3 as an important component of EGF/EGFR axis as an immediate and sustained target of EGF stimulation. Implications for these findings will be important in better understanding EGFR biology.

Keywords: Sorting Nexin 3 (SNX3), Epidermal Growth Factor Receptor, Breast Cancer

ÖZ

SNX3'ÜN EGFR-POZİTİF MEME HÜCRELERİNDE ERKEN VE UZUN DÖNEM EGF UYARIMINA DUYARLILIĞI

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Sorting nexin 3 (SNX3), endositik trafik ağında yer alan Sorting Nexin ailesinin bir üyesidir. SNX3 yalnızca endozomların fosfatidil inositol-3-fosfata bağlanmasına yardımcı olan ve erken endozomlarda retromer kompleksinin oluşumunda rol oynayan bir PX alanına sahiptir. Endositozla hücre içine alınan reseptörler, plazma zarına ve Trans-Golgi Ağı'na geri dönüştürülür veya lizozomlarda yıkıma uğrar. Epidermal Büyüme Faktörü Reseptörü'nün (EGFR) geri dönüştürülmesinde SNX3'ün rol oynadığı, bir epidermoid karsinom hücre hattında (A431) gösterilmiştir. Bu nedenle, SNX3'ün meme kanserlerinde EGF/EGFR ekseninin önemli bir bileşeni olduğunu varsaydık ve EGF uyarısına yanıt olarak EGFR pozitif hücrelerde SNX3 geninin nasıl düzenlendiğini araştırdık. Bu hipotezi test etmek için, EGF'ye yanıt veren bir hücre hattı olan EGFR (+) MCF10A hücrelerini kullandık. SNX3 stimülasyonunu zamana bağlı bir şekilde test ettik, EGF muamelesi yapılan hücrelerden RNA ve lizatlar topladık. EGF uyarımı üzerine SNX3 proteininin erken ve sürekli cevap olarak arttığını gözlemledik. İlginç bir şekilde, erken zaman aralığında SNX3 protein miktarındaki artış, transkripsiyonel düzenlemeden bağımsızdı. EGF stimülasyonu protein yarı ömrünü uzattı. Bununla birlikte, EGF

stimülasyonuna geç yanıt, RT-qPCR ile gösterildiği gibi transkripsiyonel artıştan kaynaklanmıştır. Ayrıca, SNX3 protein artışının, EGFR inhibitörü AG1478 ve EGFR alt yolaklarını bloke eden diğer inhibitörleri kullanarak EGFR yolağına özgü olduğunu da gösterdik. Bu gözlemler, EGFR (+) başka bir hücre hattı olan HEK293 hücrelerinde de doğrulandı. Genel olarak, bu sonuçlar SNX3'ü EGF/EGFR ekseninin önemli bir bileşeni olarak EGF stimülasyonunun erken ve sürekli hedefi olarak göstermektedir. Bu bulgular için çıkarımlar, EGFR biyolojisinin daha iyi anlaşılmasında önemli olacaktır.

Anahtar Kelimeler: Sorting Nexin 3 (SNX3), Epidermal Büyüme Faktörü Reseptörü, Meme Kanseri

To my Family…

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS

EGFR	Epidermal Growth Factor Receptor
RTK	Receptor Tyrosine Kinase
EGF	Epidermal Growth Factor
TM	Transmembrane
JM	Juxtamembrane
TKD	Tyrosine Kinase Domain
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
PIP2	Phosphatidylinositol-(4,5)-bisphosphate
mTOR	Mammalian Target of Rapamycin
WLS	Wntless
TGN	Trans-Golgi Network
CHX	Cycloheximide
ActD	Actinomycin D
EtOH	Ethanol
NaAc	Sodium Acetate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TIFF1	Trefoil Factor 1
CTNNB1	Beta catenin
ER	Estrogen Receptor
FKBP12	FKBP prolyl isomerase 1A
S6K1	S6 ribosomal protein kinase
4EBP	Eukaryotic elongation factor 4E-binding protein
TNBC	Triple Negative Breast Cancer
IBC	Inflammatory Breast Cancer
IEGs	Immediate Early Genes
TCF	Ternary Complex Factor
TGFα	Transforming growth factor alpha

CHAPTER 1

INTRODUCTION

1.1 Epidermal Growth Factor Receptor and ErbB Family

The epidermal growth factor receptor (EGFR) is a member of ErbB family of receptor tyrosine kinases (RTKs), along with ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 (Lemmon *et al.*, 2014). Members of ErbB family are expressed in almost all cell types, with the exception of hematopoietic cells (Fisher and Lakshmanan, 1990). RTKs are crucial for normal embryonic development of vertebrates since deficiency of any of the ErbB family members is generally lethal in mice (Sibilia *et al.*, 2007; Wee and Wang, 2017).

As the first identified member of RTK family, EGFR regulates a wide variety of signal transduction events that control key processes of life; such as proliferation, differentiation, and migration (Wieduwilt and Moasser, 2008; Ceresa and Peterson, 2014). Activating mutations in the EGFR gene, alterations in the signaling cascade, deregulated expression and degradation are reported in several cancer types including glioblastomas, head and neck, lung, colon and breast cancers. Therefore, EGFR is considered as a target for anti-cancer therapies (Yarden and Pines, 2012).

Epidermal growth factor (EGF) is the most extensively studied EGFR ligand but in mammals six other ligands including, heparin-binding EGF-like (HBEGF), transforming growth factor alpha (TGFα), amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), and epigen (EPG) have been identified (Cohen, 1962; Shoyab *et al.*, 1988; Massague, 1990; Toyoda *et al.*, 1995; Strachan *et al.*, 2001; Higashiyama *et al.*, 1991; Shing *et al.*, 1993). All of these ligands are produced as membrane bound precursors and are transformed into active growth factors upon cleavage by cell surface proteases (Harris *et al.* 2003; Adrain and Freeman, 2014).

These ligands stimulate specific intracellular trafficking cascades and cellular responses *in vitro* and *in vivo* (Singh and Harris 2005; Wilson *et al.* 2009; Yang *et al.*, 2017). In some cases, these different biological outcomes are attributed to the differences in strength of the receptor-ligand interaction, which determines whether the ligand dissociates from the receptor (as in case of TGFα) or not (as in case of EGF) under the mild acidic conditions of the endosomes, favoring the recycling or degradation of the EGFR, respectively. In other cases, the different signaling features of the different ligands are referred to their capability to differentially stabilize EGFR dimers (Frenceh *et al.*, 1995; Wee and Wang, 2017).

Like all RTKs, EGFR comprises an extracellular region responsible for ligand recognition, a single spanning transmembrane (TM) domain, and an intracellular region including juxtamembrane (JM) region, the tyrosine kinase domain (TKD) and a C-terminal regulatory tail carrying the tyrosine residues which are phosphorylated upon ligand binding (Lemmon and Schlessinger, 2010). The extracellular region of EGFR contains four domains: Domain I and III show sequence similarity and are the primary ligand binding regions. Domain II and IV are cysteine-rich domains and have roles in receptor dimerization (Lemmon *et al.*, 2014).

Based on the data obtained from monomeric and dimeric structures of the EGFR, two models have been proposed for the activation of the receptor by ligand binding; the "ligand induced dimerization" and the "rotation" models. According to the ligand induced dimerization model (Figure 1.1), in the absence of bound EGF, the EGFR localizes at the cell surface mainly in a monomeric form. In this closed confirmation, TKD remains inactive and the β-hairpin of the domain II (the dimerization arm) interacts with domain IV to create a tethered autoinhibitory configuration. Binding of the EGF to domains I and III triggers a conformational change which results in the extension of the extracellular region and exposure of the dimerization arm. With the exposed dimerization arm, the extracellular region of the EGFR dimerizes (Burgess *et al.* 2003; Kovacs *et al.*, 2015). Dimerization of the extracellular regions brings the TKDs into a close proximity to form an asymmetric dimer which leads to

kinase activation by trans-autophosphorylation (Zhang *et al.* 2006; Lemmon *et al.*, 2014).

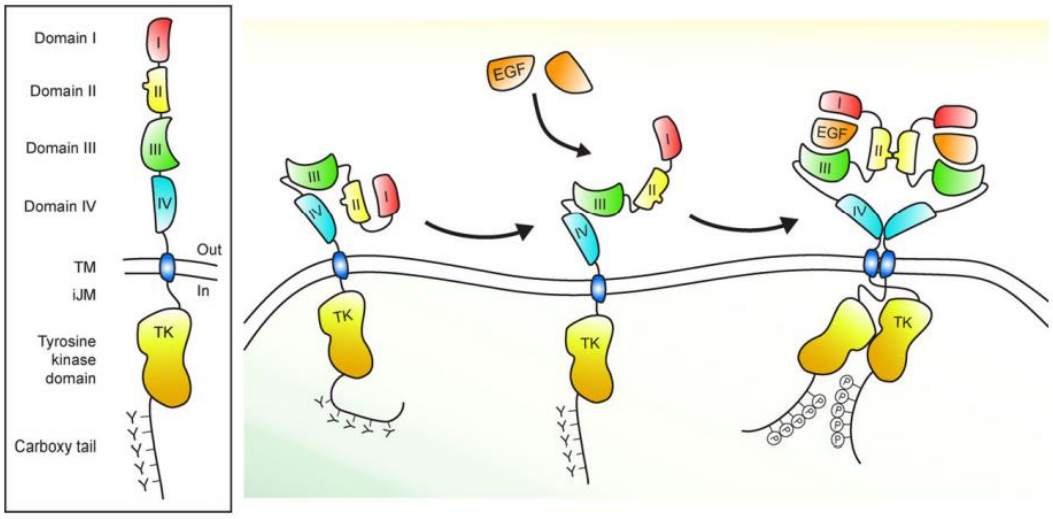


Figure 1.1. Representation of ligand-induced dimerization of EGFR. The EGFR extracellular region has 4 domains (I, II, III and IV) followed by transmembrane (TM) region, intracellular juxtamembrane domain (iJM), tyrosine kinase domain (TK) and carboxy tail. Upon EGF binding to domain I and III, a conformational change occurs and extracellular region of EGFR dimerizes. TDKs come into close proximity which leads to kinase activation via trans-autophosphorylation (Sigismund *et al.*, 2018).

On the other hand, according to the rotational model, unoccupied EGFR exists in a dimeric form which is stabilized by the interactions between the extracellular domain IV, the transmembrane domains and the intracellular domains (Figure 1.2). In this inactive form, the TKDs exist in a symmetric configuration. The extracellular domains of the dimer shift between a “tethered” and an “extended” form through the interaction between domains II and IV. Among these configurations, EGF has a higher affinity for the extended structure, and binding of the ligand stabilizes the ligand binding domain for exposure of the β-hairpin. Interactions between the two β-hairpins induces rotation of the transmembrane domains. This rotation results in

dissociation of the symmetric inactive kinase dimer and creates an active asymmetric kinase dimer. In the active asymmetric configuration of the dimer, both in "ligand-induced dimerization model" and "rotational model", the C-lobe of the activator kinase domain activates the receiver kinase domain by interacting the N-lobe of the latter (Bae and Schlessinger, 2010; Kovacs *et al.*, 2015; Lemmon *et al.*, 2014; Bessman *et al.*, 2014; Purba *et al.*, 2017).

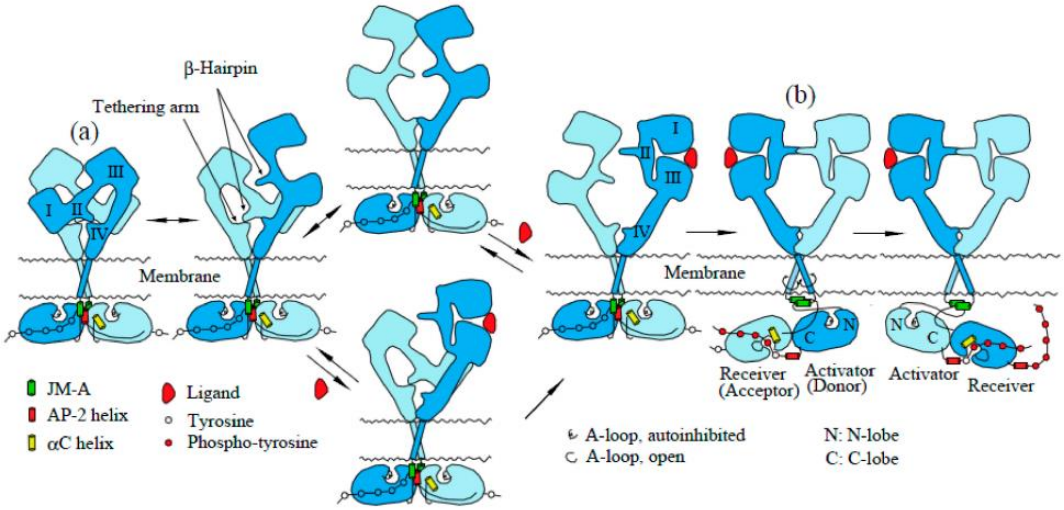


Figure 1.2. Rotational model for the activation of EGFR by ligand binding. Structures (a) and (b) indicate a tethered inactive dimer and an extended active dimer, respectively. EGFR exists in dimeric form, with the intracellular domains, transmembrane domains, and extracellular C-terminal regions of Subdomain IV interacting to stabilize it. Interactions with the AP-2 helix further stabilize the intracellular kinase domains with a symmetric configuration. Through the interaction of Subdomains II and IV, the extracellular domain of the receptor dimer adopts flexible configurations between "tethered" and "extended" forms. The ligand has a high affinity for the "extended" structure and stabilizes the extracellular ligand binding domain, allowing the "β hairpin" to be exposed. Interaction between the dimer's two "β hairpins" causes rotation of the transmembrane domains, resulting in the dissociation of the intracellular symmetric inactive kinase dimer and the formation of an asymmetric active kinase dimer. The C-lobe of the "activator" kinase domain interacts with the N-lobe of the "receiver" kinase domain to activate the latter in this asymmetric active configuration (Purba *et al.*, 2017).

Phosphorylated tyrosine residues located in the C-terminal regulatory tail of an activated EGFR serves as a docking site for endocytic adaptors and signaling molecules which in turn induces receptor endocytosis and intracellular signaling, respectively (Lemmon and Schlessinger, 2010).

1.2 EGF Stimulation and Signal Transduction

EGF is a known stimulant of cell growth, differentiation, proliferation, migration (Wee and Weng, 2017) and a suppressant of apoptosis (Leu *et al.*, 2000). EGF stimulation causes diverse effects in cells as was demonstrated by global phosphorylation of 2244 proteins at 6600 sites (Olsen *et al.*, 2006; Wee and Wang, 2017). Moreover, EGF treatment results in significant differences in expression of more than 3000 mRNAs and over 500 proteins in human mammary epithelial cells (HMEC) (Waters *et al.*, 2012). Moreover, the activated signaling network involves numerous ligands, other receptors, enzymes, ion channels, transcription factors, G protein subunits, and adaptor proteins (Oda *et al.*, 2005). Evidently, EGF has a wide-ranging effect on cells and the EGFR-mediated signal transduction can be complex. The complex signaling networks downstream of EGFR can be summarized within the following main pathways which include PI3K-AKT-mTOR, Ras-ERK MAPK, PLC-γ1-PKC, JAK-STAT, SRC and JNK (Figure 1.3).

For all of these pathways, EGFR-mediated signal transduction consists of the following steps; i) activation of EGFR as a result of ligand binding, ii) receptor dimerization, iii) C-terminal tail trans-phosphorylation, iv) recruitment of signaling proteins or adaptors and v) propagation of the signal in the cell for the regulation of expressions of various genes (Wee and Wang, 2017).

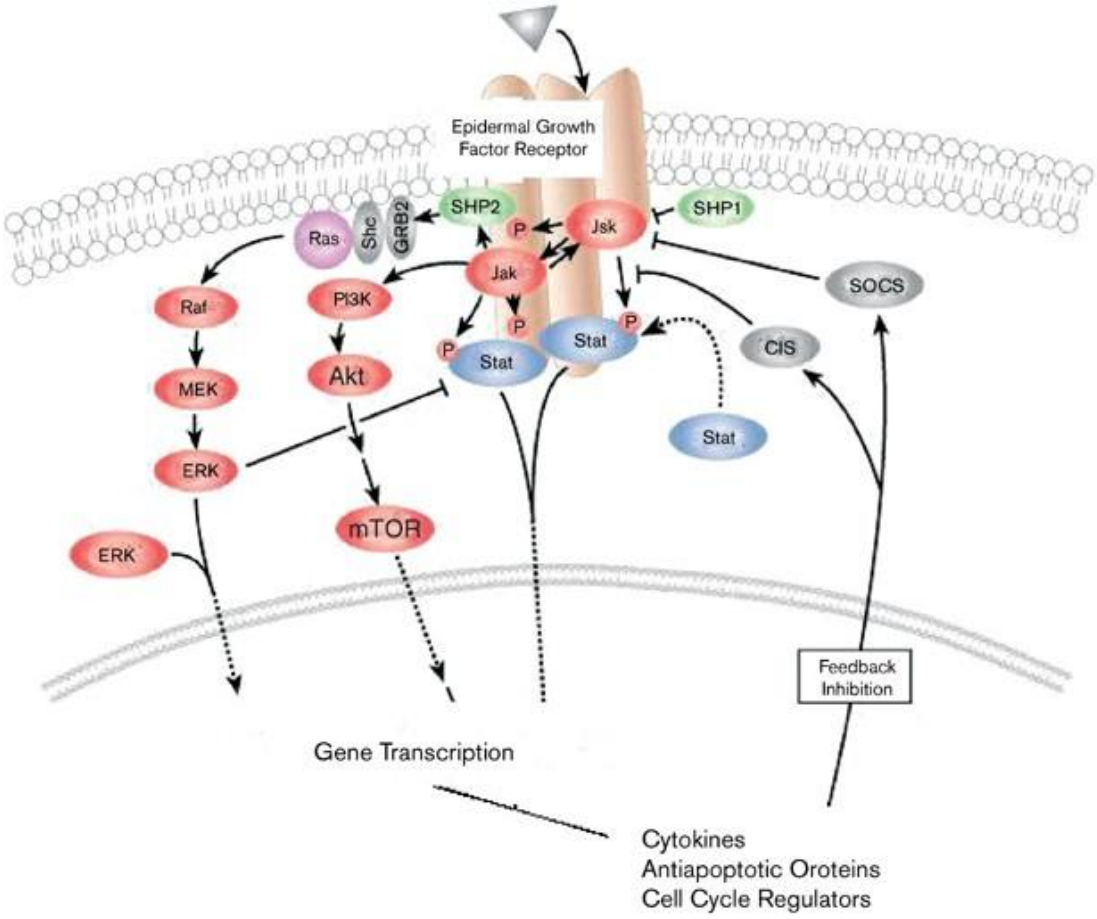


Figure 1.3. Main signaling cascades activated by EGFR. The EGFR phosphorylates and activates the Ras-Raf-MAP kinase, PI-3K/Akt, and Stat/Jak pathways in response to ligand activation. This, in turn, activates transcription factors and modulates cell cycle, growth, apoptosis, and angiogenic processes (El-Rayes and LoRusso, 2004).

1.2.1 Activation of the RAS-ERK MAPK Pathway

Following trans-phosphorylation of the EGFR upon ligand binding, GRB2 (Growth factor receptor-bound protein 2) recruits SOS (son of sevenless) to cell membrane, through SHC (Src homology/collagen). SOS is a guanine nucleotide exchange factor (GEF) for RAS small guanosine triphosphatase (GTPase), and activates RAS by inducing GDP to GTP exchange (Boriack-Sjodin *et al.*, 1998). RAS can then interact

with RAF-1. Phosphorylation of RAF-1 is an important event for MEK1/2 binding (Xiang *et al.*, 2002; Zang *et al.*, 2008; Diaz *et al.*, 1997; Fabian *et al.*, 1993). RAF-1 directly activates MEK1/2 through phosphorylation (Bondzi *et al.*, 2000), which in turn phosphorylates and activates ERK1/2 (Dhanasekaran and Premkumar Reddy, 1998). ERK1/2 translocate to the nucleus, where transcription factors, such as ELK1, are phosphorylated/stabilized to transactivate immediate early genes, for example, *MYC* (Bertotti and Sassi, 2015).

1.2.2 Activation of the PI3K-AKT-mTOR Pathway

The recruitment of PI3K (Phosphoinositide 3-kinase) differs between ErbB family members following ligand stimulation. PI3K binds directly with HER3 and HER4 (Soltoff *et al.*, 1994; Kim *et al.*, 1994), but indirectly with EGFR and HER2 (Soltoff and Cantley, 1996). Indirect interactions for ERBB1 and ERBB2 to PI3K are mediated by the adaptor protein GAB1 (GRB2-associated binder), which contains the canonical PI3K-binding sites (Mattoon et al, 2004). Ligand binding to EGFR elicits phosphorylation of multiple tyrosine residues on the receptor's C-terminus, which act as docking sites for proteins harboring phosphotyrosine-interacting domains (Pritchard *et al.*, 2008). Phosphorylation of EGFR allows binding of Grb2. Then the adaptor protein Gab1, which is essential for EGF-mediated activation of Akt (Mattoon *et al.*, 2004), is recruited via its PRD domain to the SH3 domain of Grb2 (Lock *et al.*, 2000). Then, PI3K binds directly to Gab1 (Holgado-Madruga *et al.*, 1996; Mattoon *et al.*, 2004) or interacts with Gab1 through other proteins that are recruited to phosphorylated Gab1 (Wu *et al.*, 2001). PI3K catalyzes the formation of phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) from phosphatidylinositol-(4,5)-bisphosphate (PIP2). PIP3 binds and recruits the kinases PDK1 and Akt, which leads to Akt phosphorylation on T308 and S473 by PDK1 and mTORC2, respectively. Both of these phosphorylation events are required for full Akt activation (Matheny and Adamo, 2009; Garay *et al.*, 2015). Activated Akt phosphorylates mTOR (mammalian target of rapamycin), E3 ubiquitin-protein ligase

MDM2, and XIAP (X-linked inhibitor of apoptosis protein). On the one hand, mTOR promotes protein synthesis through the phosphorylation of the ribosomal protein S6 (Burnett *et al.*, 1998; Isotani *et al.*, 1999). On the other hand, MDM2 and XIAP act as antiapoptotic cues through inhibition of P53 and CASP, respectively (Ogawara *et al.*, 2002; Zhou *et al.*, 2001; Feng *et al.*, 2004). The mechanistic Target of Rapamycin (mTOR) is a serine/threonine protein kinase in the PI3K-related kinase (PIKK) family that forms the catalytic subunit of two distinct protein complexes, known as mTOR Complex 1(mTORC1) and 2 (mTORC2) (Saxton and Sabatini, 2017). mTORC1 plays a central role in suppressing autophagy, promoting lipid and nucleotide synthesis and protein synthesis (Chung *et al.*, 1992). mTORC1 functions as a downstream effector for many frequently mutated oncogenic pathways, including the PI3K/Akt pathway as well as the Ras/Raf/Mek/ Erk (MAPK) pathway (Eng *et al.*, 1984).

Overall, EGF stimulation causes a diverse response in downstream signaling pathways which may lead to altered transcriptional, post-transcriptional and post-translational changes. It is important to understand these targets of EGF stimulation as EGFR is an important prognostic and therapeutical target in several cancers including breast cancer (Masuda *et al.*, 2012). In this thesis, we describe *SNX3* (Sorting Nexin 3) as a novel target of EGF stimulation in EGFR+ breast cancers.

1.3 Sorting Nexins and SNX3

Sorting nexins (SNXs) constitute a large group of proteins with more than 30 members (Cullen, 2008) which are characterized by a phospholipid-binding PX domain (Teasdale and Collins, 2012). The SNX family is divided into subfamilies depending on the presence or absence of additional domains and three of these subfamilies include members which interact with retromers (Lucas *et al.*, 2016) (Figure 1.4).

1) SNX-PX subfamily member SNX3 consists of only a PX domain (Strochlic *et al.*, 2007; Harrison *et al.*, 2014); 2) SNX-BAR subfamily members such SNX1, SNX2, SNX5, SNX6 and SNX32 have a BAR domain in addition to the PX domain (Rojas *et al.*, 2007; Wassmer *et al.*, 2007); 3) the SNX-FERM subfamily member SNX17, SNX27 and SNX31 comprise FERM domains in addition to the PX domain (Steinberg *et al.*, 2013; Temkin *et al.*, 2011; Gallon and Cullen, 2015).

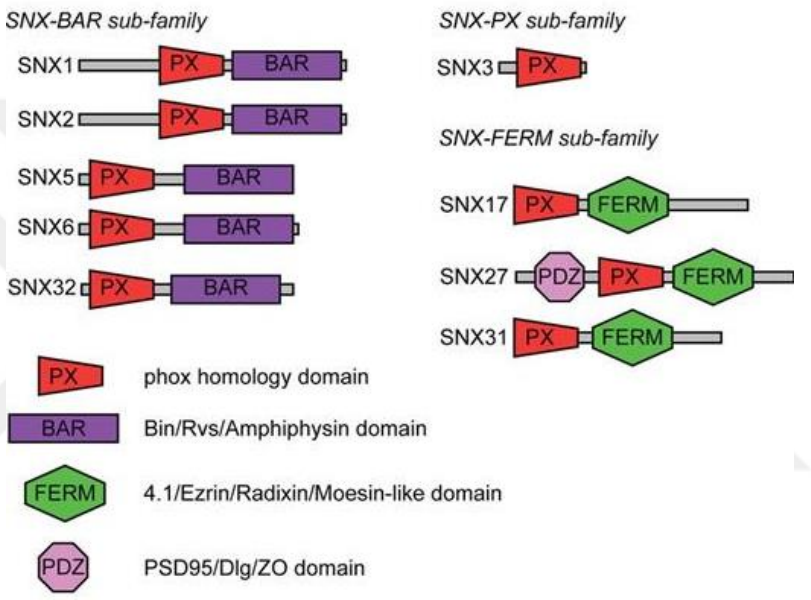


Figure 1.4. Domain architecture of sorting nexin proteins that associate with retromer. The endosomal retromer complex, rescues transmembrane proteins from the lysosomal degradative pathway and facilitates their recycling to other cellular compartments. Retromer interacts with a variety of associated proteins, including members of the sorting nexin (SNX) family (Gallon and Cullen, 2015).

Within the SNX protein family, SNX3 is gaining attention due to its roles in receptor recycling. Among other SNXs, only SNX3 is indispensable for recognition and recycling of Wntless (WLS) in *Drosophila* (Zhang *et al.*, 2011) and *C. elegans* (Harterink *et al.*, 2011). WLS is a cargo receptor that carries WNT ligands from trans-Golgi network (TGN) throughout the secretory pathway (Yu *et al.*, 2014).

Another study showed that SNX3 facilitates the recycling of transferrin receptor and thus is required for the proper delivery of iron to erythroid progenitors (Chen *et al.*, 2013).

SNX3 is involved in an early step of endocytic pathway with its role in recruiting VPS26-VPS35-VPS29 retromer trimer to early endosomes (Lenoir *et al.* 2018). The PX domain of SNX3 enables its binding to phosphatidylinositol-3-phosphate (PI3P), which is the predominant phospholipid species found on early endosomes (Xu, 2001; Strochlic *et al.*, 2007; van Weering 2012; Marat and Haucke, 2016). SNX3 bound to the early endosome, recruits retromer-trimer consisting of VPS26, VPS29 and VPS35 proteins. Recruitment of this complex results in recycling of endocytosed receptors, transporters, and adhesion molecules, to the plasma membrane or to the trans-Golgi network (TGN) (Arighi *et al.*, 2004; Seaman, 2004; Seaman *et al.*, 1998; Steinberg *et al.*, 2013; Temkin *et al.*, 2011). Alternatively, early endosomes mature into late endosomes that eventually fuse with lysosomes which results with the degradation of cargo receptors (Burd and Cullen, 2014; Bakker *et al.*, 2017). A group of SNX proteins including SNX1, SNX2, SNX5, SNX6, SNX12 and SNX16 have been implicated in lysosomal degradation of EGFR (Kurten *et al.*, 1996; Choi *et al.*, 2004; Gullapalli *et al.*, 2004; Liu *et al.*, 2006; Cavet *et al.*, 2008; Priya *et al.*, 2017). SNX3 has also been linked to EGFR recycling in A431 cells which is an epidermoid carcinoma cell line (Chiow et.el., 2012). However, *SNX3* gene regulation has not been investigated in full.

1.4 Aim of the Study

Interesting roles of SNX3 are beginning to emerge as a result of structural and biochemical studies. The current view of SNX3 function is recycling of endocytosed receptors including EGFR as was shown in an epidermoid carcinoma cell line (A431). Based on literature and findings from our laboratory, we hypothesized SNX3 to be an important component of EGF/EGFR axis in breast cancers and investigated here how *SNX3* gene is regulated in response to EGF stimulation in

EGFR positive cells. We provide evidence that SNX3 is regulated both at the transcriptional and protein level and is an immediate and sustained target of EGF/EGFR pathway.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines and Treatments

MCF10A cells were grown in 1:1 Dulbecco's Modified Eagle Medium (DMEM): Nutrient Mixture F-12 (Ham's) (01-170-1A, BI) supplemented with 10 μg/mL insülin (I9278, Sigma-Aldrich), 1% penicillin/streptomycin (P/S) (03-031-1B, BI), 0.5 μg/mL hydrocortisone (H4001, Sigma-Aldrich), 5% horse serum (04-004-1A, BI), 0.1 μg/mL cholera toxin (C8052, Sigma-Aldrich), and 20 ng/mL EGF (PHG0313, Thermo Fisher Scientific). HEK293 and MCF7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (01-052-1A, BI) supplemented with 10% Fetal Bovine Serum (FBS) (S1810-500, Biowest), 1% L-Glutamine (01-115-1A, BI), 1% Sodium Pyruvate Solution (03-042-1B, BI) and 1% Penicillin/Streptomycin (Pen/Strep) (03-031-1B, BI). Cells were incubated at 37^0C with 95% humidified air and 5% CO2 and were grown as monolayers. All cell lines were incubated at 37°C with 95% humidified air and 5% CO_2.

For EGF treatment, cells were serum-starved and EGF was given to cells in serum-free media for indicated time points. Cells were treated with 30 μg/ml cycloheximide (CHX) (66-81-9, Tocris) for 3,6,12 and 24 hours. For combined treatment of CHX and EGF, MCF10A cells were serum starved and treated with 20 ng/ml EGF and/or with 30 μg/ml CHX for 6 hours and lysates were collected.

MCF10A cells were treated with 1 μM MG132 (M8699, Sigma-Aldrich) for 6 hours and lysosomal inhibitor cocktail (10 μM Leupeptin (L2884, Sigma-Aldrich), 1 μM E-64 Protease inhibitor (E3132, Sigma Aldrich), 1.5 μM Pepstatin A (77170, Sigma Aldrich) for 24 hours. For combined treatment with actinomycin D and EGF, MCF10A cells were serum starved and pre-treated with 10 μg/ml actinomycin D

(1229, Tocris) for 1 hour then with 20 ng/ml EGF added for 12 hours.

2.2 Blocking signaling pathways

To block EGFR, PI3K/Akt and MAPK/Erk and pathways; AG1478 (1276, Tocris), MK2206(S1078, MedChemExpress) and PD0325901 (S1036, MedChemExpress) were used respectively.

MCF10A cells were serum starved and pre-treated with 25 μM AG1478 for 12 hours and pre-treated with 1 μM MK2206 and 1 μM PD0325901 for 4 hours or with 20 ng/ml EGF for 30 minutes. Inhibitor pre-treated cells were then treated with 20 ng/ml EGF for 30 minutes and lysates were collected. For inhibitor and EGF treatments, HEK293 cells were serum starved and pre-treated with 1 μM AG1478 or 1 μM MK2206 or 1 μM PD0325901 for 4 hours or with 50 ng/ml EGF for 15 minutes. Inhibitor pre-treated cells were then treated with 50 ng/ml EGF for 15 minutes and lysates were collected. For Everolimus (tlrl-eve, InvivoGen) and EGF treatments, MCF10A cells were serum starved and pre-treated with indicated concentrations of Everolimus for 4 hours and then with 20 ng/ml EGF for 30 minutes.

2.3 Preparation of CD-FBS for Hormone Treatment Experiments

Preparation of CD-FBS were performed according to the protocol provided by Dr. Mesut Muyan (METU, Ankara). Briefly; 10 g of dextran-coated charcoal (C6241, Sigma-Aldrich) were added to 500 ml FBS and incubated overnight at 4°C on a magnetic stirrer. The following day FBS-charcoal mixture were divided into two sterile centrifuge tubes and centrifuged at 10800xg for 30 minutes at 4°C. In a biological safety cabinet, the supernatants were carefully transferred into a 0.45 μm filter unit (CLS430770, Sigma-Aldrich) and filtered. The filtrate again mixed with 10 g dextran-coated charcoal and incubated at 4°C for 4 – 6 hours on a magnetic stirrer. The mixture again divided into two sterile centrifuge tubes and centrifuged at 10800xg for 30 minutes at 4°C. In a biological safety cabinet, the supernatants again

were carefully transferred into a 0.45 μm filter unit and filtered. The filtrate aliquoted into 50 ml sterile falcon tubes and stored at -20°C.

2.4 17β-Estradiol Induction

Before hormone stimulation experiments, MCF7 cells were grown in phenol red free medium (01-053-1A, BI) supplemented with 10% charcoal-dextran treated FBS (CD-FBS), 1% L-Glutamine, 1% Sodium Pyruvate solution and 1% Pen/Strep solution for 48 hours. MCF7 cells were treated with 10 nM 17 β-Estradiol (E2) (Sigma-Aldrich) or with ethanol (vehicle control) for 45 minute, 3 hours and 12 hours after reaching 70 – 80 % confluency (Lee and Sheen, 1997). For hormone induction experiments, cells were grown in phenol red-free medium supplemented with 10% charcoal stripped FBS, 1% P/S and 1% L-glutamine for 48 hours. MCF7 cells were either treated with 10 nM E2 or ethanol (vehicle control) for 12 hours and 1 μM ICI 182,780 (Tocris Biosciences) pre-treatments were done 1 hour before E2 treatment.

2.5 Expression Analysis

2.5.1 RNA Isolation

Total RNA was isolated with High Pure RNA Isolation Kit (11828665001, Roche) according to the instructions of the manufacturer. To eliminate DNA contamination, the isolated total RNA samples were incubated with DNase I (04716728001, Roche) at 37°C for 2 hours according to theindicated conditions in Table 2.1.

Table 2.1 DNase-I Treatment Reaction Conditions

Components	**Amount**
Total RNA	75 µl
10x Incubation Buffer	10 µl
DNase I recombinant, RNase-free (10 units/ul)	5 µl
Molecular Grade(mg) H_2O	10 µl
Total Reaction Volume	100 µl

At the end of the incubation period for DNase treatment, equal amount of (100 µl) Phenol: Chloroform: Isoamyl alcohol (25:24:1) (pH 4.0) was added on the samples. The tubes were vortexed at highest speed for 30 seconds and incubated for 10 minutes on ice. Following centrifugation at 14000xg for 20 minutes at 4°C, 80 µl of the upper phase, containing RNAs, were transferred into a fresh tube. 240 µl of molecular grade ethanol (EtOH) and 8 µl of 3M Sodium Acetate (NaAc) were added to samples and the mixtures were incubated at -20°C, overnight. Following the centrifugation at 14000xg for 30 minutes at 4°C, the supernatants were removed. The pellets were washed with 600 µl of 70% molecular grade EtOH. Following the centrifugation at 14000xg for 15 minutes at 4°C the supernatants were removed and the pellet, containing extracted RNAs, were dissolved in 25 µl mgH2O.

Following DNase I treatment and phenol-chloroform extraction, elimination of DNA contamination was confirmed by Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) specific Polymerase Chain Reaction (PCR) with GAPDH_F: 5'-GGGAGCCAAAAGGGTCATCA-3' and GAPDH_R: 5'-TTTCTAGACGGCAGGTCAGGT-3' primer pair. PCR performed according to the given conditions: initial denaturation at 95°C for 3 minutes, 40 cycles of 95°C for 30

seconds, 56°C for 30 seconds and 72°C for 60 seconds, and a final extension at 72°C for 5 minutes. MCF7 genomic DNA was used as positive control for PCR reactions.

Following the confirmation of DNA contamination elimination, RNA samples were spectrophotometrically quantified by using NanoDrop (MN-913, Maestrogen). Purity of RNA samples were determined by evaluation of A260/A280 and A260/A230 ratios. A representative result is given in Appendix showing a proper RNA samples with appropriate A260/A280 and A260/A230 ratios. All RNA samples that were used in this study fit the purity criteria.

2.5.2 cDNA Synthesis from RNA Samples

cDNA synthesis performed by using RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific) using oligo_dT primers and 1000 ng total RNA. Reaction conditions given in Table 2.2 were followed for cDNA synthesis.

Table 2.2 cDNA Synthesis Reaction Conditions

Components	**Amount**
Total RNA	1000 ng
Oligo(dT)$_{18}$ Primer, 100 µM	1 µl
Mg H_2O	Up to 12 µl
Mix throughly, spin down, incubate at 65 °C for 5 minutes	
10 mM dNTP Mix	2 µl
5X Reaction Buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl2, 50 mM DTT)	4 µl
Ribolock RNase Inhibitor (20U/µl)	1 µl
Revertaid Reverse Transcriptase (200U/µl)	1 µl
Total Volume	20 µl
Incubate at 42 °C for 60 minutes and 70 °C for 5 minutes. Store synthesized cDNA samples at -20 °C.	

2.5.3 Expression Analysis for *TFF1* Gene

Trefoil factor 1 (TFF1, also known as pS2) is a known E2 responsive gene (Brown *et al.*, 1984). E2 stimulated expression of this gene was evaluated by PCR as a positive control for E2 treatment experiments. The following primer pair was used for PCR analysis TFF1_F: 5'-CCATGGAGAACAAGGTGATCTGC-3' and TFF1_R: 5 -GTCAATCTGTGTTGTGAGCCGAG-3' (product size: 399 bp). PCR performed according to the given conditions: initial denaturation at 95°C for 3 minutes, 18 cycles of 95°C for 30 seconds, 61°C for 30 seconds and 72°C for 60 seconds, and a final extension at 72°C for 5 minutes.

2.5.4 RT-qPCR Analysis

MIQE Guidelines and checklist (Bustin *et al.,* 2009) were followed during expression analysis experiments including RNA isolation, RNA quantification, cDNA synthesis and RT-qPCR analysis.

Ssoadvanced Universal SYBR Green Supermix (BioRad, 1725271) and CFX Connect Real Time PCR Detection System (BioRad, cat# 1855201) was used for RT-qPCR. Fold changes were normalized against RPLP0 reference gene. For relative quantification, the reaction efficiency incorporated ΔΔCq formula was used (Fleige, S., *et al.,* 2006). The following primer pairs was used for RT-qPCR analysis for *MYC* expression (131bp); MYC_F: 5'-CAGCTGCTTAGACGCTGGATT-3', MYC_R: 5'-GTAGAAATACGGCTGCACCGA-3', for SNX3 expression (165 bp); SNX3_F: 5′-GCCTGAAATTTGGCAAGAAG-3′, SNX3_R:5′-TCTTGTCAACTGCCAAAACAA-3′; for RPLP0 expression, RPLP0_F: 5'-GGAGAAACTGCTGCCTCATA-3', RPLP0_R: 5'- GGAAAAAGGAGGTCTTC TCG-3'. For detection of SNX3 expression, following conditions were used: incubation at 94°C for 10 minutes, 40 cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. For detection of c-myc and RPLP0 expression following conditions were used: incubation at 94°C for 10 minutes, 40 cycles of 94°C for 15 seconds, 61°C for 30 seconds, and 72°C for 30 seconds.

2.6 Protein Isolation and Western Blotting

Cells were harvested and total protein was isolated using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, cat# 78501) containing protease inhibitor cocktail (Roche, complete EDTA-free, Cat# 05056489001) and phosphatase inhibitor cocktail (Roche, PhosStop, Cat# 4906845001). Concentrations of isolated proteins were measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, cat# 23227). 50 µg of proteins were denatured in 6X Laemmli buffer (12% SDS, 30% 2-mercaptoethanol, 60% Glycerol, 0.012% bromophenol

blue, 0.375 M Tris) at 95°C for 10 min. and loaded to 10% SDS-Polyacylamide gel. Proteins were transferred onto a PVDF membrane (Merck, cat# 03010040001). Skim-milk (BioRad, cat# 170-6404) or bovine serum albumin (BSA; Serva, cat# 11943.01) was used for blocking in TBS-T (Tris Buffer Saline-Tween: 20 mM Tris, 137 mM NaCl, pH: 7.6, 0.1% Tween 20). Membranes were incubated overnight at 4°C with appropriate primary antibodies and 1 hour at room temperature with appropriate HRP-conjugated secondary antibodies. For the visualization of proteins, WesternBright ECL (Advansta, cat# K12045-D50) and Chemidoc MP Imaging System (BioRad) were used.

Antibodies used in the western blotting experiments are listed in Table 2.3.

Table 2.3 List of the antibodies used in western blot analysis

Antibody Name and Catalog Information	**Dilution Information**	**Blocking Reagent**
Anti-SNX3 antibody (10772-1-AP, Proteintech)	1:500 in 0.1% TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti-phospho-EGFR (Tyr1086) antibody (2220, CST)	1:500 in 0.1% TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti-EGFR antibody (sc-373746, SCBT)	1:500 in 0.1% TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti phospho Akt antibody (Ser473) (4060S, CST)	1:500 in 0.1% TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti Akt 1/2/3 antibody (sc-81434, SCBT)	1:500 in 0.1%TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti p-Erk 1/2 Antibody (Pt202/Py204) (sc-136521, SCBT)	1:500 in 0.1% TBS-T	5% Non-Fat Dry Milk (170-6404, Bio-Rad)
Anti Erk 1/2 Antibody (sc-514302, SCBT)	1:500 in 0.1% TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti-ACTB Antibody (sc-4778, SCBT)	1:500 in 0.1% TBS-T	3% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti CTNNB1 Antibody (sc-133240, SCBT)	1:500 in 0.1% TBS-T	5% Non-Fat Dry Milk (170-6404, Bio-Rad)
Anti p53 Antibody (554169, BD Pharmingen)	1:500 in 0.1% TBS-T	5% Non-Fat Dry Milk (170-6404, Bio-Rad)
Anti-phospho-p70S6K Antibody (Thr389) (9205, CST)	1:500 in 0.1% TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Anti p70S6K Antibody (9202, CST)	1:500 in 0.1% TBS-T	5% Albumin Bovine Modified Cohn Fraction V, pH 7.0 (11943.01, Serva)
Goat-anti-rabbit HRP secondary antibody (R-05072-500, Advansta)	1:2000 in 0.1% TBS-T	Depending on primary antibody used
Goat-anti-mouse HRP secondary antibody (R-05071-500, Advansta)	1:2000 in 0.1% TBS-T	Depending on primary antibody used

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Time Dependent Upregulation of SNX3 protein in Response to EGF

SNX3 is involved in receptor recycling and has been implicated in EGFR recycling in epidermoid carcinoma (A-431) cells (Chiow *et al.*, 2012). In our laboratory, we were interested in investigating the role of SNX3 in EGFR positive breast cancer cells. In this thesis, I focused on how SNX3 gene is regulated. I first asked whether *SNX3* is an EGF/EGFR induced gene. I used human mammary MCF10A cell line which is an EGFR positive, non-tumorigenic and hormone receptor negative cell line. MCF10A is a well-established model for studying time-dependent EGF response (Golan Lavi *et al.*, 2017). We investigated time dependent regulation of SNX3 protein levels upon EGF stimulation in MCF10A. In order to detect dynamic changes in gene expression, we stimulated cells with EGF in a time course experiment and collected lysates and RNA. Shortly after EGF (15-30 minutes) stimulation, abundance of SNX3 protein was increased and remained high throughout the EGF treatment (i.e., 12 hours) (Figure 3.1).

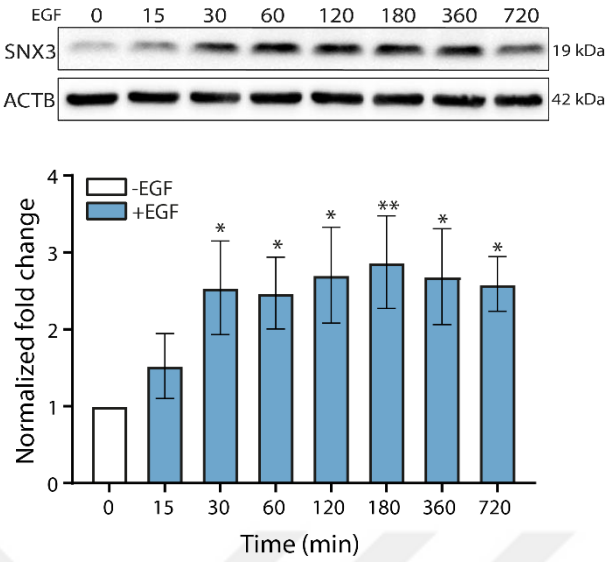


Figure 3.1. EGF stimulation and SNX3 protein levels. MCF10A cells were starved for 20 hours with 0.9 % Horse serum and 1% Pen/Strep containing DMEM-F12 and treated with 20 ng/ml (Golan-Lavi *et al.*, 2017) EGF for the indicated time points. SNX3 protein expression was measured by western blotting. ACTB was used as a loading control. Blots are representative of 3 independent treatments. Graphs show densitometric quantification of bands. The data represent the mean (SD) of 3 experiments. One-way ANOVA with Tukey's multiple comparison test was used, * indicates $p<0.05$, ** indicates $p<0.01$.

3.2 Early Response of SNX3 to EGF Stimulation

To understand how SNX3 protein abundance was increased in response to EGF, we first tested the possibility that *SNX3* is transcriptionally upregulated in early response to EGF stimulation. We treated MCF10A cells, with 20 ng/ml EGF for 30 minutes as an early time point and isolated RNA. To test the activity of EGF stimulation, *MYC* transcript levels were detected as a known early response gene to EGF stimulation (Nass and Dickson, 1998). While *MYC* transcript levels were upregulated in response to 20 ng/ml EGF stimulation for 30 minutes, *SNX3* transcript levels did not change as an early response to EGF stimulation (Figure 3.2).

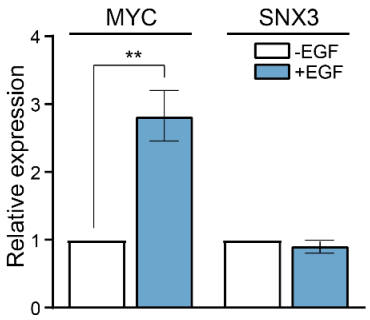


Figure 3.2. *MYC* and *SNX3* RT-qPCR results upon EGF stimulation. MCF10A cells were starved for 20 hours with 0.9 % Horse serum and 1% Pen/Strep containing DMEM-F12 then treated with 20 ng/ml EGF for 30 minutes. Cells collected after starvation were used as control. For each experiment, 3 biological replicates were used. qRT-PCR assays were performed with 3 technical replicates for each experiment. Treatments were repeated three independent times. The baseline for the control treated samples was set to 1. Every treated sample were normalized to the control (0 h) sample. Quantification was done using the reaction efficiency correction and ΔΔCq method. The data represent the mean (SD) of 3 independent treatments. Unpaired t-test was performed for statistical significance, ** indicates $p<0.01$.

These results suggested that, SNX3 protein level upregulation at early time point of EGF stimulation was not due to increased transcription.

Next, we tested whether SNX3 upregulation occured at the protein level. We measured SNX3 protein half-life after blocking translation with cycloheximide (CHX) treatment alone (Figure 3.3A) or together with EGF stimulation in a time course experiment (Figure 3.3B) and collected cell lysates for western blotting. CTNNB1 (Beta-catenin) was used as a control for the CHX treatment due to its known short half-life (Yang *et al.*, 2019). Inhibition of translation by CHX (30 µg/ml) resulted in decreased availability of SNX3 protein in cell lysates. However, treating cells with EGF along with CHX rescued SNX3 protein levels 30%, suggesting that EGF stimulation enhanced SNX3 protein half-life.

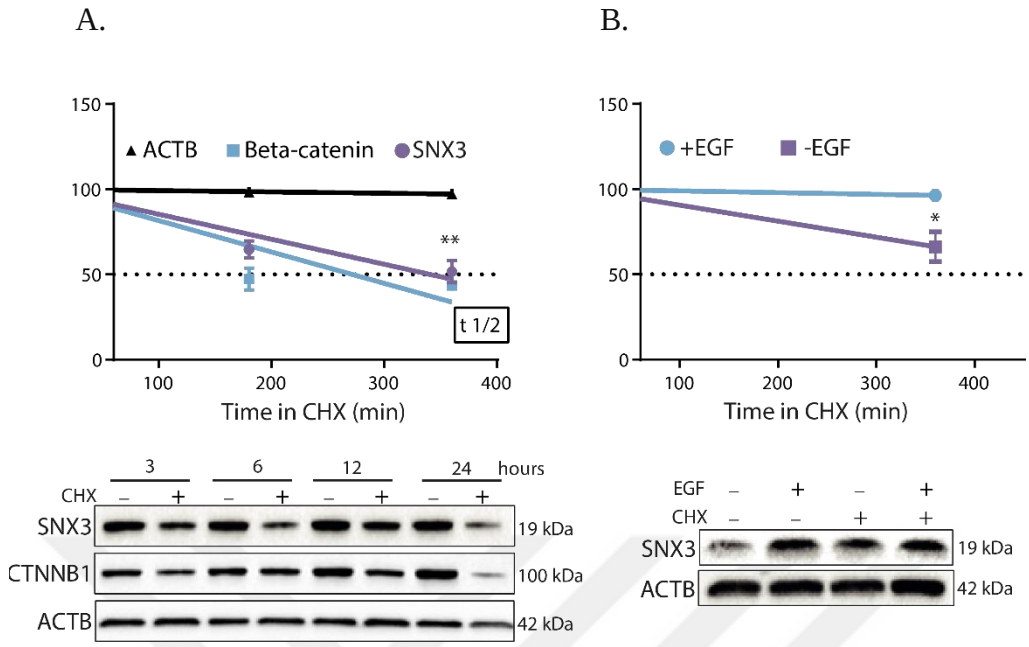


Figure 3.3. Effect of EGF on SNX3 protein stability A. MCF10A cells were treated with 30 µg/ml CHX for indicated times and lysates were collected. CTNNB1 was used as a positive control for CHX treatment. ACTB was used as loading control. B. MCF10A cells were starved for 20 hours with 0.9% Horse serum and 1% Pen/Strep containing DMEM-F12 treated with CHX and/or EGF. Cells were treated with 20 ng/ml EGF with or without 30 µg/ml CHX for 6 hours. SNX3 protein expression was measured by western blotting. The data represent the mean (SD) of 3 independent treatments. Unpaired *t*-test with Welch's correction was performed for statistical significance, * $p<0.05$, ** $p<0.01$.

Considering that lysosomal and/or proteasomal degradation may affect protein stability, MCF10A cells were treated either with proteasome or lysosome inhibitor. MCF10A cells were treated with MG132 (1 uM, Sigma 474790), a proteasome inhibitor (Figure 3.4A). MG132 had no effect on SNX3 protein levels, suggesting that, SNX3 protein may not be degraded via the proteasomal pathway, whereas a lysosomal inhibitor cocktail enhanced SNX3 protein abundance (Figure 3.4B). These results indicate that SNX3 protein may be degraded in lysosome.

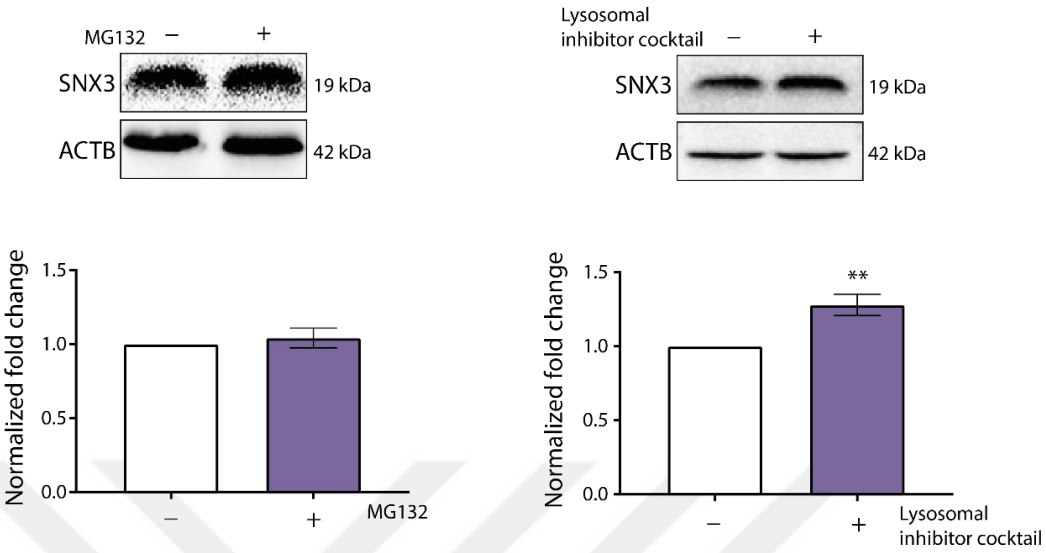


Figure 3.4. Inhibition of lysosomal or proteasomal degradation. A. MCF10A cells were treated with lysosomal inhibitor cocktail (10 µM Leupeptin, 1.5 µM Pepstatin A, 1 µM E-64) for 24 hours, B. MCF10A cells were treated with 1 µM MG132 for 6 hours and lysates were collected for western blotting with SNX3 antibody. Blots are representative of 3 independent treatments. Graphs show densitometric quantification of bands. The data represent the mean (SD) of 3 experiments. Unpaired t-test was performed for statistical significance, ** indicates $p<0.01$.

3.3 Late Response of SNX3 to EGF Stimulation

Next we investigated the mechanisms of SNX3 upregulation at later points of EGF stimulation.

MCF10A cells were treated with EGF for indicated time points. RNA was isolated from treatment groups and cDNA was synthesized. *SNX3* mRNA levels did not change until 12 hours. RT-qPCR results showed transcriptional upregulation of *SNX3* mRNA at 12 hours of EGF treatment (Figure 3.5B). Upregulation *MYC* transcript level was also detected upon EGF stimulation *(*Nass and Dickson, 1998) (Figure 3.5A).

A. B.

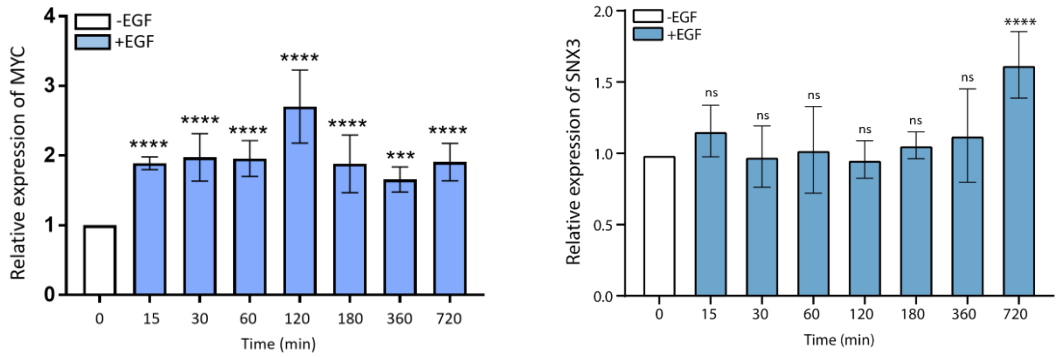


Figure 3.5. Late response to EGF stimulation**.** MCF10A cells were treated with 20 ng/ml EGF for the indicated time points. A. Expression of *MYC* and B. *SNX3* mRNA was determined by RT-qPCR and normalized against the reference gene; *RPLP0*. Stimulated samples were normalized to the unstimulated samples. n= 3 independent treatments. For statistical analysis, one-way ANOVA with Dunnett's multiple comparison test was applied. *** indicates $p<0.001$ and **** indicates $p<0.0001$, ns: non-significant.

Next, we showed that upregulation of *SNX3* mRNA in response to EGF stimulation was reversible by AG1478, a selective inhibitor of EGFR trysoine kinase activity (Levitzki & Gazit, 1995) (Figure 3.6). AG1478 blocks EGFR-mediated signaling pathway, inhibits cell proliferation of several tumours and induces apoptosis through competitive binding to ATP-binding sites to inhibit protein tyrosine kinase activity (Shushan *et al.*, 2004). RT-qPCR results showed transcriptional upregulation of *SNX3* mRNA at 12 hours of EGF treatment (Figure 3.6).

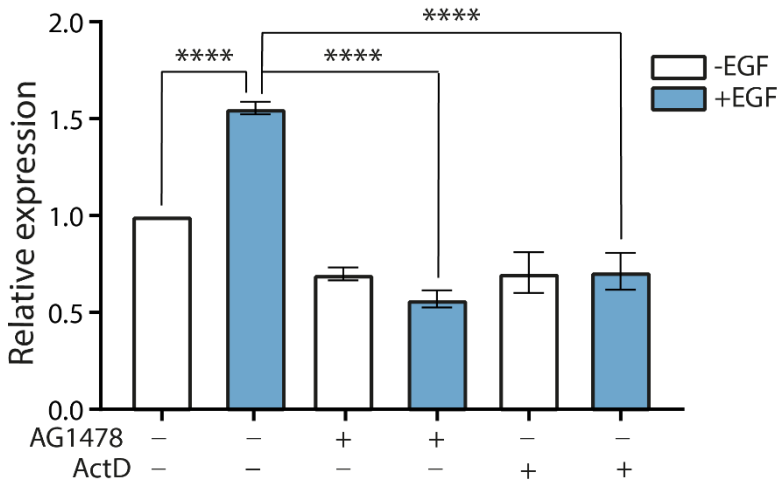


Figure 3.6. SNX3 transcriptional upregulation at later time points of EGF stimulation is EGFR specific. MCF10A cells were starved for 20 hours with 0.9% Horse serum and 1% Pen/Strep containing DMEM-F12 and pre-treated with 25 uM AG1478 for 12 hours or with 20 ng/ml EGF for 12 hours. Inhibitor pre-treated cells were then treated with 20 ng/ml EGF for 12 hours. For ActD and EGF treated samples, MCF10A cells pre-treated with 10 µg/ml ActD for 1 hour and 20 ng/ml EGF added for 12 hours. 0 h cells (Cells collected after starvation period) were used as control. For each experiment, 3 biological replicates were used. RT-qPCR was performed with 3 technical replicates for each experiment. The baseline for the control treated samples was set to 1. Relative expression of *SNX3* mRNA was normalized against the reference gene; *RPLP0* (Boussac *et al.*, 2018). Quantification was done using the reaction efficiency correction and ΔΔCq method (Fleige *et al.*, 2006). The data represent the mean (SD) of 3 independent treatments, One-way ANOVA with Tukey's multiple comparison test was used, **** indicates $p<0.0001$.

We also wanted to confirm that this upregulation was due to enhanced new transcription rather than increased mRNA stability in response to EGF stimulation. Therefore, I pre-treated MCF10A cells with actinomycin D for 1 hour and then with EGF for 12 hours. Cells were collected and RNA isolation performed and cDNA was synthesized. RT-qPCR results showed that *SNX3* transcriptional upregulation was inhibited by actinomycin D (Figure 3.6) that blocks new transcription (Bensaude, 2011).

These reults showed that SNX3 upregulation was transcriptional at later time points of EGF stimulation.

Next, we wanted to test whether any proliferative signal would cause *SNX3* transcriptional upregulation. We turned to another breast cancer related proliferating agent; estrogen. We used MCF7 breast cancer cell line which is estrogen receptor (ER) positive. I used E2 and ICI 182,780 (ER specific antagonist) to demonstrate the effect of E2 is ER specific. Efficiency of E2 treatment was tested by the expression levels of *TFF1* (Trefoil Factor 1), which is a known E2 responsive gene (Brown *et al.*, 1984). *TFF1* levels were increased as expected in E2 treated samples compared to ethanol or ICI treated cells (Figure 3.7A). E2 treatment did not affect *SNX3* mRNA (Figure 3.7B) expression levels compared to ethanol treated controls and ICI treated samples in MCF7 cells. SNX3 protein level also didn't change upon E2 treatment (Figure 3.7C).

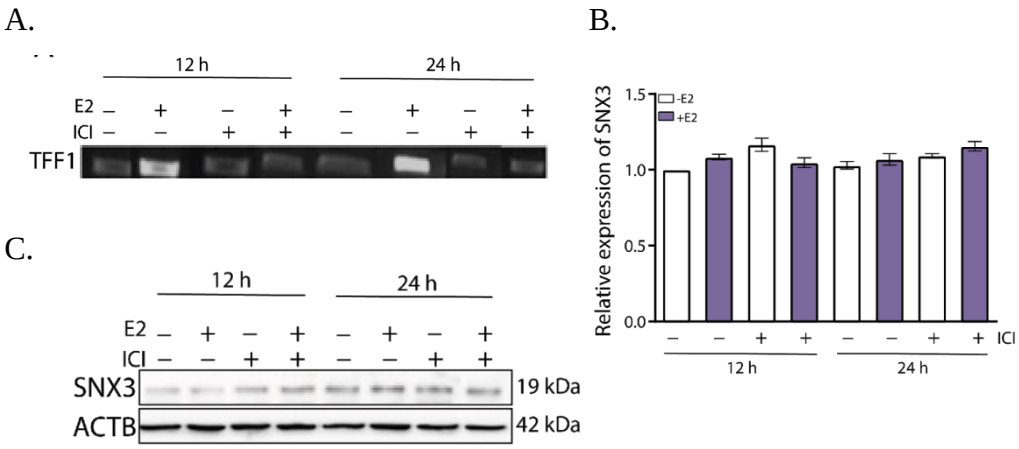


Figure 3.7. MCF7 cells were grown in phenol red free media supplemented with 10% dextran coated charcoal stripped FBS for 48 hours. Then, cells were pre-treated with 1 μM ICI 182,780 (ICI, fulvestrant, Faslodex), an estrogen receptor antagonist, for 1 hour or 10 nM E2 for 12 and 24 hours, along with the ethanol treated samples. ICI pre-treated samples were also treated with 10 nM E2 for 12 and 24 hours. A. TFF1 was used as positive control for E2 treatments. B. *SNX3* mRNA expression was quantified by RT-qPCR upon E2 treatment. C. SNX3 protein levels upon E2 and ICI treatment in MCF7 cells determined by immuno-blotting. ACTB antibody was used to test equal loading of proteins.

Overall, these results suggested that as opposed to early response, EGF stimulation causes transcriptional upregulation of *SNX3* as part of late response to EGF stimulation. This response was EGF specific as *SNX3* mRNA or protein was not upregulated in response to other proliferative signals such as estrogen in ER+ breast cancer cells.

3.4 Regulation of SNX3 by EGFR Pathway

To better delineate how SNX3 is regulated by EGF/EGFR axis, I used pharmacological inhibition approach to block EGFR and/or related downstream pathways. I treated MCF10A cells with EGFR, AKT and MEK inhibitors alone or

together with EGF stimulation for 30 minutes or 12 hours to test the early and late response.

I collected lysates from cells and performed western blotting (Fig 3.8). Western blot analysis and densitometric analysis of bands showed that increased abundance of SNX3 protein upon EGF stimulation at early and late time points was reversible by AG1478 (Levitzki & Gazit, 1995) (Figure 3.8A), by MK2206, an Akt inhibitor (Hirai *et al.*, 2010) (Figure 3.8B) and by PD0325901, a MEK inhibitor (Ciuffreda *et al.*, 2009) (Figure 3.8C). Inhibition of EGFR and downstream pathways blocked SNX3 protein upregulation. In addition to the blocking of protein increase, we did not see the transcriptional increase, which we see as a late response (Figure 3.8D, right panel).

Early and late response to EGF treatment was controlled by upregulated *MYC* mRNA levels (Figure 3.8D).

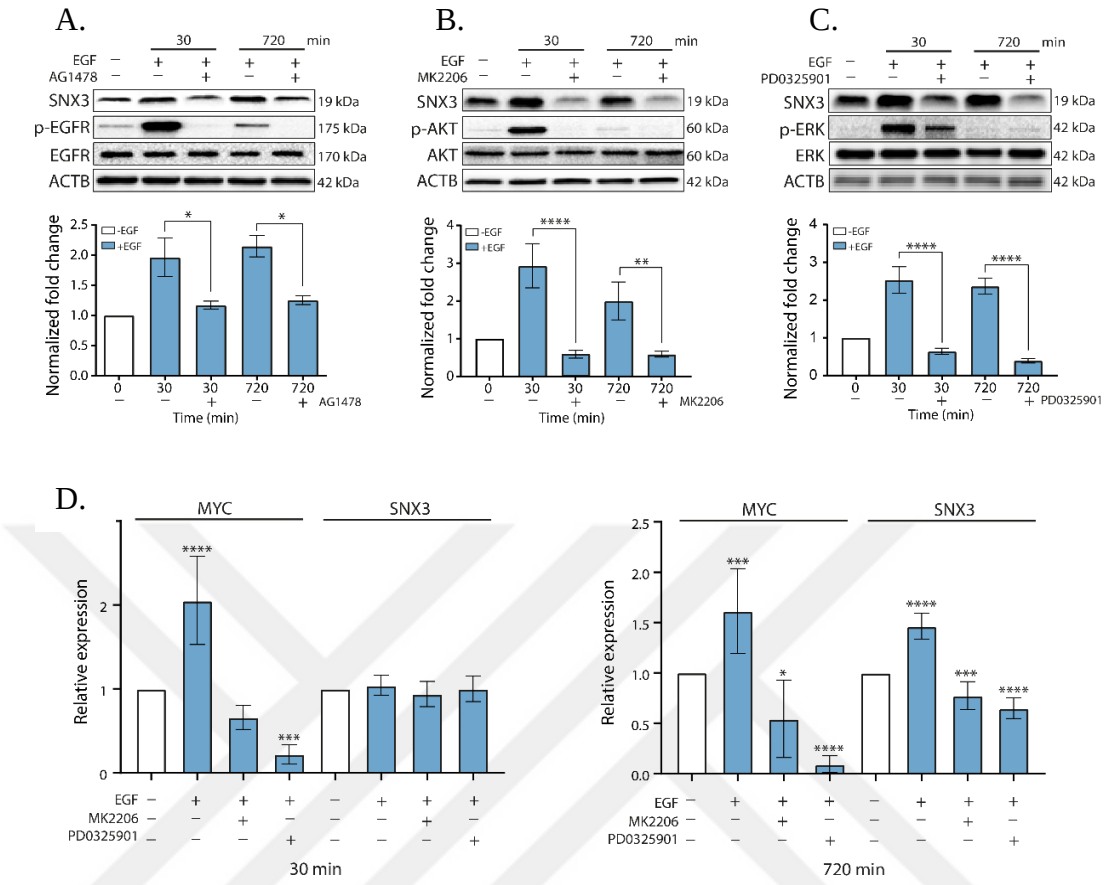


Figure 3.8. EGFR pathway regulates SNX3 expression. MCF10A cells were starved for 20 hours with 0.9% Horse serum and 1% Pen/Strep containing DMEM-F12 and pre-treated with inhibitors and/or EGF. **A.** MCF10A cells were pre-treated with 25 uM AG1478 for 12 hours **B.**1 μM MK2206 or with **C.**1 μM PD0325901 for 4 hours or with 20 ng/ml EGF for 30 minutes or 12 hours. Inhibitor pre-treated cells were then treated with 20 ng/ml EGF for 30 minutes or 12 hours and lysates were collected for western blotting. p-EGFR, p-AKT, p-ERK, EGFR, AKT and ERK levels were detected. ACTB was used as loading control for all western blots. Blots are representative of 3 independent treatments. Bars show densitometric quantification of SNX3 levels (mean (SD) of 3 experiments). One-way ANOVA with Tukey's multiple comparison test was used, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$. **D.** RT-qPCR for the expression of SNX3 for the described treatments. *MYC* expression was used as a positive control. Fold change for *SNX3* mRNA was calculated as described. The data represent the mean (SD) of 3 independent treatments. One-way ANOVA with Dunnett's multiple comparison test was used, * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

These results suggested that, SNX3 regulation was controlled via EGFR and related downstream pathways.

I also tested whether the mechanistic Target of Rapamycin (mTOR) pathway may enhance SNX3 protein synthesis via triggering *SNX3* mRNA translation efficiency upon EGF treatment (Figure 3.9). To test whether mTOR has a role in SNX3 upregulation in response to EGF stimulation, we used a mTORC1 inhibitor Everolimus (Houghton, 2010). Everolimus binds to FKBP prolyl isomerase 1A (FKBP12) and inhibits mTOR kinase activity and reduces the activity of the downstream effectors S6 ribosomal protein kinase (S6K1) and eukaryotic elongation factor 4E-binding protein (4EBP) (Atkins *et al.*, 2009). To block mTORC1 activity, I treated MCF10A cells with EGF and/or with Everolimus (Figure 3.9).

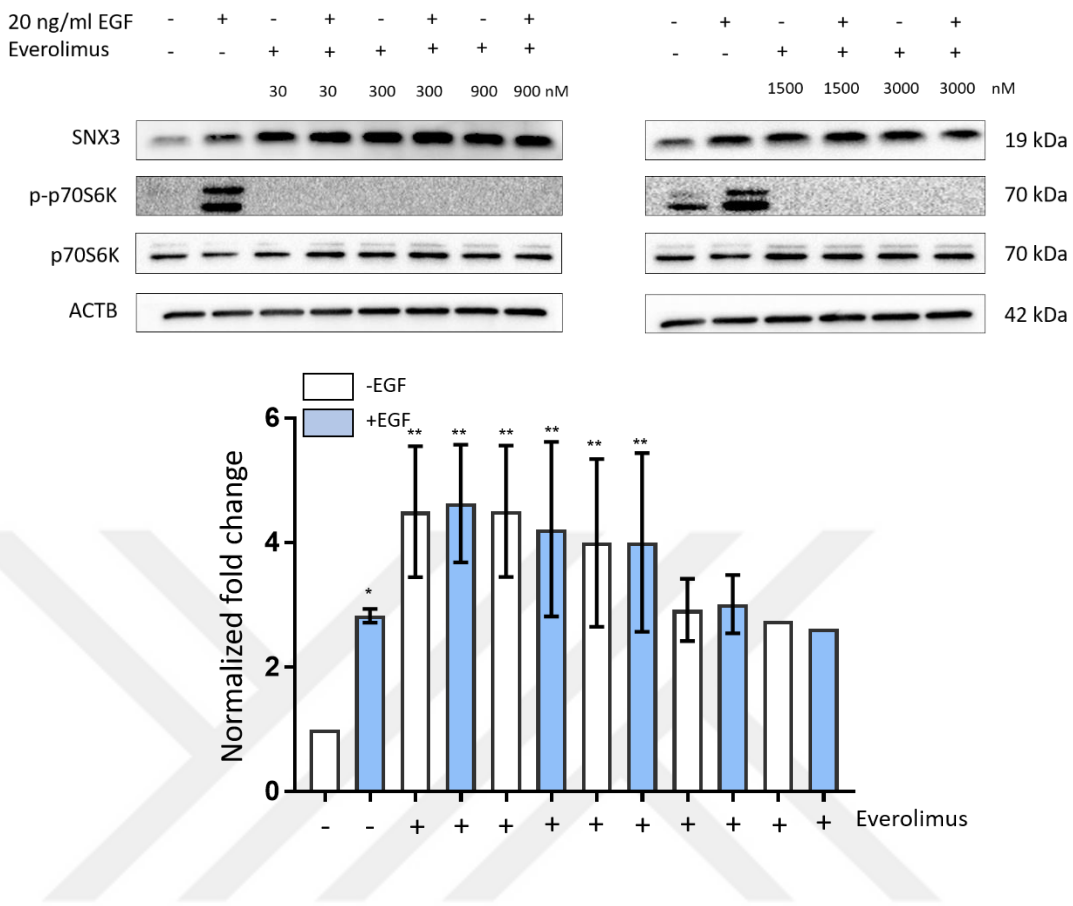


Figure 3.9. Inhibition of mTORC1pathway. MCF10A cells were starved for 20 hours with 0.9% Horse serum and 1% Pen/Strep containing DMEM-F12 and pre-treated with indicated concentrations of Everolimus and/or with 20 ng/ml EGF for 30 minutes. Inhibitor pre-treated cells were then treated with 20 ng/ml EGF for 30 minutes and lysates were collected for western blotting. p-p70S6K and p70S6K levels were detected. ACTB was used as loading control for all western blots. Blots are representative of 3 independent treatments. Bars show densitometric quantification of SNX3 levels (mean (SD) of 3 experiments). One-way ANOVA with Dunnett's multiple comparison test was used, * $p<0.05$, ** $p<0.01$.

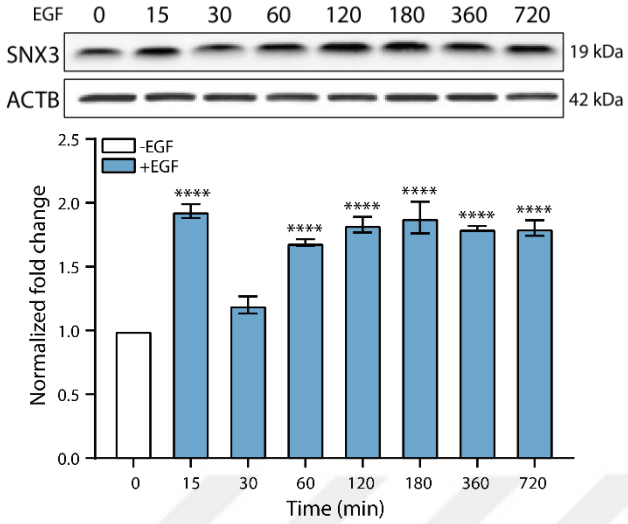
Effciency of Everolimus treatment was verified by bloked phosphorylation of S6K1 (Figure 3.9). However, unexpectedly, SNX3 protein levels were upregulated upon Everolimus treatment (Figure 3.9), an observation that can be explained by Everolimus induced autophagy and possible autophagy related roles of SNX3. Interestingly, an earlier report suggested that Snx3 and Snx6 are required for proper

autolysosomal degradation in Drosophila larval fat body cells (Maruzs *et al.*, 2015). SNX3 protein increase may be related with Everolimus induced autophagy induction and recruitment of early endosomes which contain SNX3.

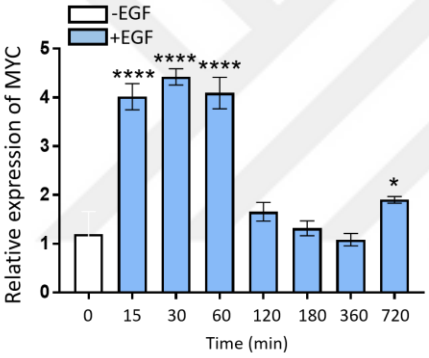
While we failed to conclude whether translation rate has an effect, our results clearly showed that early response to EGF stimulation causes an increase in SNX3 protein levels.

I also performed experiments with HEK293 cells, another EGFR positive cell line (Kramer *et al.*, 2002) whether we would see the same results as MCF10A cells. I treated HEK293 cells with EGF as indicated time points after a starvation period. Then I collected lysates and performed western blot experiments. A similar induction pattern was observed in HEK293 cells, suggesting that what we observed in MCF10A cells were not a cell line specific response (Figure 3.10A). In support of the results of MCF10A cells, upregulation of SNX3 in HEK293 cells was also reversible by blocking the EGFR and downstream pathways using the same inhibitors (Figure 3.10B, C and D). These results showed that SNX3 is a target of EGF stimulation in EGFR positive cells, first at protein level and later at transcription level.

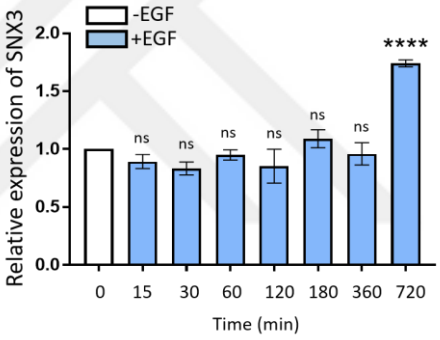
A.


EGF 0 15 30 60 120 180 360 720
SNX3
19 kDa
ACTB
42 kDa
-EGF
+EGF
Normalized fold change
Time (min)

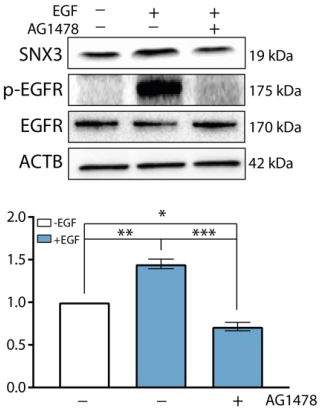
B.


-EGF
+EGF
Relative expression of MYC
Time (min)

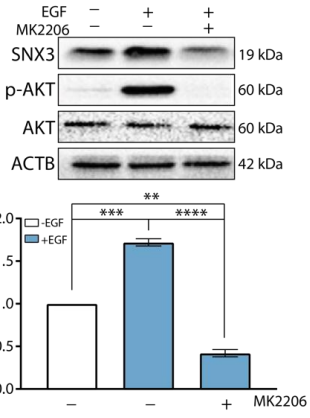
C.


-EGF
+EGF
Relative expression of SNX3
Time (min)

D.


EGF
AG1478
SNX3
19 kDa
p-EGFR
175 kDa
EGFR
170 kDa
ACTB
42 kDa
AG1478

E.


EGF
MK2206
SNX3
19 kDa
p-AKT
60 kDa
AKT
60 kDa
ACTB
42 kDa
MK2206

F.

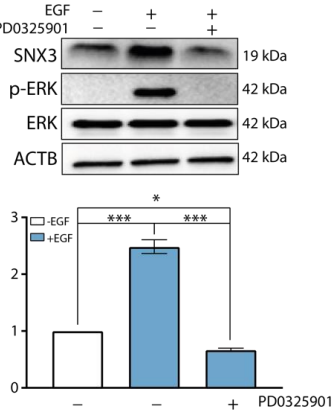

EGF
PD0325901
SNX3
19 kDa
p-ERK
42 kDa
ERK
42 kDa
ACTB
42 kDa
PD0325901

Figure 3.10. Effect of EGF stimulation and EGFR blockade on SNX3 protein levels in HEK293 cells. A. HEK293 cells were starved for 6 hours in growth factor free media and treated with 50 ng/ml EGF (Sampaio *et al.*, 2008) for the indicated time points. SNX3 protein expression was measured by western blotting. ACTB was used as a loading control. Blots are representative of 2 independent treatments. Graphs show densitometric quantification of bands. The data represent the mean (SD) of 3 experiments. One-way ANOVA with Tukey's multiple comparison test was used, **** indicates $p<0.0001$. B. Expression of *MYC* and C. *SNX3* mRNA was determined by RT-qPCR and normalized against the reference gene; *RPLP0*. Stimulated samples were normalized to the unstimulated samples. n= 3 independent treatments. For statistical analysis, one-way ANOVA with Dunnett's multiple comparison test was applied. * indicates $p<0.05$ and **** indicates $p<0.0001$. HEK293 cells were serum starved in DMEM for 6 hours and were pre-treated with D. AG1478 (25 μM), E. MK2206 (1 μM), F. PD0325901 (1 μM) for 4 hours. Cells were then stimulated with EGF (50 ng/ml) for 15 minutes and lysates were collected and blotted for p-EGFR, p-AKT, p-ERK, EGFR, total AKT and total ERK levels. ACTB was used as loading control. Bars show densitometry results of 2 independent treatments. For statistical analysis, one-way ANOVA with Tukey's multiple comparison test was used. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

CHAPTER 4

CONCLUSION

The epidermal growth factor receptor (EGFR) is one of the important targets of antitumor agents (Mendelsohn and Baselga, 2003). About half of triple negative breast cancer (TNBC) and inflammatory breast cancer (IBC) cases overexpress EGFR (Burness *et al.*, 2010). Therefore, understanding how the EGFR pathway is regulated for the treatment of breast cancer and the function of the proteins involved in the regulation of this pathway is extremely important for the treatment of breast cancers with EGFR overexpression.

Recent studies have shown that the EGFR pathway regulates epithelial-mesenchymal transition, cell migration and tumor invasion and targeting EGFR increases the chemosensitivity of TNBC cells by upregulating apoptotic signaling networks in TNBC (Masuda *et al.*, 2012). These studies suggest that EGFR-targeted therapy may be promising in TNBC, IBC, and other types of EGFR-dependent cancers. Therefore, understanding the role of regulators of the EGFR pathway is very important in the treatment of breast cancer. In this thesis, to investigate the role of SNX3 in relation to EGFR in breast cancers, I evaluated whether SNX3 is a gene regulated by the EGF signaling pathway and EGFR.

The responses of a protein in the cell to extracellular stimuli are governed by gene transcription, mRNA degradation and translation, and protein degradation (Golan-Lavi *et al.*, 2017). In this thesis, such responses of human breast cells to the Epidermal Growth Factor (EGF) were investigated over time. Therefore, the MCF10A cell line (Bessette *et al.*, 2015), an EGFR positive cell line, was used in the experiments and the time-dependent EGF (Golan-Lavi *et al.*, 2017) response was examined (Figure 3.1). According to the results from this study, EGF treatment increases SNX3 protein levels of MCF10A cells as an early response without an

increase at *SNX3* mRNA levels (Figure 3.2). It is possible to explain this situation with the more efficient translation of SNX3 mRNA as a result of EGF treatment or changes in the SNX3 protein half-life during normal cellular processes. Indeed, results from experiments with cyclohexamide (CHX), which inhibits eukaryotic translation, show that SNX3 protein stability is enhanced by EGF treatment. For example; after 6 hours of CHX treatment, the SNX3 protein level was halved, but the amount/half-life of SNX3 protein was increased when CHX and EGF were given to the cells together (Figure 3.3). Our results agree with an earlier study which suggests precise time-dependent control of protein translation and degradation to be important in shaping the observed transient pulses of protein abundance upon EGF stimulation in MCF10A cells (Golan-Lavi *et al.*, 2017). For example, TUBA1B protein stability increases after administration of EGF and CHX (Golan-Lavi *et al.*, 2017). Our results also show rescued SNX3 protein levels upon CHX and EGF treatments. It will be important to show whether lysosomal degradation of SNX3 is blocked and/or SNX3 mRNA is more efficiently translated in response to EGF stimulation. We did investigate the possibility of *SNX3* mRNA translation rate increase upon EGF stimulation. Our results showed that SNX3 protein levels did not decrease, even increased when mTORC1 pathway was inhibited via Everolimus (Figure 3.9). These results can be explained by the fact that Everolimus induced autophagy and the possible role of SNX3 in autophagy regulation. Aside from its role in lysosomal enzyme receptor recycling, retromer has been implicated in a number of vesicular trafficking events, including the early stages of autophagy and endocytosis (Carosi *et al.*, 2019). The late stages of autophagy and endocytosis are impaired in Vps26 and Vps35 deficient Drosophila larval fat body cells, but autophagosome and endosome formation is not compromised (Maruzs *et al.*,2015). It was also shown that, Snx3 and Snx6 are also required for proper autolysosomal degradation in Drosophila larval fat body cells (Maruzs *et al.*, 2015).

An increase in both mRNA and protein levels of SNX3 was observed in the late response to EGF treatment. In addition, experiments with actinomycin D showed

that the changes observed in *SNX3* mRNA levels in the late response to EGF occur transcriptionally.

Experiments with EGFR inhibitor, on the other hand, indicate that changes in SNX3 mRNA and protein levels in both early and late EGF induction are specific to EGFR. It is known that EGFR activates a number of downstream signaling cascades, including the Ras/Raf/MAPK(MEK)/ERK and PI3K/Akt/mTOR (Purba *et al.*,2017). Upon activation of MEK1/2, ERK 1 and 2 also activated by phosphorylation (Wee and Wang, 2017). Activated ERK also translocates to the nucleus, where it activates ternary complex factor (TCF) transcription factors, which are important in the induction of immediate early genes (IEGs) (Dhillon *et al.,* 2007). IEG products include c-FOS and c-MYC, which promote late-response genes (Murphy and Blenis, 2006). Nuclear ERK can also activate ELK-1, ETS, SP-1, and c-JUN (Geer *et al.*, 1995). The AP-1 (activator protein 1) complex, which includes c-FOS and c-JUN, functions as a transcription factor that binds to the CYCLIN D1 promoter to activate transcription of the important G1 driver (Herber *et al.*, 1994). EGF-activated ERK1/2 has also been shown to stabilize and induce the expression of FOXC1, a transcription factor implicated in triple-negative breast cancer (Jin *et al.*, 2014). ERK also inhibits apoptosis by phosphorylating the pro-apoptotic BH3-only protein BIM (BCL2-like 11), causing BIM ubiquitination and proteasome degradation. ERK is also known to be involved in the regulation of pyrimidine synthesis, chromatin remodeling, ribosome synthesis, and protein translation (Chambard *et al.*, 2007). Another important downstream target of EGFR pathway is AKT. In terms of cell survival, AKT inhibits apoptosis by directly phosphorylating components of the cell death machinery. AKT phosphorylates the pro-apoptotic BAD (Bcl-2-associated death promoter) protein, rendering it inactive and preventing it from binding and inhibiting BCL-XL, an anti-apoptotic protein (Datta *et al.*, 1997). AKT also inhibits caspase-9 catalysis as well as the activity of FOXO1 (Tang *et al.*, 1999). Pro-apoptotic proteins BIM and the FAS ligand are among the FOXO1 downstream gene targets (Brunet *et al.*, 1999). AKT also phosphorylates MDM2 at S166, allowing it to translocate to the nucleus, where it ubiquitinates and downregulates p53, a well-known tumor

suppressor (Ogawara *et al.*, 2002). Akt also activates mTOR and activation of mTOR increases the synthesis of CYCLIN D1, HIF1 (hypoxia-inducible factor 1), and growth factors such as VEGF (Abraham R.T., 2004). Results from our experiments suggest that SNX3 may also be a late transcriptional target of EGFR pathway. Currently, it is not clear which transcription factors are responsible for SNX3 transcription.

Our findings have implications for cellular homeostasis and signaling pathways. SNX3 is a member of sorting nexins which plays an important role in the recycling pathway. By binding to the phosphatidylinositol-3-phosphate (PI3P) of early endosomes, SNX3 participates in an early step of endocytosis and is an essential component of the VPS35:VPS26: VPS29 retromer complex. Instead of directly targeting receptors to lysosomes, SNX3 has a cargo recognition function within the retromer and is involved in receptor recycling (Lenoir *et al.* 2018). Endocytosed receptors such as Wnt receptor Wntless (Wls) and EGFR are recycled back to the trans-Golgi network or plasma membrane by retromer complexes (Zhang *et al.*, 2011; Chiow *et al.*, 2012). If endocytosed receptors in the early endosomes are not recycled, endosomes mature into late endosomes and fuse with lysosomes for degradation of the cargo (Bakker *et al.*, 2017). Loss of SNX3 expression has been shown to cause increased degradation of cargo receptors, including EGFR (Chiow *et al.*, 2012).

Overall, the findings of this thesis show that SNX3 is a novel player in the EGF/EGFR axis. EGF stimulation causes upregulation of SNX3 first at protein level and later at transcript level. Implications of SNX3 upregulation upon EGF stimulation will be highly relevant in EGFR endocytosis and recycling. Hence, considering that SNX3 is a retromer protein, investigating the role of SNX3 in EGFR recycling will be of interest, not only in breast cells but also other cancer types that are dependent on the EGFR signaling pathways.

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APPENDICES

A. Buffers and Solutions

10% Seperating Gel Mix:

30 % Acrylamide – Bisacrylamide	3.33 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml
10 % SDS	100 µl
10 % APS	100 µl
TEMED	4 µl
dH_2O	3.96 ml

5% Stacking Gel Mix:

30 % Acrylamide – Bisacrylamide	1.36 ml
1 M Tris-HCl (pH 6.8)	1 ml
10 % SDS	80 µl
10 % APS	80 µl
TEMED	8 µl
dH_2O	5.44 ml

0.1 % TBS-T (pH 7.6):

Tris	20 mM
NaCl	137 mM
Tween-20	0.1 %

6x SDS Sample Buffer:

SDS	12 %
2-mercaptoethanol	30 %
Glycerol	60 %
Bromophenol Blue	0.012 %
Tris	0.375 M

Stripping Buffer (pH 2.2):

Glycine	15 g
SDS	1 g
Tween-20	10 ml
dH_2O	up to 1 L

10x Running Buffer:

Tris	25 mM
Glycine	190 mM
SDS	0.1 %

10x Blotting Buffer (pH 8.3):

Tris	0.25 M
Glycine	1,92 M

Transfer Buffer:

Methanol	200 ml
10x Blotting Buffer	100 ml
dH2O	700 ml

10x PBS (pH 7.4):

NaCl	1.37 M
KCl	27 mM
Na_2HPO_4	100 mM

B. Markers

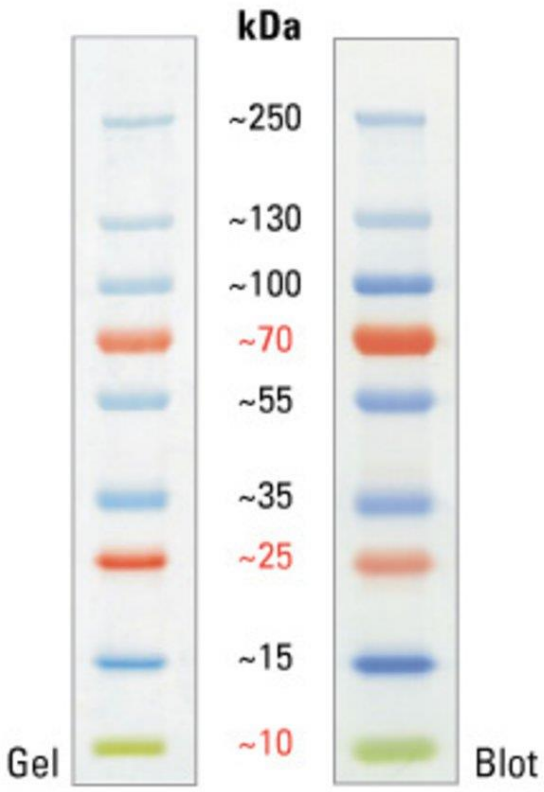

Figure B.1 Protein ladder (Thermo PageRuler Plus Prestained, Cat #: 26619).

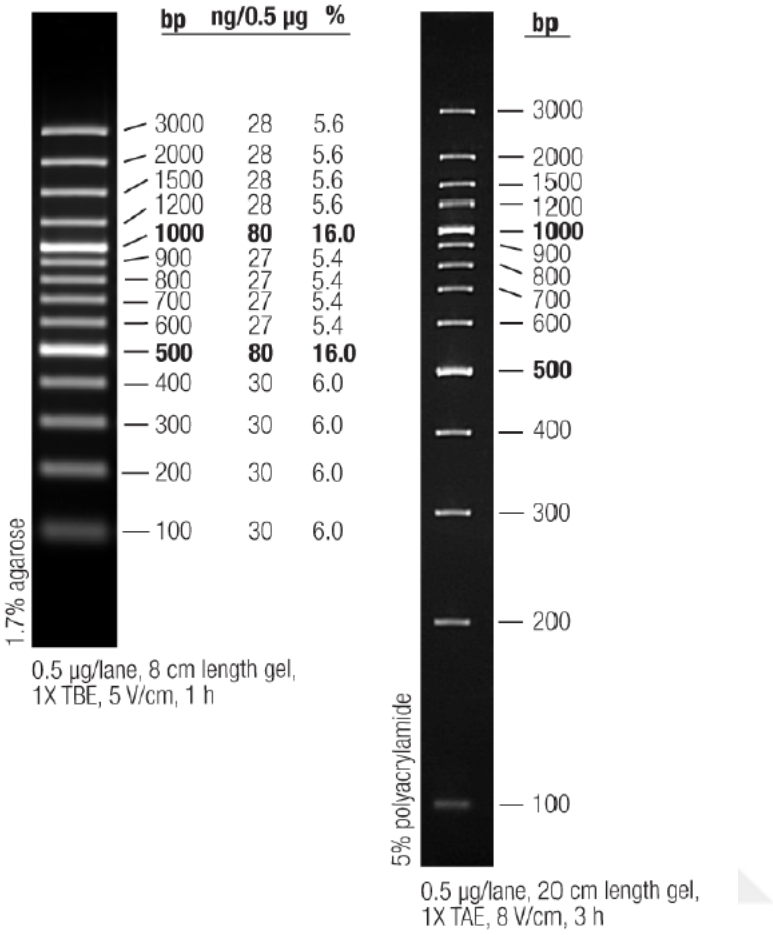

Figure B.2 DNA Ladder (Thermo GeneRuler 100 bp Plus, Cat #: SM0321).